The Ecology of Reproductive Tract Microbiota of a Polygynandrous Host, the Red Junglefowl (*Gallus gallus*)



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Acknowledgements

My thesis is dedicated to all the academics who struggle with mental health. We have got this.

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Thesis Abstract

The Ecology of Reproductive Tract Microbiota of a Polygynandrous Host, the Red Junglefowl (*Gallus gallus*)

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In a world dominated by bacteria, animals harbour microorganisms both within and on their bodies. Host-associated bacteria, or microbiota, can play a key role in host health, metabolism, immune functioning, and even behaviour. These microbiota are not stable over time—novel bacteria can be acquired from the surrounding environment and exchanged between multiple hosts. In the current literature, hypotheses have emerged that suggest the potential for host social behaviour to influence its gut microbiota, while the gut bacteria may also affect host social decision making. The focus of host microbiota research has been on bacterial communities found in host guts, but a similar interdependence may occur between host sexual behaviour and its reproductive microbiota. Studying the role of bacteria in the context of host reproduction may aid in understanding previously unexplored avenues in host evolutionary ecology. For example, reproductive bacteria may partake in host mating behaviour, play a role in sexual selection and conflict, and determine host socio-sexual network structure. Surprisingly little, however, is known about the bacterial communities in the reproductive tracts of non-human hosts. To fill this gap in knowledge, my thesis investigated the ecology of reproductive tract microbiota of the polygynandrous red junglefowl (*Gallus gallus*). By using state-of-the-art technologies developed in the field of microbial ecology, I carried out a thorough survey of bacteria associated with reproductive tracts. First, I show that both females and males harbour bacteria across the continuum of their internal reproductive tracts, and that these assemblages are distinct from the gut microbiota and spatially-structured in females, while more homogenous in males. I then explore temporal variation in fowl ejaculate microbiota, and show that while ejaculate-associated bacteria differ between young and old males, ejaculates have a consistent bacterial composition over the course of breeding season and across a sequence of matings. Finally, I focus on the association between fowl mating interactions and cloacal bacteria, and find some evidence consistent with the idea that socio-sexual interactions during experimental trials may be associated with variation in cloacal microbiota. Throughout the thesis, I report considerable intra- and inter-individual variation, highlighting the importance of including phenotypic variation in host microbiota studies. Combined, the findings of my thesis show the potential importance of studying reproductive microbiota, and encourage further interdisciplinary work to understand the role of reproductive bacteria in host evolution and ecology.

Author Contributions

Chapter II: Rosie Eccleston, Tommaso Pizzari, and Liisa Veerus conceived the idea. Tommaso Pizzari and Rosie Eccleston carried out the fieldwork. Liisa Veerus, with the guidance of Shorok B. Mombrikotb and Emma Ransome, developed the laboratory methods. Liisa Veerus performed all laboratory processing, post-sequencing sample processing, statistical analysis, and wrote the chapter. We thank Grant McDonald and Kirstie McLoughlin for helping with the fieldwork, Shorok B. Mombrikotb and Emma Ransome for their guidance with statistical analysis, and Ellen Pasternack for proofreading and comments.

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Chapter IV: Liisa Veerus, Yunke Wang, and Tommaso Pizzari conceived the idea. Yunke Wang carried out the fieldwork and ejaculate processing, Liisa Veerus collected the microbiota samples. Liisa Veerus performed all laboratory processing, post-sequencing sample processing, statistical analysis, and wrote the chapter. We thank Anamaria Sluganovic, Ella Glover, and Ellen O'Brien for helping with the fieldwork, Emma Ransome for her guidance with optimising the laboratory processing, and Hannah Ogden for proofreading and comments.

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Chapter I. General Introduction: Overview of Host-Bacteria Interdependence with a Focus on Reproductive Microbiota in the Context of Host Evolutionary Ecology

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Bacteria appeared in the Precambrian period, 3.5 billion years ago (Konhauser et al., 2002), and have since become ubiquitous across planet Earth, forming the most diverse branch of the tree of life (Hug et al., 2016). Bacteria have been found to inhabit water and soil (DeLong & Pace, 2001), and even extreme environments, such as deep-sea hydrothermal vents and ice (Mancuso Nichols, Guezennec & Bowman, 2005). Despite their widespread prevalence, humans did not discover bacteria until the 1660s, when Anton van Leeuwenhoek examined pond water through a magnifying lens. After centuries of research, it is now widely accepted that eukaryotic cells and multicellularity evolved in a bacterial world (McFall-Ngai et al., 2013; Archibald, 2015). In fact, a landmark study of human gut microbiome[1] found that more than 99% of the genes present in the gut are of bacterial, and not human origin (Qin et al., 2010), highlighting the importance of studying host-bacteria interactions and inspiring countless host microbiota studies, including this thesis. The specific aim of this introduction and thesis is to explore the relationship between multicellular animal hosts and the many bacteria that are specialised to a host-associated lifestyle, with a special focus on reproductive microbiota, and its role in host evolution and ecology.

[1]In the thesis, I define "microbiota" (plural "microbiota") as an assemblage of microorganisms, and "microbiome" (plural "microbiomes") as an assemblage of microorganisms and their genomes (Marchesi & Ravel, 2015). Both include bacteria, archaea, fungi, protists, and viruses, but I focus only on bacterial assemblages.

Animal Hosts as Bacterial Ecosystems

In a broad sense, animal hosts provide two types of environments for bacteria. First, the outer surface of the host can be colonised by aerobic bacteria that are interacting with the host, but also with the environment around them (Kong, 2011). Second, there is increasing evidence of mostly microaerophilic and anaerobic bacteria that inhabit internal host environments, including well-studied ecosystems like the human gut (Yatsunenko et al., 2012), vagina (Ravel et al., 2011), and oral cavity (Grice & Segre, 2012). A previous meta-analysis showed that each of these body sites harbours a significantly distinct bacterial composition in humans alone (Lozupone et al., 2013). Not only do humans harbour organ-specific microbiota, there is surprising variation in bacterial communities within an organ system. For example, the pH and nutrient gradient across the human gastrointestinal tract results in a continuum of distinct microbiota (Hollister, Gao & Versalovic, 2014). In total, bacterial cells are predicted to make up ~0.3% of adult human body weight (Sender, Fuchs & Milo, 2016).

The highly-specialised and localised microbiota rarely remain stable even within an individual host. Intra-individual variation has been reported in human newborns as their gut microbiota shifts from the infant to the adult state (Palmer et al., 2007). There is increasing evidence that age, diet, environment, health status, and many more abiotic and biotic aspects affect human gut microbiota (Xu & Knight, 2015; Greenhalgh et al., 2016; Rinninella et al., 2019). This variation has been found to result in inter-individual variation in gut bacterial composition. For example, family members share a core gut microbiome, but individuals were found to differ in the specific bacterial strains sequenced from their faeces (Turnbaugh et al., 2009).

Considering the astonishing intra- and inter-individual variation, studies across animal kingdom have, unsurprisingly, reported also inter-species variation in host-associated microbiota. Humans, chimpanzees (*Pan troglodytes*), gorillas (genus *Gorilla*), and bonobos

(*Pan paniscus*) all harbour a distinct gut microbiota, despite the hosts being closely related (Moeller et al., 2016). Moreover, the gut bacterial compositions in hominids show cospeciation with their hosts (Moeller et al., 2016). Indeed, a study comparing the gut microbiota of wild mice (genus *Apodemus*), voles (genus *Microtus*), and shrews (genus *Sorex*) found that species identity predicted the host gut microbiota better than the surrounding environment, with conspecifics trapped from different locations sharing a more similar gut bacterial composition, while harbouring a distinct gut microbiota from heterospecifics that were sampled in their home habitat (Knowles, Eccles & Baltrūnaitė, 2019).

In addition to inter-species variation in microbiota composition, there is also variation in host-associated bacterial richness and specificity across the animal kingdom. For example, the Hawaiian bobtail squid (*Euprymna scolopes*) has formed a symbiosis with a single strain of the luminous *Vibrio fischeri* (phylum Proteobacteria) in their light-organ (Nyholm & McFall-Ngai, 2004), only seven bacterial "species" (identity ~97%) make up the core gut microbiota of honey bees (*Apis mellifera*; Kwong et al., 2017), while some animal hosts—for example, ants, dragonflies, and oysters among others—are hypothesised not to need nor have a resident microbiota altogether (Hammer, Sanders & Fierer, 2019).

Indeed, host species and their microbiota have been found to differ so vastly that characterising host-associated bacterial communities has become an important focus in conservation biology, as the survival of a species depends not only on its habitat quality, but also its genetic and bacterial predisposition (Trevelline et al., 2019; West et al., 2019). Exploring host microbiota has become especially important and urgent at the doorstep of the climate crisis that is expected to severely disturb the associations between the host and its bacteria (Apprill, 2017).

Resident and Transient Bacteria within and on Animal Hosts

Animal hosts are a multi-level bacterial ecosystem complex, which allows interactions and transmission both within a single host and between multiple hosts. First, on the microbe-microbe level, bacteria interact with each other across the competition-cooperation gradient within and on their animal host (Flemming et al., 2016). On the host-microbe level, bacteria translocate between different host organ systems (Balzan et al., 2007), while ultimately being at the mercy of top-down regulation by the animal host (Foster et al., 2017). On the host-host level, bacteria are transferred horizontally during social and sexual interactions (Archie & Theis, 2011; Archie & Tung, 2015), and also vertically along the mother-offspring axis (Mändar & Mikelsaar, 1996; Dominguez-Bello et al., 2010). Finally, on the host-environment level, bacteria are exchanged between the host and its ecosystem. These four levels provide a dynamic bacterial pool within and outside of the host, highlighting the importance of applying a metacommunity approach to studying host bacterial communities (Miller, Svanbäck & Bohannan, 2018). Through complex, multi-level ecosystem dynamics, host-associated microbiota develop to harbour resident, core bacterial compositions that are constantly challenged and perturbed by transient bacterial strains.

Both the resident and transient bacteria living within and on their animal hosts could be pathogenic, commensal, or mutualistic in their interactions with the host. Pathogenic bacteria may cause lethal infections, while mutualistic bacteria may aid in buffering the detrimental effect of pathogens. For example, a gastrointestinal infection of *Vibrio cholerae* (phylum Proteobacteria) is suppressed by *Lactococcus lactis* (phylum Firmicutes) in mice (*Mus musculus*; Mao et al., 2018). More often than not, however, it is the commensal bacteria that constitute the majority of host microbiota, where they play a vital role in host immune functioning and metabolic pathways (Hooper & Gordon, 2001).

From 2015, the microbiology field has been shifting from operational taxonomic units (OTUs; ~97-99% identity) to amplicon sequence variants (ASVs; 100% identity) when referring to bacterial "species", revealing previously underappreciated within-"species" variation (Callahan et al., 2016a). We now know that bacteria can change their interaction type along the pathogen-mutualist gradient. For example, *Helicobacter pylori* (phylum Proteobacteria) in human guts (Lin & Koskella, 2015) and *Lactobacillus iners* (phylum Firmicutes) in human vaginas (Petrova et al., 2017) have been found to alter their within-host function according to the surrounding bacterial composition. Considering the highly-dynamic role of bacteria within and on their animal hosts, it is thus often more informative to characterise both the identity and function of distinct bacterial strains in host microbiota, if possible (Ellegaard & Engel, 2016).

Host-Microbiota Interdependence, as Illustrated by Examples from Gut Microbiota Research

Animal hosts have evolved in a bacterial world, leading to an intricate interdependence between the host and its microbiota. Host physiology and social behaviour affect its bacterial composition, while bacteria carry out functional roles in the host and affect host social decision-making. First, I focus on the host-microbe level, and discuss the effect of host physiology on its microbiota, and the numerous functions that bacteria perform within their host. Then, I shift the spotlight to the host-environment and host-host level, and discuss how animal hosts affect their microbiota via their social decision-making and behaviour, and *vice versa*. We illustrate our case with examples predominantly from the gut microbiota research, which is currently the leading focus of interest in the microbiota field.

The recent literature has revealed the intricacies of the host-microbiota interdependence within the host organism. For example, the mucosal lining of the human gastrointestinal epithelium has been found to defend against and neutralise pathogenic bacteria, such as

members of the genus *Salmonella* (phylum Proteobacteria), while providing nutrients to commensal and mutualistic bacteria (Martens, Neumann & Desai, 2018). In turn, the commensal bacteria provide a vital function within the host. The mutualistic genus *Bifidobacterium* (phylum Actinobacteria) found in mammalian guts has been found to metabolise complex carbohydrates, stabilise the host gut microbiota, and decrease the incidences of diarrhoea, inflammatory bowel disease, and colorectal cancer (O'Callaghan & van Sinderen, 2016). This highly-specialised interdependence between the host and its gut microbiota plays a role not only in host digestive functions, but has also been found to affect host brain and behaviour via the gut-brain axis (Cryan & O'Mahony, 2011; Davidson et al., 2018). Gut bacteria establish a communication between the two host organs via the vagus nerve (Lyte et al., 2006), and have been shown to play a part in developing anxiety and depression (Foster & McVey Neufeld, 2013), but also autism spectrum disorders among others (Vuong & Hsiao, 2017). In extreme cases, bacteria have become solely responsible for a function in the host. For example, fruit flies (*Drosophila melanogaster*) rely on their gut microbiome metabolites for a fully-functional developmental and metabolic homeostasis (Shin et al., 2011). A recent study in the Camponotini ant tribe (10 ant genera, including *Camponotus*, i.e. the carpenter ants) showed an obligate endosymbiosis between *Blochmannia* (phylum Proteobacteria) bacteria and the ants (Rafiqi, Rajakumar & Abouheif, 2020). The bacteria are integrated into ant embryos and play a substantial role in embryonic development (Rafiqi, Rajakumar & Abouheif, 2020).

Although the interdependence between the host and its gut microbiota is well-established and highly-adapted, the system is not immune to perturbations, and the consequences of such perturbations could be detrimental for both counterparts. Medical antibiotic treatments and antibiotics present in human food sources have a drastic effect on human gut microbiota, leading to bacterial dysbiosis, or imbalance, and consequently exposing

the host to increased disease acquisition risk (Francino, 2016). In fact, it is the studies on the effect of antibiotics on human gut microbiota that have truly revealed the far-reaching importance of host microbiota. For example, the use of antibiotics, in particular during pregnancy and early life, may lead to an increased incidence of asthma (Ni et al., 2019), autoimmune diseases (Vangoitsenhoven & Cresci, 2020), and Alzheimer's disease (Angelucci et al., 2019), although questions remain about the exact mechanisms. Thus, while antibiotics have revolutionised modern medicine, their detrimental side-effects on host microbiota in particular have inspired several counterbalancing treatments, including pre- and probiotics, and faecal transplants, that attempt to re-establish the "healthy" gut microbiota (van den Abbeele et al., 2013; Giles, D'Adamo & Forster, 2019).

In addition to host physiology, the decisions of the host, such as what to eat and with whom to interact, have been shown to affect its gut microbiota. Daily diet decisions in humans significantly impact their gut microbiota (Johnson et al., 2019a). Wood mice (*Apodemus sylvaticus*) have a seasonal diet that shifts between insects and seeds, which also affects their gut microbiota variation (Maurice et al., 2015). Similarly, herbivorous, omnivorous, and carnivorous vertebrate species have been shown to harbour different gut bacterial compositions (Youngblut et al., 2019). The considerable effect of diet on host gut microbiota has inspired research into what could be the ideal human diet for maximised health benefits (Deehan & Walter, 2016). However, host dietary choices may also be influenced by its microbiota. A study in fruit flies showed that commensal gut bacteria can induce a specific appetite when their host is lacking any essential amino acids (Leitão-Gonçalves et al., 2017), indicating a feedback loop between dietary choices and host microbiota.

Host social interactions with its conspecifics also affect its microbiota, and *vice versa*. Wild yellow baboons (*Papio cynocephalus*) that interact with each other more frequently also share a more similar gut microbiota (Tung et al., 2015). In fact, social transmission of bacteria

may play an important role in acquiring and maintaining a "healthy" gut microbiota and thus, hosts are expected to facilitate this process. For example, upon eclosion, bumble bee (*Bombus terrestris*) pupae come in contact with and acquire the bacteria present in their nestmates' faeces, which protect the bees against a gut parasite (Koch & Schmid-Hempel, 2011). Similarly, female red firebugs (*Pyrrhocoris apterus*) smear their eggshells with a mutualistic Actinobacteria strain to promote acquisition by their hatchlings (Kaltenpoth, Winter & Kleinhammer, 2009). Thus, social transmission of beneficial bacteria may be a selective force contributing to the maintenance of sociality, and has even been proposed as a reason for why some social behaviours evolved (Archie & Theis, 2011; Ezenwa et al., 2012; Archie & Tung, 2015). Not only do bacteria have an effect on host sociality in general, host microbiota may also specifically influence who interacts with whom. Previous studies have found that bacteria present in the anal gland secretions of meerkats (*Suricata suricatta*; Leclaire, Nielsen & Drea, 2014) and badgers (*Meles meles*; Sin et al., 2012), or in the gut of fruit flies (Lizé, McKay & Lewis, 2014) and leaf-cutting ants (*Acromyrmex echinatior*; Teseo et al., 2019), may provide honest cues about the identity of their host, and underly mechanisms of kin, social group, and nestmate recognition.

Just like the transfer of beneficial bacteria may promote host sociality, the spread of pathogenic bacteria could induce avoidance behaviour in host populations (Hart, 1990, 2011; Curtis, 2014; Hart & Hart, 2018). For example, uninfected black garden ants (*Lasius niger*) distance themselves from infected conspecifics, thus protecting the larger colony from the fungal pathogen (Stroeymeyt et al., 2018). In extreme cases, bacteria may affect host social behaviour for bacterial fitness benefits at the expense of the fitness of the host. For example, fruit fly olfactory cues can be hijacked by a lethal gut pathogen, *Pseudomonas entomophila* (phylum Proteobacteria), which affects the host aggregation pheromones, attracting uninfected conspecifics to the infected individual, favouring the spread of the pathogen (Keesey et al.,

2017). Regardless of whether their effect on the host is beneficial or detrimental, socially-transmitted bacteria have been found to reflect and affect host behaviour and social group structure (Griffin & Nunn, 2012; Tung et al., 2015; Grieneisen et al., 2017; Dill-McFarland et al., 2019).

Reproductive Tracts as Bacterial Ecosystems

While the focus of host microbiota studies has largely been on gut bacteria, the interest is starting to shift towards other host ecosystems as well, including the reproductive tract (Figure 1). From the gonads to genitalia, including the accessory glands, reproductive tracts support bacterial communities that are distinct from other host microbiota (Mändar, 2013; Lozupone et al., 2013; Chen et al., 2017; Goffau et al., 2019). In a broader sense, reproductive microbiota also includes bacteria that are found in and on any tissue, structure, and/or fluid that comes in contact with the gametes (Rowe et al., 2020a). There is also a distinction between female and male reproductive microbiota. For example, female (Chen et al., 2017) and male human reproductive tracts (Mändar, 2013) have been shown to harbour distinct bacterial compositions both when compared with other microbiota in humans, and each other. In addition, there is microbiota variation within the respective reproductive tracts. The female human reproductive tract may be considered a continuum of different microbiota, with the lower organs, including the vagina, showing a strong *Lactobacillus*-dominance, while the upper organs harbour a higher diversity of bacterial genera (Chen et al., 2017). Despite the higher bacterial diversity, however, the upper organs support a decreased bacterial load, or biomass, than the lower female reproductive organs (Chen et al., 2017), highlighting the specificity and complexity of host reproductive microbiota. The vast bacterial diversity across these host ecosystems is only starting to be discovered, and current research suggests that, similarly to gut microbiota, at least the female reproductive tract bacterial compositions show

impressive intra- (Gajer et al., 2012) and inter-individual (Ravel et al., 2011), as well as inter-species variation (Yildirim et al., 2014).

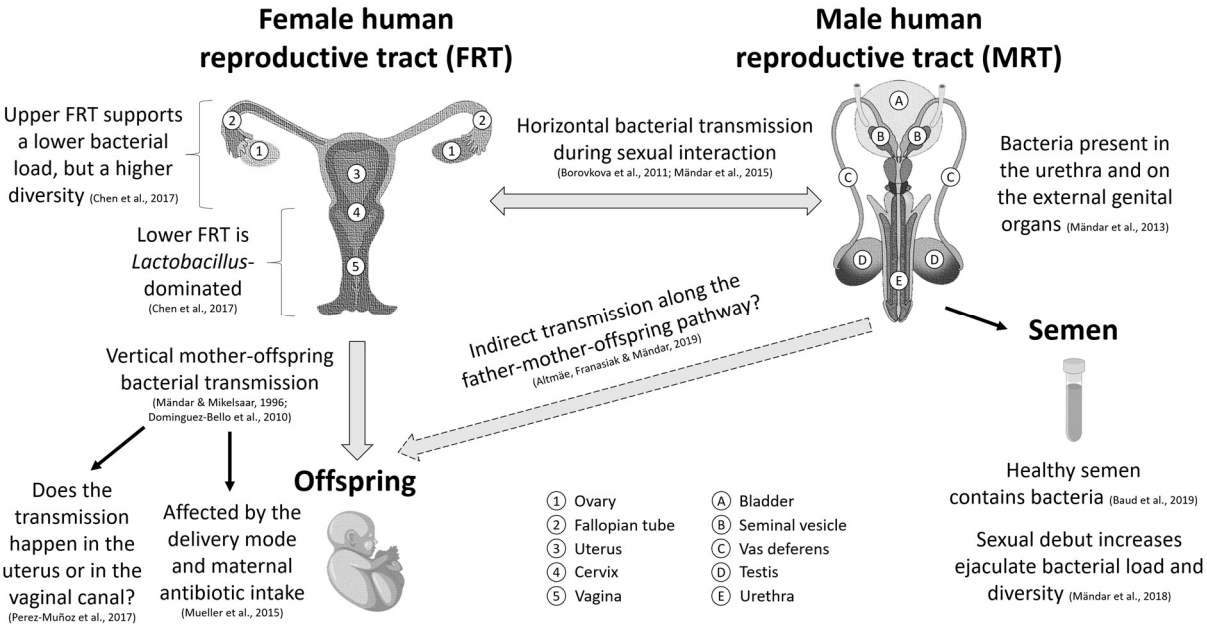

Figure 1. Current understanding of the human reproductive tract microbiota, and the potential horizontal and vertical bacterial transmission pathways. Majority of the current reproductive tract microbiota literature is about the human reproductive tract. Much of the focus has been on the vertical mother-offspring bacterial transmission pathway, which has biased the research efforts towards the female reproductive tract (FRT) microbiota. As a consequence, male reproductive tract (MRT) has received far less attention, with most studies concentrating on seminal bacteria as a proxy for the MRT microbiota. While several studies have emphasised the importance of studying the FRT and MRT microbiota in parallel (Borovkova et al., 2011; Mändar et al., 2013; Mändar et al., 2015), we find few examples of that in the literature. Due to these current limitations, several research questions remain currently unanswered. For example, (i) how divergent are the FRT and MRT microbiota under parallel standardised laboratory and computational processing; (ii) how and where exactly does the vertical mother-offspring bacterial transmission happen, is it similar across the ovoviviparity spectrum (egg-laying vs live-bearing reproduction), and do the male partners contribute anything directly/indirectly to the offspring microbiota; (iii) how well do proxy samples reflect on the entire reproductive tract microbiota, e.g. how representative is the ejaculate microbiota of the MRT microbiota; (iv) how do intra- and inter-individual, and inter-species differences affect reproductive tract microbiota? Images of the FRT (original author: Mikael Häggström), MRT (original author: Sheldahl), offspring (original author: Vecteezy), and ejaculate sample in the tube (original author: iStock) were drawn based on the vector art of the original authors.

Host phenotype, including variation in age and diet, has been found to have a considerable impact on host reproductive microbiota. For example, women in their menopause have been shown to harbour a significantly lower abundance of mutualistic *Lactobacillus* (Muhleisen & Herbst-Kralovetz, 2016), and human seminal fluid microbiota was found to differ significantly between high-fat and control diet groups, with several known commensals, such as *Bifidobacterium*, more abundant in the control group (Javurek et al., 2017). Hosts have also been shown to regulate their reproductive microbiota via hormones and antimicrobial peptides (AMPs). In women, plasma levels of sex hormones—such as oestrogen and progesterone—fluctuate over menstruation and pregnancy in concert with the AMPs to maintain a "healthy" reproductive microbiota throughout the female host's life (Yarbrough, Winkle & Herbst-Kralovetz, 2015). Similarly, AMPs, such as defensins, have been found to maintain male human, mouse, and rat (*Rattus norvegicus domestica*) reproductive microbiota (Com et al., 2003), highlighting the considerable role of host homeostasis in regulating reproductive bacterial communities.

Mating interactions between female and male hosts can also have an effect on their respective reproductive microbiota. Sexual debut in humans has been shown to increase seminal bacterial diversity and load (Mändar et al., 2018). Non-mated female and male bed bugs (*Cimex lectularius*) were reported to harbour divergent reproductive microbiota prior to mating, with mating resulting in an exchange of bacteria between the sexes (Bellinvia et al., 2020a). Indeed, while host gut (Tung et al., 2015; Perofsky et al., 2017) and skin microbiota (Walke et al., 2011) are predominantly affected by social interactions, reproductive microbiota are mostly affected by sexual bacterial transmission (Kulkarni & Heeb, 2007; White et al., 2010; Mändar et al., 2015). In internally-fertilising species, the female and male reproductive microbiota come in direct contact during mating, which allows the sexual transmission of

bacteria between mating partners. In humans, mating has been shown to lead to a shared seminovaginal microbiota between sexual partners (Mändar et al., 2015).

In addition to the onset of mating, the subsequent mating behaviour of the host has a considerable impact on their reproductive microbiota. Indeed, monogamous female California mice (*Peromyscus californicus*) harbour a distinct vaginal microbiota compared with polygamous deer mice (*Peromyscus maniculatus*) that were found to have a significantly higher vaginal bacterial diversity (MacManes, 2011). Similarly, female viviparous lizards (*Zootoca vivipara*) that mated with multiple partners were reported to have a significantly higher bacterial diversity on their cloacae than females that mated with a single male (White et al., 2011). As such, host phenotype and mating behaviours affect host reproductive microbiota in concert.

Finally, in addition to horizontal transmission, reproductive microbiota can be vertically-transmitted along the mother-offspring axis (Mändar & Mikelsaar, 1996; Dominguez-Bello et al., 2010). More specifically, mammalian foetuses first come in contact with bacteria in the maternal reproductive tract, although exactly where and how remains poorly understood. There are two mutually non-exclusive theories about the mother-to-offspring bacterial transmission. One suggests that the newborn first comes in contact with their mother's reproductive microbiota in the vaginal canal, where they acquire bacteria from the genera *Lactobacillus* and *Bifidobacterium* among others (Perez-Muñoz et al., 2017). The second proposes that the foetus acquires the first bacteria already in the uterus (Perez-Muñoz et al., 2017). However, considering that the upper female reproductive organs in humans harbour a lower bacterial load (Chen et al., 2017), one must be careful when interpreting results from low biomass samples. It is possible that the bacteria found in the upper female reproductive tract have been false positives due to laboratory contaminants (Salter et al., 2014; Perez-Muñoz et al., 2017). A carefully-conducted experiment revealed that while the human

placenta does not harbour a microbiota, pathogenic *Streptococcus agalactiae* (phylum Firmicutes) from the mother's genital tract may come in contact with the foetus via the placenta and cause sepsis (Goffau et al., 2019). Regardless of the specific mechanism, the gut microbiota of mice has been shown to be predominantly vertically-transmitted (Moeller et al., 2018). Similarly, human newborns born by Caesarean section are predominantly colonised by their mother's skin microbiota, while vaginally-born babies harbour a gut microbiota that shares key bacterial strains with their mother's vaginal microbiota (Dominguez-Bello et al., 2010). The recent literature has established that by six weeks after birth, newborns born vaginally and by Caesarean section have become more similar to each other in their gut microbiota (Chu et al., 2017). The role of vertically-transmitted bacteria, however, may be more important than originally thought, and vaginal seeding of newborns with their mother's reproductive microbiota is gaining popularity in modern medicine to promote infants acquiring their mother's vaginal bacteria (Lee et al., 2017). Considering the shared seminovaginal microbiota between mating partners (Mändar et al., 2015), there is a current consensus that the role of male reproductive tract microbiota should also be considered in the formation and development of offspring microbiota (Altmäe, Franasiak & Mändar, 2019). Despite the potential for vertical father-to-offspring bacterial transmission, however, the female reproductive microbiota is expected to play a larger role in offspring microbiota (Amato et al., 2020), as the embryo develops in the mother organism, suggesting that male reproductive microbiota most likely has a direct effect on female reproductive microbiota, but an indirect role in determining the offspring microbiota. Importantly, the vertical transmission of bacteria along the parent-offspring axis is not reserved just to the mammalian class, but has also been detected in oviparous species. The eggshell protects the developing embryo from bacteria, but it can also serve as a bacterial acquisition and infection site for red-eared slider turtle hatchlings (*Trachemys scripta elegans*; Holgersson et al., 2016). In birds, bacteria have been found to be

incorporated both within and on eggs, affecting the microbiota of the offspring (De Buck et al., 2004; Potter et al., 2017; Van Veelen, Salles & Tieleman, 2018). The divergence of reproductive microbiota from other host microbiota, its horizontal, bidirectional sexual transmission between partners, and its widespread cross-generational transmission have sparked the hypothesis that reproductive bacteria may play a key role in host evolution and ecology (Dominguez-Bello et al., 2019; Rowe et al., 2020a).

Evolutionary Significance of Reproductive Microbiota

Studies revealing the considerable effect reproductive bacteria may have on host health have inspired the hypothesis that reproductive microbiota could also affect host evolution and ecology. More specifically, reproductive microbiota may play a role in host fitness and reproductive success, offspring fitness, sexual selection and conflict, socio-sexual group structure, mating systems, and even speciation. Below, I discuss in more detail these aspects of host-reproductive microbiota interactions.

Studies of bacteria associated with host reproductive tracts traditionally focused on sexually-transmitted infections (STIs) and their pathogenesis. Studies of human STIs have found that pathogenic bacteria, such as *Neisseria gonorrhoeae* (phylum Proteobacteria; Hsu, Rice & Lieberman, 1963) and *Chlamydia trachomatis* (phylum Chlamydiae; Paavonen & Eggert-Kruse, 1999), can have lasting health implications for their hosts. In fact, reproductive microbiota dysbiosis significantly increases the risk of contracting HIV during unprotected sex (Abdool Karim et al., 2019) and developing reproductive cancers in female humans (Walther-António et al., 2016; Łaniewski, Ilhan & Herbst-Kralovetz, 2020). Similarly, an "unhealthy" male reproductive tract microbiota has been shown to cause inflammation in reproductive organs (Mändar, 2013), and play a role in prostate (Sfanos et al., 2018) and bladder cancer

(Bučević Popović et al., 2018) in humans. Combined, these results highlight the role of reproductive bacteria in both host reproductive and general health.

The advent of next-generation sequencing (NGS) in the 1990s significantly increased the throughput and reduced the price of genomic analysis, allowing exploring the whole reproductive microbiota instead of focusing on a single pathogenic bacterial strain. This breakthrough has led to the discovery that the reproductive tract also hosts commensal and mutualistic bacteria. The best known mutualistic genus in female human reproductive tract is *Lactobacillus* (Redondo-Lopez, Cook & Sobel, 1990). Lactobacilli have been reported to protect their host from *C. trachomatis* (Nardini et al., 2016) and *Escherichia coli* (phylum Proteobacteria; Ghartey et al., 2014) infections, but also lower the contraction risk of non-bacterial STIs, such as HIV (Aldunate et al., 2013). In turn, female human physiology has been shown to support the proliferation of *Lactobacillus* in their vaginal canal by breaking down glycogen in epithelial cells into simpler sugars that serve as a bacterial food source (Spear et al., 2014). Less is known about potentially beneficial bacteria in male reproductive microbiota, although *Lactobacillus* has also been associated with a "healthy" seminal microbiota (Weng et al., 2014; but see: Haines et al., 2013; Wang et al., 2020a).

In addition to host health, reproductive microbiota also plays a role in host reproductive fitness. For example, dysbiosis in female human reproductive microbiota has been shown to reduce fertilisation success *in vivo* (Amato et al., 2020) and *in vitro* (Schoenmakers & Laven, 2020). Equally, pathogenic bacteria found in human semen may have a detrimental effect on *in vitro* fertilisation (Altmäe, Franasiak & Mändar, 2019). However, the exact mechanisms of bacteria-induced infertility are poorly understood. In female hosts, bacteria are thought to affect the microclimate of the female reproductive tract. Both pathogenic bacterial strains, such as *Enterococcus faecalis* (phylum Firmicutes; Ricci et al., 2018), and mutualistic bacteria, such as *Lactobacillus crispatus* (Wang et al., 2020a), present in human vaginas have been shown to

adhere to sperm cells and reduce their motility, thus severely affecting fertilisation success. Similarly, pathogens found in human semen have been shown to affect sperm motility (Diemer et al., 1996; Huwe et al., 1998), morphology (Moretti et al., 2009), and number (Hosseinzadeh et al., 2001; Fraczek et al., 2007), and thus decrease the chances of sperm cells reaching and fertilising the egg. Considering that both fertile (Mändar et al., 2015) and infertile (Borovkova et al., 2011) human couples share a seminovaginal microbiota, ameliorating bacteria-induced infertility is complicated and demands the treatment of both partners (Plummer et al., 2018).

Not only do the reproductive microbiota of mating partners affect their own health and reproductive fitness, reproductive bacteria also have an effect on offspring fitness. Vaginal dysbiosis due to a shift from the *Lactobacillus*-dominated composition to a more diverse bacterial community has been found to significantly increase the chances of preterm birth, which is the main cause of neonatal death (Hyman et al., 2014). Additionally, inheriting a sub-optimal microbiota along the mother-offspring axis may affect offspring health. The use of antibiotics during gestation and the delivery mode are known to have a significant impact on the establishing of gut microbiota in newborns (Mueller et al., 2015). The exact links remain currently poorly understood, but the initial infant gut microbiota is thought to provide the foundation for neonatal physical, immunological, and neurological development, and predict the risk of future disease (Tamburini et al., 2016; Yang et al., 2016). In support of this, prenatal use of antibiotics by the mother is likely to have a lasting effect on the immune system of the offspring, with the child more likely to develop allergy, asthma, and obesity in later life (Milliken, Allen & Lamont, 2019). Similar patterns have been reported in non-mammalian hosts. A study in domestic chickens (*Gallus domesticus*) showed that there is a considerable genus-level inheritance of bacteria between the maternal hen and the embryo (Ding et al., 2017). The bacterial strains detected in the intestines of the embryos were predominantly

associated with metabolic pathways (Ding et al., 2017), highlighting the importance of inheriting a "healthy" maternal microbiota for offspring fitness.

Increasing evidence suggests that reproductive microbiota may also play an important role in episodes of sexual selection (Rowe et al., 2020a). Sexual selection is defined as the intra-sexual competition for the access of mating partners and reproductive opportunities (Darwin, 1871; Andersson, 1994). Across the sexually-reproducing animals, males produce smaller gametes than females (anisogamy) and tend to exhibit lower rates of parental care (Janicke et al., 2016), typically generating more intense sexual selection on males and leaving females to invest more in gamete production and offspring rearing (Agrawal, 2001; Kokko & Jennions, 2008). Sexual selection on males manifests itself as mate choice exerted by females and male-male competition. In polyandrous species, where females mate with multiple males, sexual selection continues after copulation through inter-sexual cryptic female choice and intra-sexual sperm competition (Birkhead & Pizzari, 2002; Andersson & Simmons, 2006). Females are strongly selected on to maximise their reproductive success and offspring fitness by carefully selecting reproductive partners before, during, and/or after copulation (Agrawal, 2001; Kokko & Jennions, 2008). Any of these mechanisms could be affected by bacteria.

To minimise the risk of acquiring sexually-transmitted pathogens, females are expected to prefer uninfected partners (Beltran-Bech & Richard, 2014). In support of this, mate preference in female olive baboons (*Papio anubis*) relies on the visual inspection of male baboon genitals to check whether they are free of syphilis-like ulcers caused by the pathogenic bacterium *Treponema pallidum* (phylum Spirochaetes; Paciênca et al., 2019). Pathogen-mediated mate choice is not only a prerogative of female hosts—male fruit flies also show a preference for females without a *Pseudomonas aeruginosa* infection (Wittman & Fedorka, 2014). In addition to affecting primary sexual traits, e.g. through warts, inflammation, and/or discharge (Nunn, 2003), bacteria have also been suggested to affect and/or produce metabolites

necessary for exhibiting secondary sexual traits that allow the pre-copulatory discrimination between high- and low-quality partners (Archie & Theis, 2011). A study in mallards (*Anas platyrhynchos*) found that females preferred males with a more brightly coloured bill that were also found to have an increased amount of antibacterial activity in their ejaculates (Rowe et al., 2011). Thus, the bill colouration may have served as an honest, bacteria-affected secondary sexual trait forming the basis of female pre-copulatory mate choice (Rowe et al., 2011). Another secondary sexual trait that may be affected by reproductive bacteria is odour (Archie & Theis, 2011). Olfactory cues are used across the animal kingdom to discriminate between healthy and infected individuals (Beltran-Bech & Richard, 2014). Bacterial vaginal dysbiosis is known to cause a malodour in humans (Penn & Potts, 1998) and could thus negatively affect the sex life of the human host (Bilardi et al., 2013).

The role of bacteria in pre-copulatory male-male competition is largely unknown, with empirical evidence of bacteria affecting male mating behaviour and success currently lacking. The effect of other microbes however, such as iridoviruses in insects, suggest how males may be affected by reproductive tract bacteria in pre-copulatory competition over access to females. Male Texas field crickets (*Gryllus texensis*) that are infected with the IIV-6/CrIV iridovirus show a decreased latency to courting females compared with uninfected males (Adamo et al., 2014). Modulating the male host's courting behaviour is beneficial to the iridovirus, as it is sexually-transmitted between mating partners (Adamo et al., 2014). Pre-copulatory male-male competition could thus also be mediated via behavioural changes induced by bacteria, in particular by sexually-transmitted pathogens (Klein, 2003). Previous research has found that specific bacteria present in the guts of Siberian hamsters (*Phodopus sungorus*) may make them more aggressive during non-breeding season (Ren et al., 2020). A bacteria-induced increase in aggression could provide an advantage in male-male competition (Dow & von Schilcher, 1975; Simmons, 1986). Other behaviours that may increase pre-copulatory male mating success

include being bolder (Kortet et al., 2012) and more exploratory (McCowan, Rollins & Griffith, 2014; Sanguinetti et al., 2019), although the potential connection between these host behaviours and bacteria are only just starting to be explored. Moreover, whether these behaviour-associated bacteria are also present in the reproductive microbiota and whether they are sexually-transmitted remains currently unknown.

In addition to pre-copulatory sexual selection, reproductive bacteria may play an equally important role in post-copulatory sexual selection (Birkhead & Pizzari, 2002; Andersson & Simmons, 2006). The role of bacteria in post-copulatory sexual selection is more readily understood in the context of sperm competition. Sperm competition occurs in species where females mate with multiple males (polyandry; Parker, 1970). Sperm swimming speed and in particular sperm number are key predictors of the outcome of sperm competition (Birkhead & Pizzari, 2002; Snook, 2005). Thus, any bacterial strain that affects sperm cells could also affect the outcome of sperm competition. Pathogenic seminal bacteria have the potential to influence sperm motility (Diemer et al., 1996; Huwe et al., 1998) and morphology (Moretti et al., 2009), and even sperm cell viability (Hosseinzadeh et al., 2001; Fraczek et al., 2007). On the other hand, certain ejaculate-associated bacteria, such as members of the genera *Lactobacillus* and *Bifidobacterium* found in human seminal fluid, could be beneficial, e.g. improving sperm motility and reducing detrimental reactive oxygen species in semen (Valcarce et al., 2017). The male host is expected to maintain an ejaculate microbiota that maximises its reproductive success in sperm competition (Rowe et al., 2020a). This may be achieved via the production of AMPs, including defensins in mammals (Com et al., 2003), and lysozyme (Rowe et al., 2013) in birds and insects (Otti et al., 2009). Like host-associated bacterial communities, AMPs show significant host specificity (Rowe et al., 2020b), further implying the importance of AMPs in host microbiota maintenance. However, it is important to emphasise that the production of AMPs is energetically costly and may damage the sperm cells themselves

(Boman, 2003), suggesting a trade-off between reproductive fitness and immunity in the context of male reproductive microbiota.

Cryptic female choice is a post-copulatory selection mechanism, which allows females to select and reject ejaculates during and after mating to systematically bias the outcome of sperm competition in favour of the ejaculates of preferred male phenotypes and/or genotypes (Eberhard, 1996; Firman et al. 2017). Cryptic female choice can target multiple male or ejaculate traits. For example, female red flour beetles (*Tribolium castaneum*) show post-copulatory preference for males that exhibited higher courtship behaviour intensity (Edvardsson & Arnqvist, 2000), and female guppies (*Poecilia reticulata*) show cryptic female choice for brightly-coloured males (Pilastro et al., 2004). Similarly, female black-legged kittiwakes (*Rissa tridactyla*) preferentially eject their mate's inseminations that occur early with respect to the oviposition cycle to avoid fertilisation by aged sperm, which reduces offspring viability (Helfenstein, Wagner & Danchin, 2003; White et al., 2008). Cryptic female choice may also reflect the compatibility of a female with individual male genotypes. For example, cryptic female choice may mediate inbreeding avoidance in some species (Firman et al., 2017) or promote disassortative fertilisation by male or sperm genotypes, which are dissimilar from the female at the major histocompatibility complex (Firman et al., 2017; Lenz et al., 2018; Jokiniemi et al., 2020). It is thus plausible that the presence of bacteria in ejaculates may also play a role in cryptic female choice, although it is currently unknown whether it is the bacterial composition, load, or both that may trigger post-copulatory sexual selection processes in females. It is likely that female hosts prefer a relatively bacteria-free ejaculate, which causes minimal disturbance to their "healthy" microbiota (Rowe et al., 2011). Support for this hypothesis comes from animal husbandry and human infertility treatment studies. The ejaculates of farm animals contain high bacterial diversity and load (Sannat et al., 2015), and cleaning the ejaculates of bacteria has been suggested to increase the success of artificial

insemination (Morrell et al., 2019). The use of antibiotics and steroids has also been shown to increase the odds of successful gestation in human IVF patients (Karipcin et al., 2012). However, a complete lack of reproductive microbiota may also be detrimental. The dominance of *Lactobacillus crispatus* in human vaginas has been found to increase fertilisation success (Amato et al., 2020), suggesting that a specific bacterial environment is necessary for fertilisation and gestation, and that any fluctuations caused by mating and insemination may be harmful to the female host.

Sexual selection generates potential for sexual conflict, which occurs when female and male fitness interests diverge and cannot be simultaneously maximised (Parker, 2006). A conflict between the sexes may also emerge in the context of reproductive microbiota. For example, certain bacteria may be beneficial for the female host, but detrimental for the male, or *vice versa*. In support of this theory, harbouring a vaginal microbiota primarily dominated by the genus *Lactobacillus* increases fertilisation success in humans (Amato et al., 2020; Schoenmakers & Laven, 2020), but slows down sperm cells (Wang et al., 2020a), indicating divergent optimal reproductive microbiota in female and male hosts. In some instances, harbouring any reproductive bacteria may affect female hosts more severely than males. A study in black garden ants found that male hosts did not show antibacterial activity in their sperm storage organs, while females did (Dávila et al., 2018), suggesting that female and male hosts may have different fitness interests and costs when it comes to reproductive microbiota.

Reproductive bacteria may also dictate the structure of socio-sexual groups and determine mating systems. If the sexually-transmitted bacteria are pathogenic, hosts are expected to avoid mating with the infected individuals to lower their contraction risk and buffer the potential consequences the pathogens may have on their offspring (Sheldon, 1993; Lombardo, 1998; Nunn, 2003; Beltran-Bech & Richard, 2014). Monogamy may arise, if the sexually-transmitted pathogen is lethal (McLeod & Day, 2014), although the effect of STIs on

mating systems is expected to be highly dynamic (Thrall, Antonovics & Bever, 1997; McLeod & Day, 2014). In principle, beneficial reproductive bacteria may also lead to more frequent matings with certain partners (Lombardo, Thorpe & Power, 1999; Smith & Mueller, 2015). This phenomenon may operate via at least two pathways. First, some beneficial sexually-transmitted bacteria may produce metabolites necessary for the production of secondary sexual traits that reflect the quality of the host. Being a carrier of such bacteria would thus lead to an increased number of partners and matings (Smith & Mueller, 2015). Second, the successful transmission of beneficial reproductive bacteria may rely on the number of matings, with more frequently mating individuals more likely to acquire the beneficial strain (Lombardo, Thorpe & Power, 1999). Beneficial sexually-transmitted bacteria could thus bias the host population towards polygamy to promote the spread of mutualistic bacteria (Smith & Mueller, 2015). Thus, the structure of the host socio-sexual group and the mating system of a population may develop to reflect the selective pressures generated by the reproductive microbiota of the hosts. A study examining four Australian avian species of different mating systems found that monogamous bird hosts harboured lower bacterial diversity on their cloacae that may lower their risk of STIs in comparison with polygamous birds (Poiani & Gwozdz, 2002). In monogamous systems, mating partners are expected to become increasingly more similar to each other in their reproductive microbiota, while significantly differing from other breeding pairs (Rowe et al., 2020a). Such pattern has been reported in the socially- and genetically-monogamous black-legged kittiwakes (White et al., 2010). Polygamous hosts are expected to converge on a shared, population-wide reproductive microbiota, as has been found in the socially-monogamous, but genetically-polygamous North American tree swallows (*Tachycineta bicolor*; Hernandez et al., 2020).

Finally, reproductive microbiota may also play a role in speciation (Shropshire & Bordenstein, 2016). It is possible that an increased local pathogen abundance may lead to

extreme avoidance behaviour between the infected and uninfected hosts, potentially resulting in reproductive isolation and speciation. Similarly, host-specific commensal reproductive bacteria may orchestrate a speciation event. For example, commensal bacteria are hypothesised to affect the fruit fly sex pheromones via modulating their cuticular hydrocarbons (Ringo, Sharon & Segal, 2011). Fruit flies with different sex pheromone profiles may start mating assortatively, leading to sexual isolation and potentially speciation between the non-mating groups (Ringo, Sharon & Segal, 2011). Considering that reproductive bacteria have been shown to affect *in vivo* (Amato et al., 2020) and *in vitro* (Schoenmakers & Laven, 2020) fertilisation success in humans, it is not unlikely that some reproductive microbiota are too incompatible to allow sperm-egg fusion, leading to reproductive isolation and speciation.

Combined, theory and empirical work discussed above suggest the potential for reproductive microbiota to play a fundamental role in the evolutionary ecology of hosts. At present, however, empirical evidence for the role of reproductive bacteria in host evolution and ecology is largely lacking.

*The Thesis and the Study System: the Polygynandrous Red Junglefowl (*Gallus gallus*)*

The overall objective of my thesis is to investigate the ecology of reproductive tract microbiota, and explore patterns of intra- and inter-individual variation in a polygynandrous avian host, the red junglefowl, *Gallus gallus*.

The red junglefowl is the main wild ancestor of the domestic chicken (*Gallus domesticus*; Kanginakudru et al., 2008; Lawal et al., 2020; Wang et al., 2020b). Red junglefowl form multi-male, multi-female groups structured in sex-specific hierarchies and characterised by a polygynandrous mating system, where both sexes have the opportunity to mate with multiple partners. Mating success varies naturally between individual birds (Collias & Collias, 1996; Kim & Zuk, 2000), creating opportunity for pre- and post-copulatory episodes of sexual

selection. The fowl (i.e. red junglefowl and the related domestic chicken) have been established as a sexual selection model system (Pizzari & McDonald, 2019).

To illustrate, female fowl mate with multiple males during the breeding season (Ligon & Zwartjes, 1995) and show strong pre-copulatory mate choice for healthy, younger, and more dominant and aggressive males (Zuk et al., 1990a, 1990b, 1990c; McDonald et al., 2017). Female birds have also been found to have sperm storage and selection mechanisms for post-copulatory cryptic female choice (Pizzari & Birkhead, 2000; Dean, Nakagawa & Pizzari, 2011; Løvlie et al., 2013).

Male red junglefowl engage in pre-copulatory male-male competition (Ligon et al., 1990; Zuk et al., 1990c) and exhibit different personality traits that affect their mating success (Roth, 2019). Sperm competition outcome in fowl is predominantly determined by the relative number of inseminated sperm (Birkhead et al., 1999; Pizzari et al., 2008), and males that mate repeatedly with the same female tend to fertilise a larger share of the eggs (Collet et al., 2012; McDonald et al., 2017; Pizzari & McDonald, 2019). Male fowl have also been reported to alter their sperm number (Pizzari et al., 2003) and seminal fluid volume (Cornwallis & O'Connor, 2009) to increase their fertilisation success in post-copulatory sperm competition.

Previous research described in Eccleston (2017) has established that the ejaculates of red junglefowl harbour a culturable microbiota that varies across individual males. Artificial insemination experiments showed that these ejaculate-associated bacteria have the potential to disturb the cloacal microbiota of female red junglefowl. However, questions remain whether female and male red junglefowl also harbour bacteria in their internal reproductive tracts, and what are the dynamics of sexual bacterial transmission in socio-sexual groups of polygynandrous fowl.

For my thesis, I studied a captive red junglefowl population at the John Krebs Field Station of the University of Oxford in Wytham, which was established in 2006. The population

is kept in outdoor aviaries and habituated to humans, allowing me to conduct standardised experiments under controlled conditions. Additionally, previous work provides a wealth of information on different aspects of the biology of this population, such as patterns of behavioural variation, mechanisms of sexual selection, and mating strategies. To illustrate, intra-sexual competition (Carleial, McDonald & Pizzari, 2020), age (McDonald et al., 2017), relatedness (Pizzari, Løvlie & Cornwallis, 2004; Tan et al., 2017), MHC-profiles (Løvlie et al., 2013), personality profiles (Roth, 2019), seminal proteomics (Borziak et al., 2016), and socio-sexual interactions (McDonald et al., 2013) of individual birds have been tracked and studied for years. This information enabled me to better contextualise my work and investigate the relationship between reproductive microbiota and host phenotype, behaviour, and socio-sexual interactions.

The Outline and the Specific Aims of the Thesis

My thesis comprises four integrated data chapters and aims to explore the ecology of reproductive tract microbiota of the polygynandrous red junglefowl, accounting for intra- and inter-individual variation. The thesis combines theory and empirical evidence from the fields of microbial ecology and evolutionary biology, and relies on a multitude of technical skills, including fieldwork, laboratory NGS-processing, bioinformatics, and performing statistical analysis by borrowing tools from ecological mixed-effects modelling and social network analysis among others.

First, I characterise the reproductive tract microbiota of female (**Chapter II**) and male fowl (**Chapter III**) in detail, establishing a point of comparison for the experimental chapters to follow. Then, I study the microbiota present in fowl ejaculates, and how that varies across male age, over the course of breeding season, and across a sequence of matings (**Chapter IV**).

Finally, I investigate the role of mating interactions and individual variation in fowl cloacal microbiota composition (**Chapter V**).

More specifically, in **Chapter II**, I set out to explore the bacterial communities across the female fowl oviduct. Using 16S SSU rRNA gene NGS, I characterise the microbiota present in lavage and tissue samples collected from functionally-distinct sections of the female fowl reproductive tract. The two sampling methods allow me to distinguish between bacteria that are specialised to inhabiting reproductive tract fluids and bacteria that are structurally-bound to reproductive tissues. Previous studies suggest that different bacterial strains are found in tissue and lumen samples (Mao et al., 2015; Mitra et al., 2017).

In **Chapter III**, I aim to characterise the microbiota along the continuum of the male red junglefowl reproductive tract by collecting tissue samples and using largely the same methodology as in **Chapter II** to provide qualitatively comparable results. Studies in humans have shown that despite sharing a considerable amount of bacterial genera, female and male reproductive tracts harbour divergent bacterial communities that decrease in biomass in the upper organs of the tracts (Mändar, 2013; Chen et al., 2017). Both **Chapter II** and **Chapter III** serve a role in gaining a better understanding of reproductive microbiota in non-human systems, and provide me with an overview of fowl reproductive tract bacterial ecology, allowing me to compare other samples, such as ejaculates and cloacal wipes, with the entire female and male red junglefowl reproductive tract microbiota.

Ejaculate characteristics vary with male fowl age, during a breeding season, and across successive mating opportunities, and in **Chapter IV**, I investigate whether similar patterns of variation are also present in the ejaculate bacterial assemblages of red junglefowl. First, I establish whether ejaculate microbiota differs from the bacterial compositions found in female (**Chapter II**) and male fowl reproductive tracts (**Chapter III**). Then, I explore ejaculate microbiota variation associated with male age. Older male fowl produce fewer sperm and have

a different seminal fluid proteome compared with younger males (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016), and this variation could also affect ejaculate microbiota. Finally, I investigate the bacterial richness, diversity, composition, and load variation in ejaculates collected over the course of breeding season and across a sequence of matings. Avian cloacal (Escallón, Belden & Moore, 2019; Kreisinger et al., 2015) and faecal microbiota (Lewis, Moore & Wang, 2016) show seasonal variation, but temporal variation in ejaculate microbiota is currently understudied. In addition, I explore the covariation between sperm velocity/number, and ejaculate bacterial load/richness. Ejaculate-associated bacteria are generally expected to have a negative effect on sperm cells (Diemer et al., 1996; Hosseinzadeh, Pacey & Eley, 2003; Fraczek et al., 2007).

After establishing what bacteria inhabit the female and male fowl reproductive tracts, and how they compare to the bacteria found in red junglefowl ejaculates, I explore what happens when the two reproductive microbiota come in direct contact. More specifically, in **Chapter V**, I aim to understand the role of mating interactions and individual phenotypic variation in cloacal microbiota assembly. I do this by collecting cloacal wipe samples from non-virgin birds before joining and after exiting experimental groups of different sex ratios, where their mating interactions are monitored over the course of six days. By incorporating social network analysis and prior knowledge of fowl age and personality profiles, I explore the potential for sexual bacterial transmission in this particular host species. Having multiple mating partners has been shown to diversify the reproductive microbiota of wild vertebrates (Poiani & Gwozdz, 2002; MacManes, 2011; White et al., 2011), and intensified social interactions between two conspecifics have been reported to homogenise their gut microbiota (Tung et al., 2015; Perofsky et al., 2017; Raulo et al., 2018). I investigate whether the same patterns hold true for cloacal microbiota in fowl socio-sexual networks. Combined, my thesis

provides a comprehensive, experimentally-standardised survey of reproductive tract bacteria in the polygynandrous red junglefowl.

References

Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

Chapter II. The Female Reproductive Tract Microbiota of the Polyandrous Red Junglefowl (*Gallus gallus*) is Ecologically Distinct and Spatially Structured

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Bacteria associated with the female reproductive tract (FRT) may have major implications for host health and fitness. For example, imbalance of FRT microbiota can cause severe inflammation and negatively affect female reproductive success. Species where both females and males mate with multiple partners (polygynandry) are especially vulnerable to the detrimental effects of sexually-transmitted pathogens due to the increased sexual transmission of bacteria between multiple mating partners. Surprisingly little, however, is known about the ecology of FRT microbiota in polygynandrous species. To fill this gap in knowledge, we studied the FRT microbiota of the polygynandrous red junglefowl, *Gallus gallus*—the main wild ancestor of the domestic chicken, *Gallus domesticus*—by collecting lavage and tissue samples to characterise bacterial communities across different sections of the FRT and controls from the digestive tract. We found that the microbiota of fowl FRT is distinct from that of the digestive tract, with the microbiota of the cloaca, where both tracts converge, harbouring an intermediate bacterial community. Analysis of tissue samples revealed spatial structure within the FRT, with total ASV richness and bacterial diversity decreasing in the upper FRT. Lavage samples, on the other hand, showed relatively uniform bacterial richness and diversity across the FRT, suggesting that fowl FRT may support both locally-structured, tissue-dwelling bacteria and more homogenous, excretion-bound bacteria. Additionally, individual phenotype was found to affect female red junglefowl microbiota, with females that suffered from cloacal discharge and those with a regressed ovary (associated with the non-breeding status) exhibiting lower overall total ASV richness and bacterial diversity. Combined, our results provide a novel characterisation of the avian FRT microbiota, starting a discussion on the role of reproductive tract bacteria in polygamous female host health and fitness.

Introduction

Increasing evidence indicates that host fitness is dependent on a "healthy" microbiota. To illustrate, studies in humans have revealed that the gut microbiota is dominated by commensal bacteria that play a key role in host metabolism and immune functioning (Hooper & Gordon, 2001). Mutualistic bacteria—such as the genus *Bifidobacterium* (phylum Actinobacteria)—protect human hosts from adverse bacterial invasions, while pathogenic bacteria—such as some serovars of *Escherichia coli* (phylum Proteobacteria)—are known agents in lethal gut infections (Kamada et al., 2013). Other potential host ecosystems—for example, the female reproductive tract (FRT) that has been shown to support different bacteria than the digestive tract in humans (Lozupone et al., 2013; Chen et al., 2017)—have received less attention.

While there are limited studies of non-human animal host reproductive tract microbiota, female human reproductive bacteria have already been shown to significantly affect host health. For example, the genus *Lactobacillus* (phylum Firmicutes) is dominant in "healthy" human vaginas, stabilising the environment and protecting the host against pathogenic bacteria (Nardini et al., 2016), HIV (Aldunate et al., 2013), pre-term birth (Hyman et al., 2014), and infertility (Borovkova et al., 2011). In turn, the human vaginal canal promotes the proliferation of *Lactobacillus* (Spear et al., 2014), suggesting that female humans may have evolved to support this interdependence with beneficial bacteria. On the other hand, detrimental bacteria may cause inflammation in the FRT and partake in the development of gynaecological cancers (Walther-António et al., 2016; Łaniewski, Ilhan & Herbst-Kralovetz, 2020).

Additionally, the FRT is of special interest, as it offers scope for both horizontal bacterial transmission between sexually-interacting partners—resulting in a shared seminovaginal microbiota in humans (Mändar et al., 2015) and other internally-fertilising species—and also vertical bacterial transfer along the mother-offspring axis. The latter

phenomenon was first demonstrated in humans, showing that vaginally-delivered newborns, but not infants born by Caesarean section, harboured gut bacterial communities that were similar to their mother's vaginal microbiota (Dominguez-Bello et al., 2010). Evidence for cross-generational bacterial transmission has also been shown in mice (*Mus musculus*) where most gut bacteria are in fact vertically-, and not horizontally-acquired (Moeller et al., 2018).

Maintenance of reproductive microbiota is thus of particular importance in females, especially as the FRT is susceptible to bacterial perturbations associated with menstruation, intromission, insemination, fertilisation, gestation, and birth or oviposition (Gajer et al., 2012; Mändar et al., 2015; Petrova et al., 2015; Freitas et al., 2017; Amato et al., 2020). Indeed, evidence in humans (Borovkova et al., 2011), birds (Kulkarni & Heeb, 2007; White et al., 2010), and bed bugs (Bellinvia et al., 2020a) suggests that mating has the potential to increase bacterial diversity in the FRT. Furthermore, promiscuous female deer mice (*Peromyscus maniculatus*; MacManes, 2011), polygamous female viviparous lizards (*Zootoca vivipara*; White et al., 2011), and female humans with multiple partners (Jespers et al., 2015) have been found to harbour a more diverse reproductive microbiota than less promiscuous females, suggesting that females that mate with multiple partners (polyandry) may be more at risk of contracting an STI (Ashby & Gupta, 2013). Female hosts, in particular polyandrous females, would thus benefit from both physiological and behavioural reproductive tract microbiota maintenance—for example by avoiding partners that transmit detrimental bacteria, while preferentially-mating with "healthy" partners—allowing hypotheses about the FRT microbiota in the context of host fitness, sexual selection, and sexual conflict (Rowe et al., 2020a).

While polyandrous host species characterised by intense sexual selection lend themselves as interesting model systems, few attempts have been made to explore their FRT microbiota. To fill this gap in knowledge, we set out to characterise the FRT microbiota of a polygynandrous bird species, the red junglefowl, *Gallus gallus*. Since Darwin, birds have been

long-established study systems in pre-copulatory sexual selection research (Darwin, 1871; Andersson, 1994), and with the increasing accessibility of molecular tools, they have also become models to study post-copulatory sexual selection (Birkhead & Møller, 1998; Firman et al., 2017). With the advent of mass sequencing, questions were raised about avian microbiota, and its potential role in host reproduction and evolution (Sheldon, 1993).

Our study system, the red junglefowl—the main wild ancestor of the domestic chicken (*Gallus domesticus*; Kanginakudru et al., 2008; Lawal et al., 2020; Wang et al., 2020b)—lives in groups that exhibit dynamic hierarchical social structure (Collias & Collias, 1996; Kim & Zuk, 2000). Female fowl are coerced to copulate with multiple males during the breeding season (Ligon & Zwartjes, 1995), and frequent matings with multiple partners could increase the reproductive bacterial diversity in females (Westneat & Rambo, 2000; Poiani & Gwozdz, 2002; Kulkarni & Heeb, 2007; White et al., 2010; Eccleston, 2017), putting them at risk of contracting sexually-transmitted infections (STIs), which could affect both female and offspring health (Keller et al., 1995; De Buck, et al., 2004). The fitness costs of polyandry may be buffered by females via imposing intense pre- and post-copulatory sexual selection on males (Pizzari & McDonald, 2019). For example, female red junglefowl were found to reject the advances of males with a smaller comb—a known male trait under female pre-copulatory sexual selection (Zuk et al., 1990c)—, which also served as a secondary sexual trait indicating higher intestinal nematode load (Zuk et al., 1990b). Therefore, by performing pre-copulatory sexual selection, female fowl can avoid increased pathogen loads. Females also have mechanisms for post-copulatory sexual selection (Pizzari & McDonald, 2019), and exhibit prolonged sperm storage and practise cryptic female choice, by for example, preferring the ejaculates of healthy, young males that have a different major histocompatibility complex (MHC) than their own (Løvlie et al., 2013; McDonald et al., 2017). Whether reproductive bacteria partake in cryptic female choice remains currently unknown. Finally, ageing female

fowl become less polyandrous (McDonald et al., 2019), which could in turn affect their FRT microbiota.

While preliminary studies of both avian host evolutionary mechanisms and microbiota have laid an excellent foundation, we had three specific aims to provide a more comprehensive understanding of the FRT microbiota by investigating the bacteria present across the oviduct of the polygynandrous red junglefowl, including patterns of intra- and inter-individual variation. First, we characterised the red junglefowl FRT and cloacal microbiota composition, and compared it with digestive tract microbiota. In humans, for example, vaginal microbiota is distinct from faecal and skin microbiota (Lozupone et al., 2013), but whether that is the case across taxa remains unknown. To further complicate the matters, avian reproductive, digestive, and urinary tracts are connected via a shared outlet, the cloaca (Figure 1). This overlap could lead to shared bacterial strains, despite the different functions of the tracts. As the effect of digestive tract microbiota on avian FRT and cloacal bacterial assemblages (and *vice versa*) was previously unknown, we decided to harvest comparative samples from the colon and faeces. Second, we explored the patterns of spatial structure within FRT microbiota. A study in humans showed that bacteria are present across the FRT, with organ-specific microbiota providing important functions in the host, such as vaginal lactobacilli protecting the female from opportunistic bacterial invasions (Chen et al., 2017). However, there may also be spatial structural differences within the same functionally-distinct sections of the tract. For example, a study in dairy cattle (*Bos taurus*) reported a difference between gastrointestinal tract lumen and tissue bacterial compositions (Mao et al., 2015). We therefore decided to use two sampling methods (lavage and tissue samples) across the functionally-distinct FRT sections to test whether similar patterns hold true in female fowl reproductive tracts. Third and finally, we investigated the effect of individual variation in health status, reproductive status, and age class on FRT microbiota.

We hypothesised that the female red junglefowl reproductive and digestive tract differ in their bacterial communities due to functional differences, while the shared outlet cloaca harbours an intermediate microbiota between the two tracts. We expected that there is a longitudinal decrease in bacterial richness and diversity ascending the oviduct, as is the case with avian gastrointestinal tract microbiota (Drovetski et al., 2018), with lavage and tissue sampling methods capturing different bacterial strains. Previous studies have shown that human vaginal microbiota is affected by host race (Ravel et al., 2011), menstruation (Gajer et al., 2012), age (Muhleisen & Herbst-Kralovetz, 2016), and mating partners (Muzny et al., 2013) among others. Similarly, avian cloacal microbiota has been found to be affected by host health (Benskin et al., 2009), breeding season (Escallón, Belden & Moore, 2019), and age (van Dongen et al., 2013), suggesting that individual phenotype may also influence the FRT microbiota. We thus hypothesised that individual variation—health status in particular—would affect fowl FRT microbiota, and expected to find known avian pathogens, such as *Shigella* (phylum Proteobacteria; Shi et al., 2014) and/or *Salmonella* (phylum Proteobacteria; Keller et al., 1995; De Buck et al., 2004), in symptomatic females.

Materials and Methods

Study Population

The captive red junglefowl population at the John Krebs Field Station of the University of Oxford in Wytham was used for FRT dissections (Figure 1). Prior to humane culling by Schedule 1 (Home Office, 2014), the experimental birds (n=27) were kept in single-sex flocks and housed in covered outdoor aviaries, where they were given food and water *ad libitum*.

All female fowl were sexually-mature. Some birds were symptomatic with white cloacal discharge that remained undiagnosed by veterinarians. We decided to account for this in our study, as the symptoms could have been caused by a bacterial infection. We therefore

classified birds based on their health status, resulting in eight "healthy" and 19 "symptomatic" females. Dissections of the oviduct revealed that 14 of the symptomatic birds also had necrotic tissue in their isthmus (Figure 1).

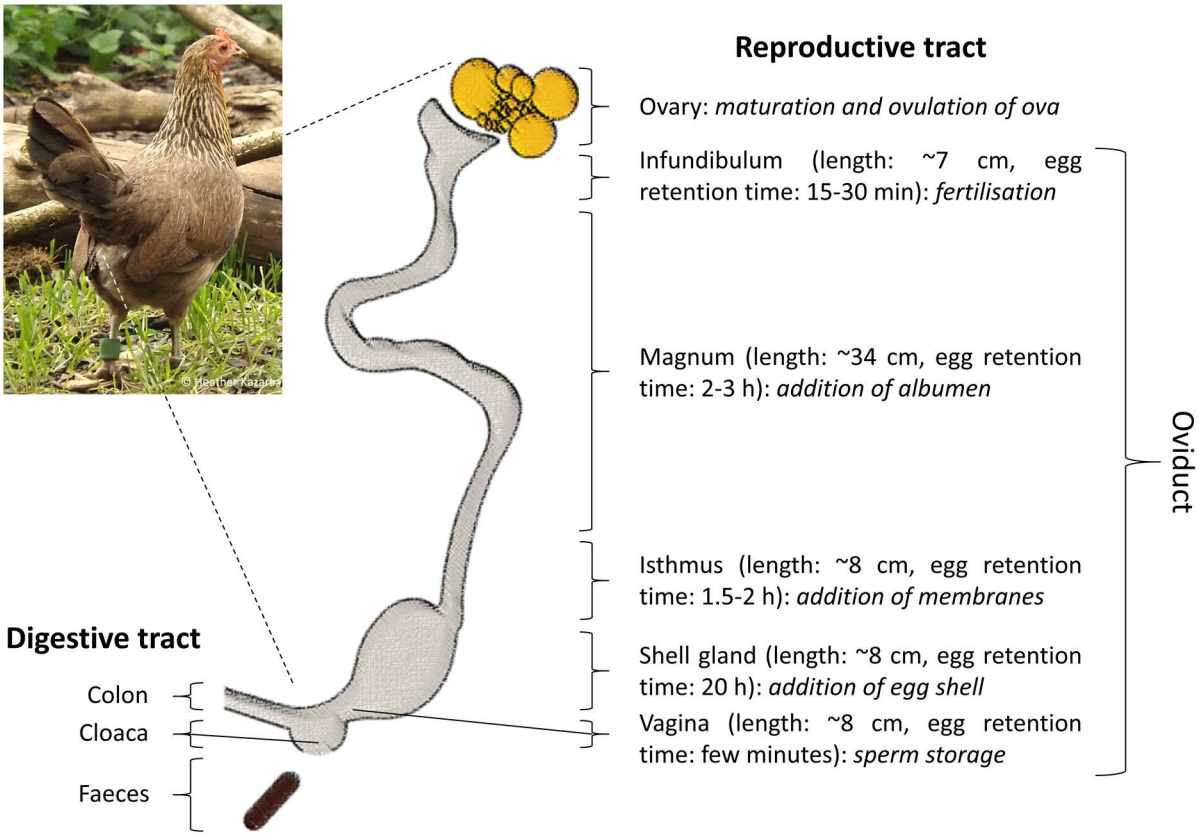


Figure 1. The developed ovary and oviduct of a female red junglefowl. Each FRT section is indicated with its relative size, egg retention time, and *function* (Sasanami, 2017). The cloaca—the shared outlet—was analysed separately from the FRT and digestive tract, as it was expected to harbour a mixed bacterial community. All indicated sections, except for the ovary, were sampled and analysed in this chapter. The diagram of the ovary and oviduct was drawn based on a photo by John Anderson, and the photo of a female red junglefowl in Wytham was taken by Dr Heather Kazara and is used with permission.

Ovary and oviduct of female fowl change in size between the breeding and non-breeding season. As the dissections were carried out at the end of the breeding season, some birds had a developed ovary, while others had transitioned to the regressed status of non-breeding season. We therefore categorised birds based on their reproductive status, resulting in six female fowl with a "developed ovary" and 21 with a "regressed ovary".

Individual birds wore unique Darvic rings that were used for identification and determining bird age class. The female fowl were categorised into two age classes, including "young" (1–3 years) and "old" (4–9 years) birds, based on a previous study on a feral population of domestic chickens, suggesting that female reproductive performance (fecundity and egg size) peaks at the age of 4, and starts declining after that (Cornwallis, Dean & Pizzari, 2014). Our study included 18 young and nine old females. All experimental work was conducted under the regulation of a Home Office project license (PIL: P50402706).

Dissections in the Field

After the breeding season of 2015, 27 female red junglefowl were sacrificed over three culling sessions (experimental period: 15 and 29 October, 5 November). The experimental fowl were immediately dissected by three members of the Pizzari Group. Two experimenters removed the female oviducts from the carcasses, and one experimenter cut the oviduct into functionally-distinct FRT sections (Figure 1).

The dissecting team handled the female fowl practising clean laboratory etiquette to minimise contaminating the samples (Salter et al., 2014). The experimenters wore laboratory coats and changed nitrile gloves between carcasses. Dissection equipment was sterilised with 70% ethanol and 1% Virkon® (DuPont, Wilmington, Delaware, USA) between each bird dissection. All dissections were carried out on ethanol-sterilised boards covered with plastic pouches, which were changed between carcasses.

Fowl oviducts were removed in their entirety and cut into six functionally-distinct sections: the cloaca, vagina, shell gland, isthmus, magnum, and infundibulum (Table 1; Figure 1). First, each organ was internally washed with sterile Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, St. Louis, Missouri, USA) to collect a lavage sample. Then, the internal walls of the organs were harvested for tissue samples. The weight of tissue samples was not

standardised during collection due to time constraints and increased contamination risk in the field. However, the tissue collection site per functionally-distinct section was kept relatively constant across carcasses by eyeing the reproductive tracts. Comparative digestive tract lavage and tissue samples were collected from the colon, and a faecal sample was stored per bird when possible (Table 1; Figure 1). Samples were kept on dry ice in individual, sterile tubes until they were frozen at -80°C approximately 2–3 hours after the dissections.

Table 1. Study sample size (n=356). Lavage samples are indicated by lilac, tissue samples by lime green, and faecal samples by peach. Samples were collected under time constraint to minimise potential environmental contamination. Therefore, not all culled birds were harvested for lavage, tissue, and faecal samples. Lavage samples (n=189) were harvested from 27 birds**,** tissue samples (n=147) from 21 birds, and faecal samples (n=20) from 20 birds. 14 birds were harvested for a full sample set of lavage, tissue, and faecal samples.

Faeces	Colon	Cloaca	Vagina	Shell gland	Isthmus	Magnum	Infundibulum
n=20	n=27	n=27	n=27	n=27	n=27	n=27	n=27
	n=21	n=21	n=21	n=21	n=21	n=21	n=21

Tissue Sample Preparation in the Laboratory

Post-fieldwork laboratory processing was carried out in 2017–2018 at Silwood Park, Imperial College London. We performed additional tissue sample handling to standardise sample weights across the data set and minimise inhibitors—in particular host DNA—in downstream laboratory processes. Preliminary experiments showed that including deeper layers of the epithelium resulted in non-specific and/or failed PCR amplification potentially due to the increased input of host cells, thus introducing inhibiting host DNA that we hypothesised caused the DNA amplification error. The secondary dissections were carried out on ice and in a biological safety cabinet to protect the samples from degradation and contamination. Individual, sterile Petri dishes, scalpels, and forceps were used per tissue sample. The uppermost epithelial layer inside each section was scraped off under a microscope, collecting a standardised ~10 mg tissue sample per functionally-distinct section.

DNA Extraction and Quantification

DNA from lavage and tissue samples was extracted with an unopened QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany). Sample handling was carried out with sterile equipment in a pre-PCR biological safety cabinet. Working surfaces were sterilised with DNAZap™ PCR DNA Degradation Solutions (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 70% ethanol.

Lavage samples were processed according to the manufacturer's protocol for blood samples and tissue samples with the manufacturer's tissue protocol. Prior to lysis, samples were handled on ice to minimise compromising their bacterial compositions. To increase yield from Gram-positive bacteria (Gill et al., 2016), we used the manufacturer's protocol with an additional lysis step. Lysozyme from chicken egg white (20 mg/ml; Sigma-Aldrich) was mixed with the ATL Buffer and the samples were incubated at 37°C for 30 minutes, after which the manufacturer's protocol proceeded with the Proteinase K step. The second incubation step at 56°C was carried out for an hour, which was enough to disintegrate all biological matter.

DNA from faecal samples was extracted with an opened MO BIO PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California, USA; now sold by QIAGEN as the DNeasy® PowerSoil Kit), following the manufacturer's protocol. We had to opt for another DNA extraction kit for the faecal samples as preliminary experiments revealed that the DNA concentration and PCR amplification were low and not comparable with the rest of the biological samples when using the QIAGEN DNeasy® Blood & Tissue Kit with faeces.

Controls from both DNA extraction kits were collected for downstream contamination analysis (QIAGEN DNeasy® Blood & Tissue DNA extraction kit, n=6 (at least one per four 96-well plates); and MO BIO PowerLyzer PowerSoil faecal DNA extraction kit, n=1). All samples were quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific)

with a Qubit® Fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol. All samples were diluted to a standardised DNA concentration (0.5 ng/µl for lavage samples and 5 ng/µl for tissue samples) and stored at -20°C.

Amplification Polymerase Chain Reactions (PCRs)

Before and after each set-up, the PCR hood was cleaned with DNAZap™ PCR DNA Degradation Solutions and 70% ethanol, followed by 30 minutes of UV-sterilisation. The consumables were UV-sterilised separately for 30 minutes. PCRs were set up on ice with sterile, filtered pipette tips and reagents taken from unopened aliquots.

The V4 region (~254 base pairs) of the bacterial 16S SSU rRNA gene was amplified, following the guidelines of the Earth Microbiome Project (Thompson et al., 2017) and Walters et al. (2015). Reactions were prepared on 96-well plates, and in three replicates to minimise amplification bias and increase yield from rare bacterial strains. 5 ng or up to 5 µl (if the DNA template concentration was too low) of DNA was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus* (Sigma-Aldrich), 5 µl of *Taq* DNA Polymerase PCR Buffer 10X (Sigma-Aldrich), 0.5 µl of Bovine Serum Albumin (Sigma-Aldrich), 1 µl of 10 nM dNTP mix (Thermo Fisher Scientific), 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) to make up the rest of the volume.

A PCR negative control per 96-well plate was prepared and sealed before template DNA entered the PCR hood. A positive standard, ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, California, USA) was added to each set-up to assess amplification success.

Parameters for the amplification PCRs were as follows: initial three-minute denaturing at 94°C followed by 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all replicate plates.

Amplification PCR products were tested on a 1% agarose gel, stained with GelRed™ (Biotium, Fremont, California, USA) according to the manufacturer's protocol, in a 1×TBE buffer at 70 volts for 45 minutes.

We detected some amplification product in PCR negative controls (Supplementary Gel Images 1A). We therefore prepared additional PCR negative controls independent of the 96-well plate set-up, following the same protocol and using the exact same PCR solution, but amplified for 30 cycles. These PCR negatives were clean (Supplementary Gel Images 1A & B) and two of them were included in downstream contamination analysis, resulting in two 30-cycle amplification PCR controls and 12 35-cycle amplification PCR controls. We decided to continue with the 35-cycle-protocol, however, as 30 cycles was not enough to amplify DNA from our biological samples (Supplementary Gel Images 1B & C).

Additional pipeline optimising experiments revealed that the contamination came from *Taq* DNA Polymerase from *Thermus aquaticus* (Supplementary Gel Images 1D), which was added to the PCR solution from previously unopened aliquots, suggesting the contamination potentially originated from manufacturing.

Clean-Up, Indexing PCRs, and Normalisation

Replicate biological samples were pooled and cleaned of primer dimers using the ZR-96 DNA Clean-up Kit™ (Zymo Research), following the manufacturer's protocol. A clean-up kit control was taken for downstream contamination analysis.

The samples were dual-indexed with the unique combinations proposed by Kozich et al. (2013). 1 µl of amplification PCR product was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus*, 5 µl of *Taq* DNA Polymerase PCR Buffer 10X, 1 µl of 10 nM dNTP mix, 0.125 µM (final concentration) of forward and reverse primer (Supplementary Table 1) each, and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the volume.

An indexing PCR negative control was processed per 96-well plate (n=4). DNA extraction kit controls (QIAGEN DNeasy® Blood & Tissue DNA extraction kit, n=6; and MO BIO PowerLyzer PowerSoil faecal DNA extraction kit, n=1), amplification PCR negative controls (n=2+12), positive standards (n=2), and a clean-up kit control (ZR-96 DNA Clean-up Kit™, n=1) were indexed as biological samples.

Parameters for the indexing PCRs were as follows: initial two-minute denaturing at 95°C followed by eight cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all set-ups.

Indexing PCR products were tested on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 70 V for 45 minutes.

Samples were standardised according to the manufacturer's protocol of the SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific), and pooled in equal volumes per plate. The sequencing libraries were stored in DNA LoBind Tubes (Eppendorf, Hamburg, Germany) at -20°C. Immediately prior to sequencing, the libraries were quantified using the Qubit™ methodology.

Next-Generation Sequencing (NGS)

NGS was carried out at the sequencing facility at Silwood Park (Imperial College London). A 400 pM library and a 40 pM PhiX control (Illumina, Inc., San Diego, CA, USA) were prepared and denatured with 2 µl of 2 M NaOH (Sigma-Aldrich) for 5 minutes at room temperature, followed by immediate neutralising with 2 µl of 2 M HCl (Sigma-Aldrich). An 8 pM library with a 10% PhiX control was loaded into the V2 chemistry MiSeq cartridge (Illumina, Inc.) to achieve 2*150 base pair (bp) sequencing reads across 192 samples.

In total, two consecutive sequencing runs were needed to process the samples (356 biological and 28 control samples). A mix of lavage, tissue, and faecal samples was included in both runs.

Bioinformatic Data Processing

Sample demultiplexing was carried out automatically by BaseSpace (Illumina, Inc.). Paired-end FASTQ files were processed with *dada2* (Callahan et al., 2016a; Version 1.12.1) in R (R Core Team, 2019; Version 3.6.1). Reads were quality-checked and filtered using standard parameters (maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2). ~10% of sequencing reads were lost. The error rates were calculated based on a random subset of samples (randomize=TRUE). Sample inference was carried out via pseudo-pooling (pool="pseudo"). minOverlap=5 was used to obtain merged, denoised sequences. Only reads of 250–256 bp were retained, eliminating non-specific amplicons. Chimera check removed ~79% of the merged sequence variants, which accounted for ~7% of the total merged sequence reads. SILVA species assignment reference database (Yilmaz et al., 2014; Version 132) was used for exact taxonomic matching (100% identity) to the species level, when possible.

The processed data set with controls included 383 samples, 7886 amplicon sequence variants or ASVs (150±7 S.E. per sample), and 16,229,353 reads (42,374±1896 per sample).

The clean-up kit control (ZR-96 DNA Clean-up Kit™, n=1) failed to sequence and produce paired-end FASTQ files. The data set was further cleaned of potential contaminants, using *decontam* (Davis et al., 2017). Contaminants were detected based on ASV prevalence in control samples versus biological samples, using a stringency threshold of 0.58 that split ASVs into contaminants (586 ASVs) and non-contaminants (7300 ASVs).

Additionally, biological samples with less than 100 sequencing reads were excluded (n=14). These were all lavage and tissue samples collected from bird K6 (Table 2). ASVs that were present in the taxonomy, but not in the data set due to data filtering (n=189) were excluded. Finally, ASVs that failed to classify at the phylum level (n=43), or that classified as chloroplasts (n=39) or mitochondria (n=11; de la Cuesta-Zuluaga & Escobar, 2016) were removed.

The final data set without controls included 342 biological samples (Table 2), 7018 ASVs (143±7 per sample), and 14,034,490 reads (41,037±1800 per sample). Mean relative bacterial abundances found in negative controls and positive standards were visualised with *ggplot2* (Wickham, 2016) and are presented in the appendices (Supplementary Figure 1).

Statistical Analysis and Data Visualisation

Statistical analysis and data visualisation were carried out in R (Version 3.6.1). Significance threshold was set to $p<0.05$ in all tests.

Hereafter, we define female red junglefowl reproductive tract (lavage and tissue samples harvested across the FRT; n=230), cloaca (cloaca lavage and tissue samples; n=46), and digestive tract (colon lavage and tissue samples, and faecal samples; n=66) as "host ecosystems"; we define lavage samples (all lavage samples harvested across FRT, cloaca, and digestive tract; n=182), tissue samples (all tissue samples harvested across FRT, cloaca, and digestive tract; n=140), and faecal samples (n=20) as "sampling methods"; and we define

vagina lavage, shell gland lavage, isthmus lavage, magnum lavage, infundibulum lavage, cloaca lavage, colon lavage, vagina tissue, shell gland tissue, isthmus tissue, magnum tissue, infundibulum tissue, cloaca tissue, colon tissue, and faeces as "sample type" (Table 2). We first tested higher-level variation across host ecosystems and sampling methods. Then, we compared the variation across specific sections and faeces by using sample type as a variable to even out sample sizes per group and avoid rank deficiency in statistical modelling.

Table 2. Final sample size (n=342) presented across sample type. Lavage samples are indicated by lilac, tissue samples by lime green, and faecal samples by peach. All lavage and tissue samples from bird K6 had too few sequencing reads to be statistically analysed. A faecal sample from bird K6 was included in the study. In the final data set, we had lavage samples (n=182) from 26 birds**,** tissue samples (n=140) from 20 birds, and faecal samples (n=20) from 20 birds. 13 birds had a full data set of lavage, tissue, and faecal samples.

Faeces	Colon	Cloaca	Vagina	Shell gland	Isthmus	Magnum	Infundibulum
n=20	n=26	n=26	n=26	n=26	n=26	n=26	n=26
	n=20	n=20	n=20	n=20	n=20	n=20	n=20

We decided *a priori* the order of health status ($n_{healthy}$=103 and $n_{symptomatic}$=239) > reproductive status ($n_{developed\ ovary}$=54 and $n_{regressed\ ovary}$=288) > age class (n_{young}=224 and n_{old}=118) in sequential statistical models, such as PERMANOVA, based on their predicted effect on female fowl microbiota.

(1) *Characterising bacterial compositions of functionally-distinct FRT sections*. FRT, cloacal, and digestive tract microbiota composition variation was analysed using β-diversity modelling, which allowed us to compare local bacterial diversity to regional bacterial diversity (Whittaker, 1960). *DECIPHER* (Wright, 2016) and *phangorn* (Schliep, 2011; Version 2.5.5) were used to align and create a phylogenetic tree respectively, as proposed by Callahan et al. (2016b). Weighted (wUF) and unweighted UniFrac (uwUF) distances (Lozupone & Knight, 2005) were calculated with *phyloseq* (McMurdie & Holmes, 2013), using the trimmed data set

(singletons—defined as ASVs present only once across all samples—were removed: 513 ASVs, i.e. 7.3% of the ASVs) that was first agglomerated to either bacterial phylum or genus level, and then normalised via proportions (McMurdie & Holmes, 2014; Callahan et al., 2016b; McKnight et al., 2019). Using the same normalised-by-proportions data set, we also calculated Bray-Curtis dissimilarities and Jaccard (dissimilarity) indices at both the phylum and genus level with *vegan* (Oksanen et al., 2019) for comparative β-diversity analysis.

We did not study bacterial strain-level variation as genus was the finest phylogenetic level that allowed us to assign a taxonomic identity to the majority of our sequencing reads (4804 out of 7018 ASVs, i.e. 68.5%, were assigned a genus identity). This was due to the current bacterial strain databases having limited information about non-human host microbiota, and because of the poor species- and strain-assignment potential of 16S SSU rRNA gene hypervariable region V4 (Gohl et al., 2016; Johnson et al., 2019b). Preliminary statistical tests revealed that strain-level β-diversity analyses with both ANOSIM and PERMANOVA had near-identical results with the genus-level β-diversity analyses, with the strain-level analyses showing slightly lower p-values that did not affect the significance patterns that we were able to observe at the genus-level. This assured us to proceed with our approach, knowing that our results at the phylum and genus level are biologically relevant, and allowing us to pinpoint specific key taxa that contribute to the variation.

Microbiota composition variation between host ecosystems ($n_{\text{reproductive tract}}$=230, n_{cloaca}=46, or $n_{\text{digestive tract}}$=66) and sampling methods ($n_{\text{lavage sample}}$=182, $n_{\text{tissue sample}}$=140, or $n_{\text{faecal sample}}$=20) was tested using separate ANOSIM models (anosim(); permutations=1000) from vegan, with the permutations constrained within strata=bird ID (n=27).

To compare microbiota composition variation between sample type, PERMANOVA (adonis(); permutations=10,000) from *vegan* was performed, setting sample type ($n_{\text{vagina lavage}}$=26, $n_{\text{shell gland lavage}}$=26, $n_{\text{isthmus lavage}}$=26, $n_{\text{magnum lavage}}$=26, $n_{\text{infundibulum lavage}}$=26, n_{cloaca}

$_{lavage}$=26, $n_{colon\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, $n_{cloaca\ tissue}$=20, $n_{colon\ tissue}$=20, or n_{faeces}=20), health status ($n_{healthy}$=103 or $n_{symptomatic}$=239), reproductive status ($n_{developed\ ovary}$=54 or $n_{regressed\ ovary}$=288), and age class (n_{young}=224 or n_{old}=118) as fixed effects, with the permutations constrained within strata=bird ID (n=27). β-diversity modelling based on Bray-Curtis dissimilarities explained the largest amount of variation at the genus level and revealed significant differences between sample type microbiota compositions (Table 3B). The significant factors of this PERMANOVA were followed up with beta dispersion tests (betadisper() followed by permutest(); permutations=1000) from *vegan* to control for significant dispersion differences.

Stacked bar plots of mean relative bacterial abundances were created with *ggplot2*. Bacterial phyla and genera that contributed <1% across sample type were considered rare and thus excluded from the figure. To visualise pairwise differences between sample type, a heatmap with average linkage hierarchical clustering was created with *Heatplus* (Ploner, 2019) based on genus-level Bray-Curtis dissimilarities.

Differential bacterial abundances across sample type were tested using *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was agglomerated to the phylum or genus level, filtered to include only taxa that contributed >1% to the mean relative abundances across sample type, and tested with differentialTest(test="Wald", fdr_cutoff=0.05), while controlling for bacterial abundance across health status, reproductive status, and age class, and variance across sample type, health status, reproductive status, and age class. Results were visualised with *corncob*.

(2) *Characterising bacterial richness and diversity of functionally-distinct FRT sections.* Spatial structure in fowl FRT microbiota was analysed with α-diversity modelling, which allowed us to explore local bacterial diversity (Whittaker, 1960). Three different α-diversity measures were calculated using the untrimmed, i.e. including singletons, data set.

Total ASV richness estimates were calculated with *breakaway* (Willis et al., 2018), and Shannon's and inverse Simpson's diversity index estimates with *phyloseq*. Normality of each α-diversity metric was assessed with a Shapiro-Wilk test, and suitable data distributions were determined from histograms, which were then confirmed with *fitdistrplus* (Delignette-Muller & Dutang, 2015), using Cullen and Frey graphs (bootstrap=500). Total ASV richness estimates and inverse Simpson's diversity index estimates followed a gamma distribution (link="log"). Shannon's diversity index estimates followed a Gaussian distribution (link="log").

α-diversity variation between host ecosystems ($n_{\text{reproductive tract}}$=230, n_{cloaca}=46, or $n_{\text{digestive tract}}$=66) and sampling methods ($n_{\text{lavage sample}}$=182, $n_{\text{tissue sample}}$=140, or $n_{\text{faecal sample}}$=20) was tested with separate models. Total ASV richness variation was compared using betta_random() from *breakaway*, with bird ID (n=27) as a random factor. Shannon's and inverse Simpson's diversity index estimates were compared with Kruskal-Wallis chi-squared tests. If Kruskal-Wallis chi-squared tests indicated significance, the groups were compared pairwise with Wilcoxon rank sum tests that were adjusted for false discovery rate (fdr).

Total ASV richness variation between sample type was statistically compared with betta_random() from *breakaway*, setting sample type ($n_{\text{vagina lavage}}$=26, $n_{\text{shell gland lavage}}$=26, $n_{\text{isthmus lavage}}$=26, $n_{\text{magnum lavage}}$=26, $n_{\text{infundibulum lavage}}$=26, $n_{\text{cloaca lavage}}$=26, $n_{\text{colon lavage}}$=26, $n_{\text{vagina tissue}}$=20, $n_{\text{shell gland tissue}}$=20, $n_{\text{isthmus tissue}}$=20, $n_{\text{magnum tissue}}$=20, $n_{\text{infundibulum tissue}}$=20, $n_{\text{cloaca tissue}}$=20, $n_{\text{colon tissue}}$=20, or n_{faeces}=20), health status (n_{healthy}=103 or $n_{\text{symptomatic}}$=239), reproductive status ($n_{\text{developed ovary}}$=54 or $n_{\text{regressed ovary}}$=288), and age class ($n_{\text{young}}$=224 or n_{old}=118) as fixed effects, and bird ID (n=27) as a random effect.

Generalised linear mixed-effects models (GLMMs) with Laplace approximation were fitted to Shannon's and inverse Simpson's diversity index estimates with *lme4* (Bates et al., 2015), setting sample type, health status, reproductive status, and age class as fixed effects, and bird ID as a random effect. Goodness of fit was assessed from Q-Q and residual plots. Function

Anova() from *car* (Fox & Weisberg, 2019) was used for deviance analyses (type II Wald chi-square tests). Specific trends and random effect statistics were obtained from model summaries. Total ASV richness was visualised with *breakaway*, and Shannon's and inverse Simpson's diversity index estimates with *ggpubr* (Kassambara, 2020).

(3) *Effect of individual phenotype on female fowl microbiota.* We first inferred the overall effect of health status, reproductive status, and age class on red junglefowl microbiota from β- and α-diversity modelling performed in aims (1) and (2) respectively, where the individual phenotype variables were included models to control for their effect.

As we were additionally interested in the effect of health status, reproductive status, and age class solely on the FRT microbiota, we excluded the digestive tract controls and created a reduced data set of only FRT and cloaca samples ($n_{vagina\ lavage}$=26, $n_{shell\ gland\ lavage}$=26, $n_{isthmus\ lavage}$=26, $n_{magnum\ lavage}$=26, $n_{infundibulum\ lavage}$=26, $n_{cloaca\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, or $n_{cloaca\ tissue}$=20). We decided to include the cloaca as it is a part of the FRT, and we were interested in pinpointing the potential bacterial infection in the symptomatic birds with white cloacal discharge. We explored differentially-abundant bacterial phyla and genera across individual phenotype variables using *corncob*, as in aim (1). We modelled differential bacterial abundances across health status ($n_{healthy}$=84 or $n_{symptomatic}$=192), reproductive status ($n_{developed\ ovary}$=42 or $n_{regressed\ ovary}$=234), and age class (n_{young}=181 or n_{old}=95) separately, while controlling for bacterial abundance across sample type and the two other individual phenotype variables (e.g. when testing for differentially-abundant bacteria in symptomatic vs healthy birds, we would control for differentially-abundant bacteria across fowl reproductive status and age class), and variance across sample type, health status, reproductive status, and age class. Results were visualised with *corncob*.

Results

(1) *Characterising Bacterial Compositions of Functionally-Distinct FRT Sections*

Female red junglefowl reproductive tract, cloaca, and digestive tract harboured significantly different microbiota (Figures 2A, 3A; Supplementary Table 2A). Based on mean relative abundances, the FRT (68.2±1.8% S.E) and cloaca (59.3±5.1%) harboured predominantly Proteobacteria, while the most common phylum in the digestive tract was Firmicutes (42.5±3.5%; Figure 2A). The FRT harboured a high relative abundance of *Escherichia/Shigella* (phylum Proteobacteria; 56.3±2.1%; distinguishing between the two genera is not possible based on the V4 hypervariable region), while female fowl digestive tract had an increased relative abundance of the genus *Fusobacterium* (phylum Fusobacteria; 25.1±3.3%; Figure 3A).

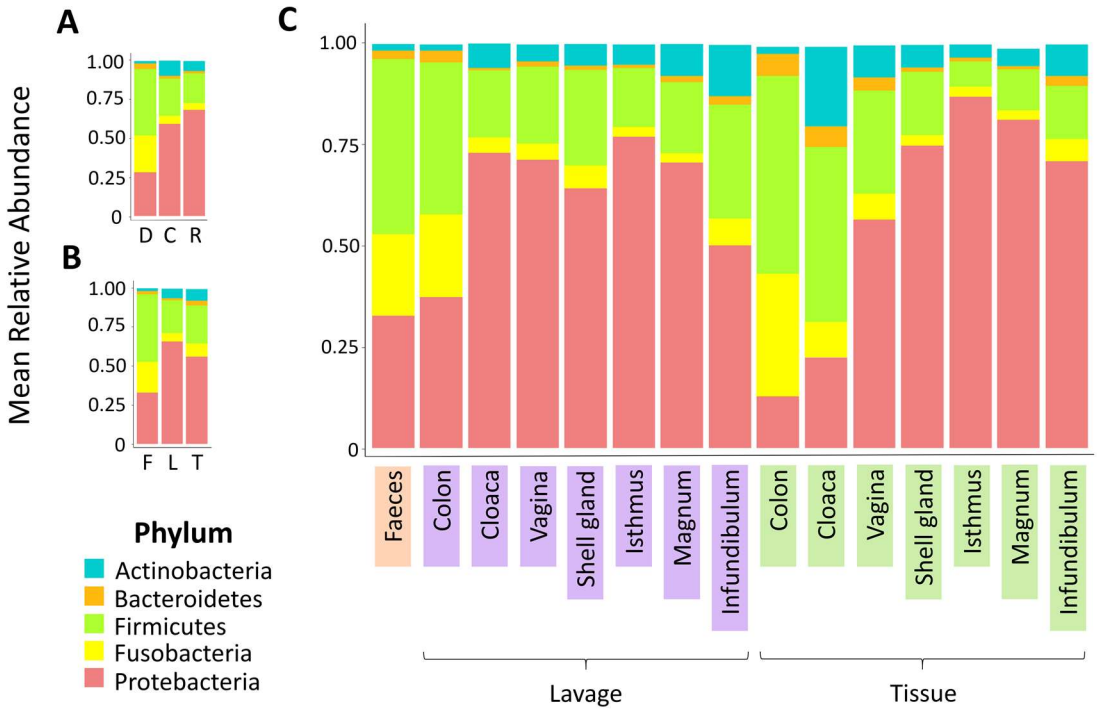


Figure 2. Stacked bar plots depicting the mean relative abundances of bacterial phyla per host ecosystem (D=digestive tract (n=66), C=cloaca (n=46), R=reproductive tract (n=230); A), sampling method (F=faecal sample (n=20), L=lavage sample (n=182), T=tissue sample (n=140); B), and sample type (n_{faeces}=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20; C). Functionally-distinct sections of the FRT (C) are presented from left to right in an anatomically-ascending order, starting with the cloaca. Each bacterial phylum is indicated by a unique colour. Phyla that contributed <1% to the mean relative abundances were excluded from the figures.

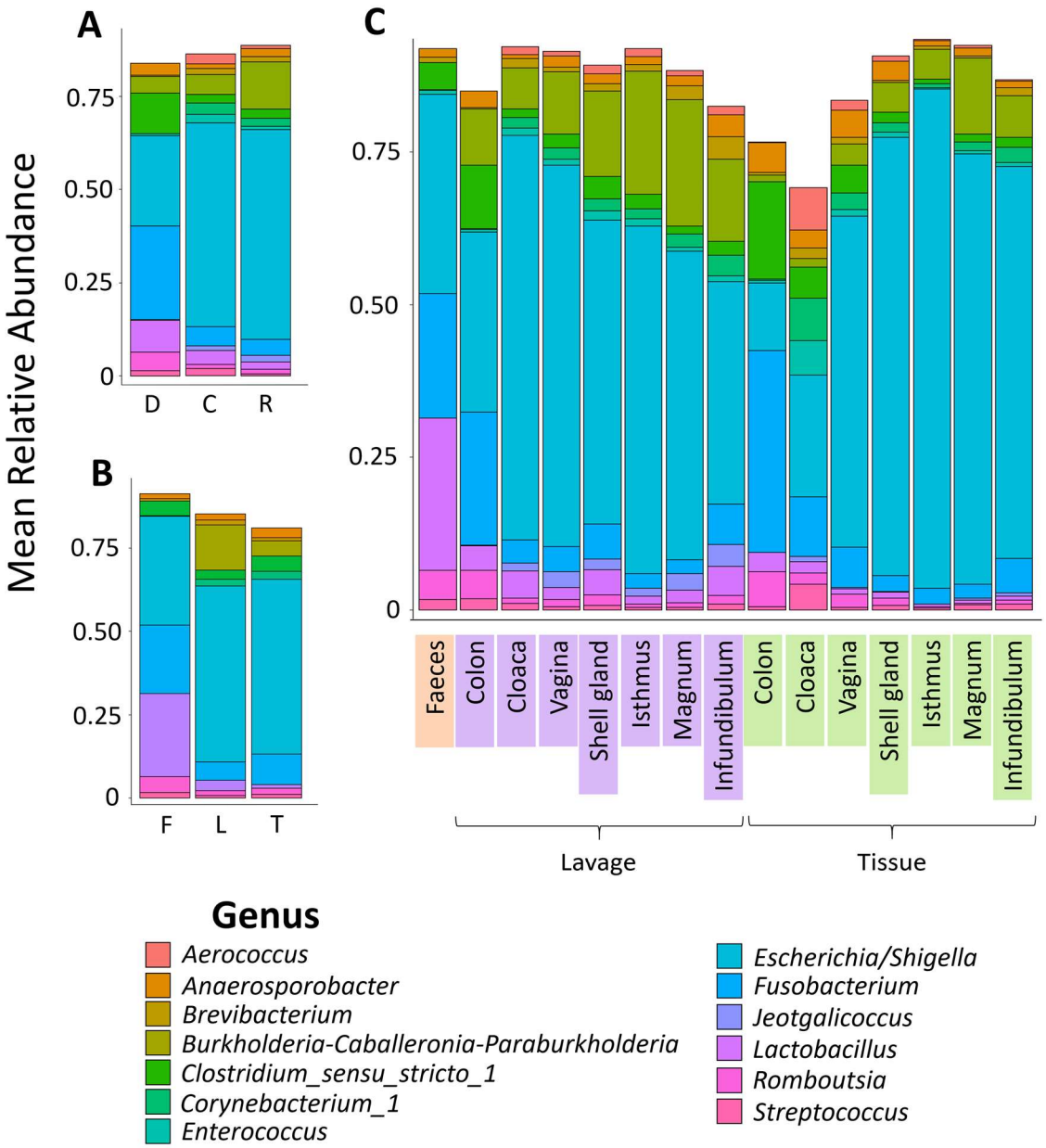


Figure 3. Stacked bar plots depicting the mean relative abundances of bacterial genera per host ecosystem (D=digestive tract (n=66), C=cloaca (n=46), R=reproductive tract (n=230); A), sampling method (F=faecal sample (n=20), L=lavage sample (n=182), T=tissue sample (n=140); B), and sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20; C). Functionally-distinct sections of the FRT (C) are presented from left to right in an anatomically-ascending order, starting with the cloaca. Each bacterial genus is indicated by a unique colour and genera are separated by black lines for clarity. Genera that contributed <1% to the mean relative abundances were excluded from the figures.

Different sampling methods also captured distinct microbiota compositions (Figures 2B, 3B; Supplementary Table 2B). Lavage and tissue samples captured similar bacterial

assemblages, while faecal samples harboured less Proteobacteria (32.9±6.2%) and Actinobacteria (2.1±1.2%), and more Firmicutes (43.2±7.1%) and Fusobacteria (19.8±5.7%) in comparison (Figure 2B). Lavage samples captured more *Burkholderia-Caballeronia-Paraburkholderia* (phylum Proteobacteria; 13.5±1.3%) than other sampling methods, while faecal samples were rich in *Lactobacillus* (phylum Firmicutes; 24.9±7.1%) and *Fusobacterium* (20.4±5.8%; Figure 3B).

Sample types were significantly different from each other in their microbiota composition (Table 3). Based on genus-level Bray-Curtis dissimilarities, sample type explained almost 26% of the microbiota variation detected (Table 3B), with cloaca tissue harbouring an intermediate bacterial community between FRT and digestive tract bacterial assemblages (Figure 4). The significant differences between sample type genus-level Bray-Curtis dissimilarities, however, were due to significant beta dispersion differences (pseudo-$F_{14, 327}$=14.74, $p<0.001$).

Bacterial phylum and genus abundances varied across different sample type (Table 3; Figures 2C, 3C; Supplementary Figure 2). Five phyla and 12 genera were significantly differentially-abundant across sample type (Supplementary Figure 2). Of note, functionally-distinct FRT sections harboured significantly more *Escherichia/Shigella*, *Corynebacterium_1* (phylum Actinobacteria), and *Jeotgalicoccus* (phylum Firmicutes; Supplementary Figure 2B). Colon lavage and tissue samples had significantly more *Clostridium_sensu_stricto_1* (phylum Firmicutes), and faeces were significantly enriched with *Lactobacillus* (Figure 3C; Supplementary Figure 2B). Cloaca tissue harboured the highest number of rare genera (30.9±0%) that each contributed <1% to the mean relative abundances (Figure 3C).

Table 3. [PERMANOVA] Microbiota composition variation between sample type ($n_{vagina\ lavage}$=26, $n_{shell\ gland\ lavage}$=26, $n_{isthmus\ lavage}$=26, $n_{magnum\ lavage}$=26, $n_{infundibulum\ lavage}$=26, $n_{cloaca\ lavage}$=26, $n_{colon\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, $n_{cloaca\ tissue}$=20, $n_{colon\ tissue}$=20, or n_{faeces}=20), health status ($n_{healthy}$=103 or $n_{symptomatic}$=239), reproductive status ($n_{developed\ ovary}$=54 or $n_{regressed\ ovary}$=288), and age class (n_{young}=224 or n_{old}=118) at bacterial phylum (A) and genus level (B), using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=10,000) were constrained within strata=*bird ID* (n=27). Significant p-values are in bold.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**10.98**	**14**	**<0.001**	**27.6%**
Health status	**6.29**	**1**	**0.004**	**1.1%**
Reproductive status	**5.27**	**1**	**0.006**	**0.9%**
Age class	**3.99**	**1**	**0.021**	**0.7%**
Bird ID	**3.74**	**23**	**<0.001**	**15.5%**
Residuals		301		54.2%
Unweighted UniFrac				
Sample type	**3.52**	**14**	**<0.001**	**11.9%**
Health status	**4.97**	**1**	**<0.001**	**1.2%**
Reproductive status	**4.08**	**1**	**0.001**	**1.0%**
Age class	0.59	1	0.742	0.1%
Bird ID	**2.35**	**23**	**<0.001**	**13.1%**
Residuals		301		72.7%
Bray-Curtis DS				
Sample type	**16.24**	**14**	**<0.001**	**36.1%**
Health status	**5.30**	**1**	**0.010**	**0.8%**
Reproductive status	**4.48**	**1**	**0.017**	**0.7%**
Age class	3.09	1	0.055	0.5%
Bird ID	**3.87**	**23**	**<0.001**	**14.1%**
Residuals		301		47.8%
Jaccard Index				
Sample type	**3.94**	**14**	**<0.001**	**13.0%**
Health status	**6.71**	**1**	**<0.001**	**1.6%**
Reproductive status	**4.70**	**1**	**<0.001**	**1.1%**
Age class	0.52	1	0.813	0.1%
Bird ID	**2.46**	**23**	**<0.001**	**13.3%**
Residuals		301		70.9%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**10.28**	**14**	**<0.001**	**24.8%**
Health status	**8.14**	**1**	**<0.001**	**1.4%**
Reproductive status	**11.27**	**1**	**<0.001**	**1.9%**
Age class	**6.38**	**1**	**<0.001**	**1.1%**
Bird ID	**4.82**	**23**	**<0.001**	**19.1%**
Residuals		201		51.7%
Unweighted UniFrac				
Sample type	**3.44**	**14**	**<0.001**	**11.5%**
Health status	**3.81**	**1**	**<0.001**	**0.9%**
Reproductive status	**3.61**	**1**	**<0.001**	**0.9%**
Age class	**2.91**	**1**	**0.002**	**0.7%**
Bird ID		**23**	**<0.001**	**14.0%**
Residuals		301		72.0%
Bray-Curtis DS				
Sample type	**11.37**	**14**	**<0.001**	**26.0%**
Health status	**6.77**	**1**	**<0.001**	**1.1%**
Reproductive status	**13.84**	**1**	**<0.001**	**2.3%**
Age class	**6.44**	**1**	**<0.001**	**1.1%**
Bird ID	**5.42**	**23**	**<0.001**	**20.4%**
Residuals		301		49.1%
Jaccard Index				
Sample type	**3.00**	**14**	**<0.001**	**10.2%**
Health status	**3.77**	**1**	**<0.001**	**0.9%**
Reproductive status	**3.18**	**1**	**<0.001**	**0.8%**
Age class	**3.24**	**1**	**<0.001**	**0.8%**
Bird ID	**2.55**	**23**	**<0.001**	**14.3%**
Residuals		301		73.0%

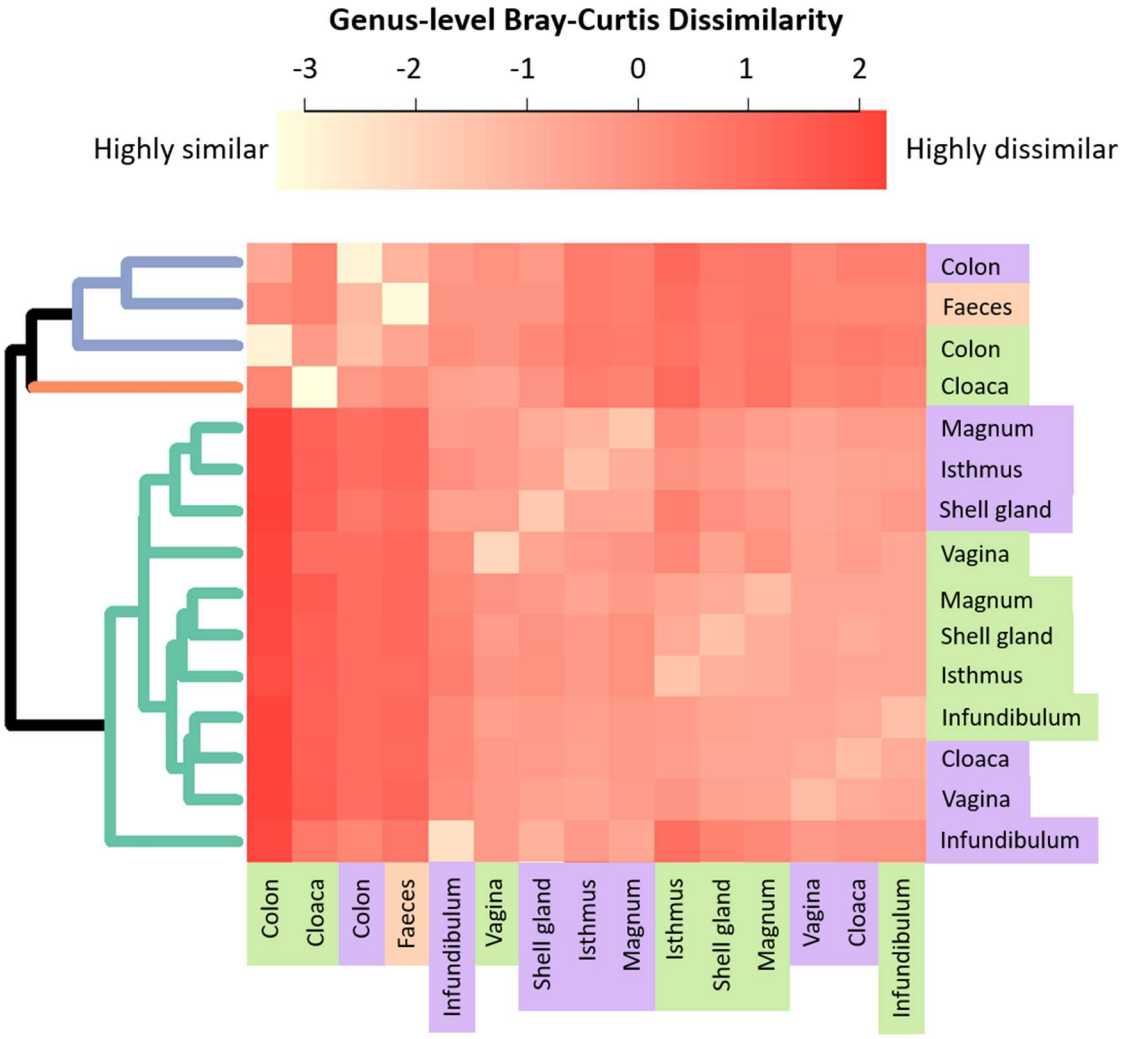


Figure 4. A heatmap with average linkage hierarchical clustering, depicting genus-level Bray-Curtis dissimilarities between sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20). Lavage samples are indicated by lilac, tissue samples by lime green, and faeces by peach. Spectrum of similarity to dissimilarity is presented by a range from light yellow to light red. Three separate clusters (dendrogram cutting height=0.50) are highlighted by unique colours.

(2) *Characterising Bacterial Richness and Diversity of Functionally-Distinct FRT Sections*

Host ecosystems were found to harbour significantly different total ASV richness. Cloacal samples (intercept; β±S.E.=178.0±5.2, p<0.001) had the highest total ASV richness, while FRT (β=-74.7±6.2, p<0.001) and digestive tract (β=-33.6±11.8, p=0.005) harboured significantly fewer unique bacterial strains. Shannon's diversity also differed significantly between host ecosystems (χ^2=25.3, df=2, p<0.001), with cloaca and digestive tract harbouring similar diversity (p=0.45), while the FRT had significantly lower diversity than either of them

($p<0.001$). Inverse Simpson's diversity comparisons confirmed these significant differences between host ecosystems ($\chi^2=39$, df=2, $p<0.001$). The cloaca and digestive tract were similar to each other ($p=0.56$), both exhibiting significantly higher bacterial diversity than FRT ($p<0.001$).

The three sampling methods captured significantly different bacterial richness, but not diversity. Lavage samples ($\beta=42.4\pm7.6$, $p<0.001$) captured significantly higher total ASV richness than tissue samples ($\beta=-14.4\pm8.5$, $p=0.09$) and faecal samples (intercept; $\beta=109.8\pm7.6$, $p<0.001$). Shannon's diversity ($\chi^2=4.3$, df=2, $p=0.12$) and inverse Simpson's diversity ($\chi^2=4.5$, df=2, $p=0.11$) were similar across sampling methods.

Total ASV richness estimates per sample type indicated differences across fowl FRT, with tissue samples from the upper FRT exhibiting significantly lower total ASV richness estimates (Table 4, Figure 5), while lavage samples across the functionally-distinct FRT sections harboured uniform total ASV richness (Table 4, Figure 5). Infundibulum lavage samples showed increased bacterial richness (Table 4; Figure 5). Colon lavage and cloaca tissue exhibited higher total ASV richness than FRT samples and faeces (Table 4; Figure 5). Similar patterns in spatial structure were detected across sample type when comparing Shannon's ($\chi^2=91.06$, df=14, $p<0.001$) and inverse Simpson's diversity ($\chi^2=181.73$, df=14, $p<0.001$) estimates (Supplementary Tables 3, 4; Supplementary Figures 3, 4).

(3) *Effect of Individual Phenotype on Female Fowl Microbiota*

Fowl health status, reproductive status, and age class had a significant overall effect on microbiota composition in red junglefowl (Table 3). Beta dispersion tests that were performed using genus-level Bray-Curtis dissimilarities—based on which the largest amount of variation was explained at the genus level β-diversity modelling (Table 3B)—indicated no significant dispersion differences between fowl health status (pseudo-$F_{1, 340}=0.01$, $p=0.925$), reproductive

status (pseudo-$F_{1, 340}$=1.34, p=0.246), nor age class (pseudo-$F_{1, 340}$=0.93, p=0.327). The largest amount of variation in microbiota composition was explained by bird ID (mean±S.E.=15.4±1.3%; Table 3).

Table 4. [betta_random] Total ASV richness estimates per sample type ($n_{vagina\ lavage}$=26, $n_{shell\ gland\ lavage}$=26, $n_{isthmus\ lavage}$=26, $n_{magnum\ lavage}$=26, $n_{infundibulum\ lavage}$=26, $n_{cloaca\ lavage}$=26, $n_{colon\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, $n_{cloaca\ tissue}$=20, $n_{colon\ tissue}$=20, or n_{faeces}=20), health status ($n_{healthy}$=103 or $n_{symptomatic}$=239), reproductive status ($n_{developed\ ovary}$=54 or $n_{regressed\ ovary}$=288), and age class (n_{young}=224 or n_{old}=118). The *intercept* is the total ASV richness estimate for faeces collected from young, healthy birds with a developed ovary. Functionally-distinct sections of the FRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold.

	Total ASV Richness	Estimate	S.E.	p-value
	Intercept	**175.2**	**5.0**	**<0.001**
Lavage	Colon	**40.9**	**19.2**	**0.033**
Lavage	Cloaca	14.2	19.0	0.456
Lavage	Vagina	3.6	19.0	0.848
Lavage	Shell gland	11.5	19.1	0.545
Lavage	Isthmus	-27.0	18.1	0.135
Lavage	Magnum	6.3	17.9	0.725
Lavage	Infundibulum	**50.2**	**19.2**	**0.009**
Tissue	Colon	10.3	21.1	0.625
Tissue	Cloaca	**79.5**	**21.0**	**<0.001**
Tissue	Vagina	6.2	20.4	0.761
Tissue	Shell gland	**-72.5**	**20.4**	**<0.001**
Tissue	Isthmus	**-80.3**	**20.3**	**<0.001**
Tissue	Magnum	**-83.1**	**20.3**	**<0.001**
Tissue	Infundibulum	**-57.5**	**20.4**	**0.005**
	Symptomatic	**-30.9**	**5.5**	**<0.001**
	Regressed ovary	**-27.7**	**5.4**	**<0.001**
	Old	12.3	12.3	0.318

Symptomatic birds and females with a regressed ovary exhibited significantly lower overall total ASV richness (Table 4). Shannon's and inverse Simpson's diversity were not considerably affected by fowl health status (Shannon's diversity: χ^2=0.94, df=1, p=0.332; inverse Simpson's diversity: χ^2=0.29, df=1, p=0.589), reproductive status (Shannon's

diversity: χ^2=8.72, df=1, p=0.003; inverse Simpson's diversity: χ^2=2.91, df=1, p=0.088), nor age class (Shannon's diversity: χ^2=0.25, df=1, p=0.620; inverse Simpson's diversity: χ^2=0.17, df=1, p=0.679). However, birds with a regressed ovary had a significantly decreased overall Shannon's diversity (Supplementary Table 3).

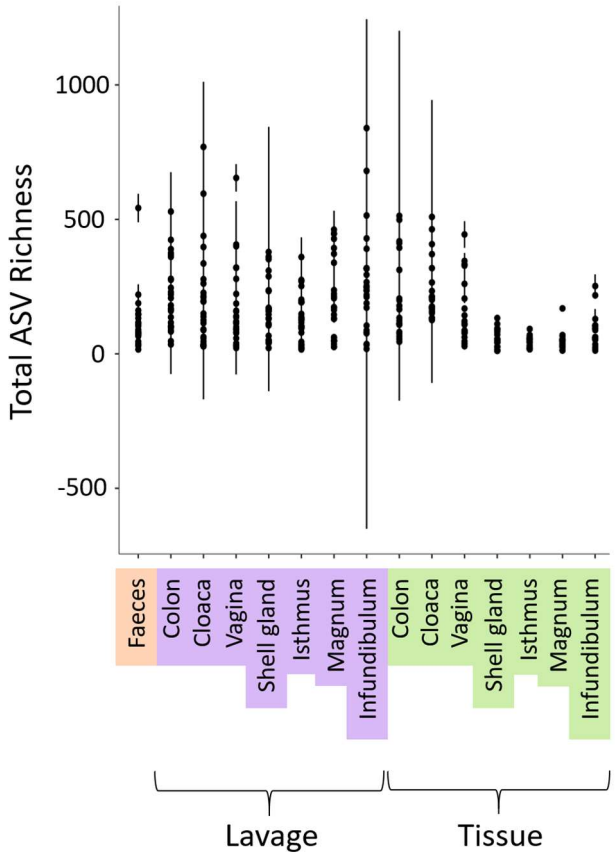


Figure 5. Total ASV richness estimates per sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20). Dots with standard errors are individual samples (n=342). Functionally-distinct sections of the FRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

Firmicutes were significantly more abundant in symptomatic birds (β±S.E.=1.91±0.40, t=4.82, p<0.001). Female fowl with a regressed ovary had significantly more Proteobacteria (β=0.60±0.19, t=3.16, p=0.002), and significantly less Fusobacteria (β=-1.09±0.40, t=-2.76, p=0.006) and Actinobacteria (β=-0.54±0.23, t=-2.33, p=0.021). Old females (4–9 years) harboured more Bacteroidetes (β=0.87±0.23, t=3.72, p<0.001), while having significantly less Actinobacteria (β=-0.56±0.19, t=-2.93, p=0.004).

We also identified bacterial genera that were significantly differentially-abundant in the FRT and cloaca across health status, reproductive status, and age class (Figure 6). Of note, *Fusobacterium* was more abundant in symptomatic birds (Figure 6A), while *Escherichia/Shigella* was significantly enriched in birds with a regressed ovary (Figure 6B). The abundance of *Corynebacterium_1* differed across the three tested phenotypic variables (Figure 6).

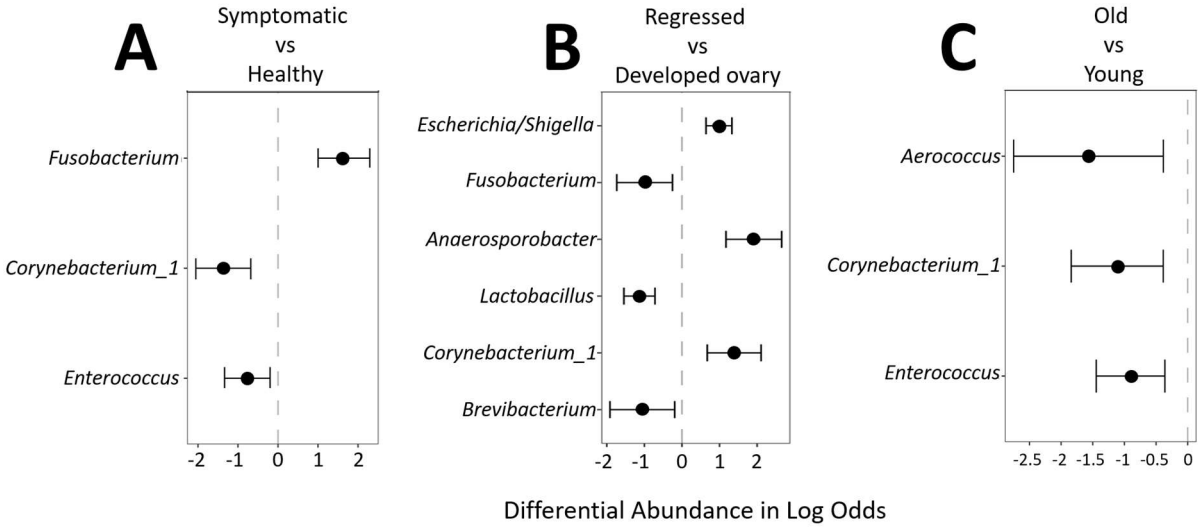


Figure 6. Differential abundance (in log odds) of bacterial genera in the FRT and cloaca across health status ($n_{healthy}$=84 or $n_{symptomatic}$=192; A), reproductive status ($n_{developed\ ovary}$=42 or $n_{regressed\ ovary}$=234; B), and age class (n_{young}=181 or n_{old}=95; C). The grey dashed line represents the point of comparison, which is either healthy female fowl (A), females with a developed ovary (B), or young birds (C). Data points with standard error bars depict the log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds indicate the significant under-representation of the bacterial genus.

Discussion

Pathogenic FRT bacteria may have severe implications for host health and fitness. Because of the male-to-female directionality of ejaculation and insemination, female hosts are expected to receive numerous bacteria during mating (Kulkarni & Heeb, 2007; White et al., 2010; Mändar et al., 2015). Considering that some of these bacteria may be pathogenic, polyandrous females are hypothesised to be especially vulnerable, as they mate with multiple partners that may each transmit bacteria to their FRT. Little, however, is known about the

ecology of FRT microbiota in polyandrous hosts. We fill this gap in knowledge by investigating the FRT microbiota of the polygynandrous red junglefowl.

We found that the FRT and digestive tract microbiota were ecologically distinct, with the shared outlet cloaca harbouring an intermediate bacterial community. Tissue, but not lavage samples, from the upper FRT harboured significantly lower total ASV richness and bacterial diversity in comparison with the lower FRT. Symptomatic birds and females with a regressed ovary (associated with the non-breeding status) harboured significantly lower overall total ASV richness and diversity. Fowl health status, reproductive status, and age class had a significant effect on FRT and cloacal bacteria.

FRT microbiota of red junglefowl was dominated by Proteobacteria, followed by Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes. Little is known about FRT microbiota in other avian species, but studies of wild bird cloacal microbiota have reported the presence of Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Kreisinger et al., 2015; Escallón et al., 2017; Escallón et al., 2019). Our results are congruent with these findings, with cloaca tissue harbouring a high abundance of Actinobacteria. Female fowl digestive tract was rich in Firmicutes, followed by Proteobacteria, Fusobacteria, Bacteroidetes, and Actinobacteria, which is similar to the domestic chicken gut microbiota (Yeoman et al., 2012; Oakley et al., 2014; Shaufi et al., 2015).

Differences between FRT and digestive tract microbiota were especially evident at the genus level. FRT was dominated by *Escherichia/Shigella* and *Burkholderia-Caballeronia-Paraburkholderia*, while digestive tract harboured more *Fusobacterium* and *Clostridium_sensu_stricto_1*. *Escherichia/Shigella* and *Clostridium* have been identified as potentially opportunistic pathogens in avian hosts (Benskin et al., 2009), while *Fusobacterium* has been found in the domestic chicken faeces (Oakley et al., 2014). We have to acknowledge here that *E. coli* and *Burkholderia* have been reported as potential laboratory contaminants

(Salter et al., 2014), and some members of the genera also identified as contaminants in our experiment (Supplementary Figure 1B). Nevertheless, we believe that our results are robust as we practised sterile laboratory etiquette and stringent removal of contaminants based on a rigorous system of negative controls (Supplementary Figure 1A & B), and were able to detect all bacterial strains associated with the positive standard (Supplementary Figure 1D).

Bacterial crossovers between reproductive and digestive tract may be common in avian systems, as the two tracts are connected by a shared outlet, the cloaca. Despite sharing phyla and genera, the bacterial abundances varied in the red junglefowl FRT and digestive tract, resulting in significantly distinct bacterial communities. Cloaca tissue revealed that the shared organ harboured an intermediate bacterial assemblage between the two tracts. It is worth mentioning that the cloaca is in direct contact with social and sexual partners (Kulkarni & Heeb, 2007), and the surrounding environment (Lucas & Heeb, 2005), which could further diversify its already distinct bacterial assemblage.

Due to the male-to-female directionality of ejaculation, females are expected to be more vulnerable to the detrimental effects of sexual bacterial transmission. Compared to their mating state, female black-legged kittiwakes (*Rissa tridactyla*) were found to revert back to lower cloacal bacterial diversity when cloacal contact between mating partners was experimentally blocked (White et al., 2010), suggesting potential for a microbiota maintenance mechanism, and costs for harbouring a diverse or sub-optimal cloacal microbiota in females. Indeed, failure to maintain a "healthy" cloacal microbiota could affect the fitness of the female host and its offspring. For example, the presence of *Enterococcus faecium* (phylum Firmicutes) on the cloacae of pied flycatchers (*Ficedula hypoleuca*) has been positively associated with nestling growth (Moreno et al., 2003), and losing such commensal strains may be detrimental for female reproductive fitness.

We found bacteria across the FRT, with bacterial diversity decreasing in the upper sections. Shell gland, isthmus, and magnum tissues harboured significantly lower total ASV richness and bacterial diversity. These results are congruent with spatial structure observed in the avian gastrointestinal tract that harbours lower bacterial diversity in the upper tract (Drovetski et al., 2018). While the decreased diversity could be explained by the upper tract being more internal and thus more difficult to reach for bacteria, we propose that it may also be due to top-down microbiota maintenance by the host.

The descending fertilised egg spends the most time in magnum, isthmus, and shell gland, where the egg white, two membranes, and eggshell are added respectively (Figure 1), and the fertilised egg remains unprotected prior to the formation of these physical barriers. To illustrate, the eggshell has been shown to protect the fertilised egg from external bacterial infections via surface nanostructure (D'Alba et al., 2014) and antimicrobial eggshell proteins (Wellman-Labadie, Picman & Hincke, 2008)—although few pathogenic bacteria, such as *Chlamydia psittaci* (phylum Chlamydiae) in birds, can still penetrate the eggshell (Ahmed et al., 2017). Some pathogens, e.g. strains of *Salmonella*, may even be incorporated within the eggshell before the shell even forms (De Buck et al., 2004). The exact process of incorporating bacteria inside the eggshell is poorly understood, but ascending bacterial infections in the oviduct could be the cause, as has been proposed for *Salmonella enteritidis* (Keller et al., 1995). The female host should therefore attempt to eradicate the ascending pathogens, while maintaining a "healthy" microbiota in the reproductive tract sections where the fertilised egg is still unprotected and susceptible to pathogen invasions—a pattern that is congruent with our results.

Eradicating pathogens may be facilitated by the mucosal barrier that covers the internal walls of the FRT and prohibits bacterial invasion (Linden et al., 2008). Additionally, AMPs that limit bacterial growth, such as lysozyme, are synthesized in the magnum and also

incorporated into egg albumen (Hincke et al., 2000). Similar bacterial growth limiting properties have been reported for antimicrobial histones found in the oviducts of White Leghorn hens (Silphaduang et al., 2006). After the shell gland section, bacterial diversity increased in the lower FRT of red junglefowl, supporting our hypothesis that the female host is maintaining its reproductive tract microbiota that may come in prolonged contact with the unprotected egg. In comparison, colon harboured significantly higher bacterial diversity than the FRT sections, further suggesting that the FRT has a more maintained microbiota than the digestive tract. Additional research is necessary, however, to understand how microbiota present in the functionally-distinct FRT sections are maintained, and whether female reproductive bacteria, both beneficial and detrimental, may be incorporated within and on eggs. A study in woodlarks (*Lullula arborea*) and skylarks (*Alauda arvensis*) reported that eggshells are unlikely to operate as cross-generational bacterial carriers in these specific species (Van Veelen, Salles & Tieleman, 2018). On the other hand, the pathogenic *Enterococcus faecalis* was found to be vertically-transmitted via eggshells in broiler chickens (Olsen, Christensen & Bisgaard, 2012). Combined, our results and the previous literature encourage further investigation of the mother-to-offspring bacterial transmission in oviparous hosts.

The reported bacterial diversity variation across the FRT was only evident from tissue, not lavage samples, which revealed a relatively uniform bacterial diversity across the oviduct. Lavage samples also captured higher bacterial diversity than other sampling methods, which is most likely due to biological, rather than methodological reasons. In gut microbiota research, different bacteria have been found to inhabit the luminal excretions captured by lavage samples and the epithelium captured by tissue samples (Mao et al., 2015). It has been proposed that the movement of cilia present on the mucosal surface of the avian reproductive tract limits the epithelial attachment of bacteria, thus increasing the bacterial load in the lumen, while decreasing it in and on the epithelium (Sasanami, 2017). Epithelium-bound bacteria are further

selected for and against by host secretions, limiting the number and diversity of tissue-dwelling bacteria (Schluter & Foster, 2012). Selected tissue-bound bacteria may then form protective biofilms, creating a layer between the host and invasive bacteria (Ventolini, Mitchell & Salazar, 2015). Combined, these mechanisms may aid in maintaining the low epithelium-associated bacterial diversity in the upper FRT of red junglefowl, although further research is necessary to understand whether the FRT microbiota are maintained by the host for adaptive reasons. Nevertheless, our results suggest that the FRT of red junglefowl provides two niches that support divergent bacterial strategies. "Resident" bacterial strains—captured with tissue samples—attach to the FRT walls, while "tourist" bacteria—captured with lavage samples—are present in the luminal excretions that may be flushed up the FRT with ejaculates and down the tract with the descending egg. As such, our study highlights the importance of using multiple sampling methods when exploring host-associated bacterial communities.

Finally, we found that individual variation in health status, reproductive status, and age class significantly affected fowl microbiota. Healthy and symptomatic birds differed significantly in their microbiota composition, with symptomatic birds harbouring a significantly decreased overall total ASV richness. A low-diversity, *Lactobacillus*-dominated vaginal microbiota is considered "healthy" in humans, and invasive pathogens have been found to significantly increase vaginal bacterial diversity (Ceccarani et al., 2019). On the other hand, "healthy" vaginal microbiota in other primate species is highly diverse (Yildirim et al., 2014). Healthy female fowl seem to support a diverse FRT microbiota in comparison with symptomatic birds. We also report that *Fusobacterium mortiferum* was more abundant in birds with the symptomatic white cloacal discharge. *F. mortiferum* has been shown to inhibit the pathogenic *Salmonella enteritidis* in poultry caeca (Portrait, Cottenceau & Pons, 2000), and its increased abundance could have served a pathogen-buffering role in symptomatic females.

Our results suggest that birds with a regressed ovary harboured a significantly distinct microbiota with lower overall total ASV richness and Shannon's diversity. The observed decrease in bacterial diversity could be due to the shift in female host behaviour and physiology between breeding and non-breeding season. In non-mating females, ovulation and mating halt, decreasing the amount of physical movement and excretions in the oviduct. As a result, the oviduct decreases significantly in size in the non-breeding season. Decreased surface area may leave less space for bacterial communities, and the halt in mating could reduce the number of sexually-acquired bacteria in the oviduct. We found that *Escherichia/Shigella* was more abundant in female fowl with a regressed ovary. *Shigella* causes shigellosis in chickens, which leads to a symptomatic cloacal discharge mixed with blood and pus, and may be lethal in severe cases (Shi et al., 2014). Shigellosis could have also been the cause of the white cloacal discharge in symptomatic red junglefowl. The reason why we did not detect significant differential abundance of *Escherichia/Shigella* between healthy and symptomatic birds may have been because all the female birds were in fact carriers of *Escherichia/Shigella*, with some of them asymptomatic, and we failed to correctly categorise the birds in our study.

An additional analysis of cloacal wipe samples collected from female fowl outside of this study and data set (**Chapter V**) that either exhibited the symptomatic white cloacal discharge or not (Supplementary Methods 1) revealed an increased abundance of *Gallibacterium anatis* (phylum Proteobacteria) on the cloacae of symptomatic birds (Supplementary Figure 5). *G. anatis* causes salpingitis (inflammation of the oviduct) and peritonitis (ectopic yolk in the body cavity) in domestic chickens (Neubauer et al., 2009). Necrosis in the isthmus tissue of symptomatic females may have been caused by a *G. anatis* infection. Interestingly, *E. coli* and *G. anatis* have been found to co-infect the chicken oviduct (Neubauer et al., 2009), which may have been the case in our study population as well.

We also detected differential abundance of the potentially mutualistic *Lactobacillus kitasatonis* (Messaoudi et al., 2011), and pathogenic *Corynebacterium_1* (van Dongen et al., 2019) and *Enterococcus cecorum* (Jung et al., 2018) across individual health status, reproductive status, and age class. Young (1–4 years), healthy birds with a developed ovary harboured significantly more *Corynebacterium_1* than old (4–9 years), symptomatic birds with a regressed ovary. A strain of *Corynebacterium* was found to be sexually-transmitted and increase incidences of egg hatching failure in female black-legged kittiwakes (van Dongen et al., 2019). If *Corynebacterium_1* is also sexually-transmitted in the red junglefowl, the detected patterns may be mediated by lower ovulation and mating rates in older birds (Holmes, Flückiger & Austad, 2001; Cornwallis, Dean & Pizzari, 2014). Further research is needed to investigate the specific ecology and in particular function of red junglefowl reproductive bacteria.

Takeaways

- Red junglefowl FRT, cloaca, and digestive tract support distinct microbiota, despite being anatomically connected
- Lower bacterial diversity in the upper FRT revealed by tissue sampling may suggest reproductive microbiota maintenance by the female host
- Individual variation in health status, reproductive status, and age class has a considerable effect on female fowl microbiota, and highlights the importance of incorporating phenotypic variation in host-microbiota studies
- Comparisons with male reproductive tract microbiota would allow a more informed understanding of the role of reproductive bacteria in polygynandrous host health and fitness

References

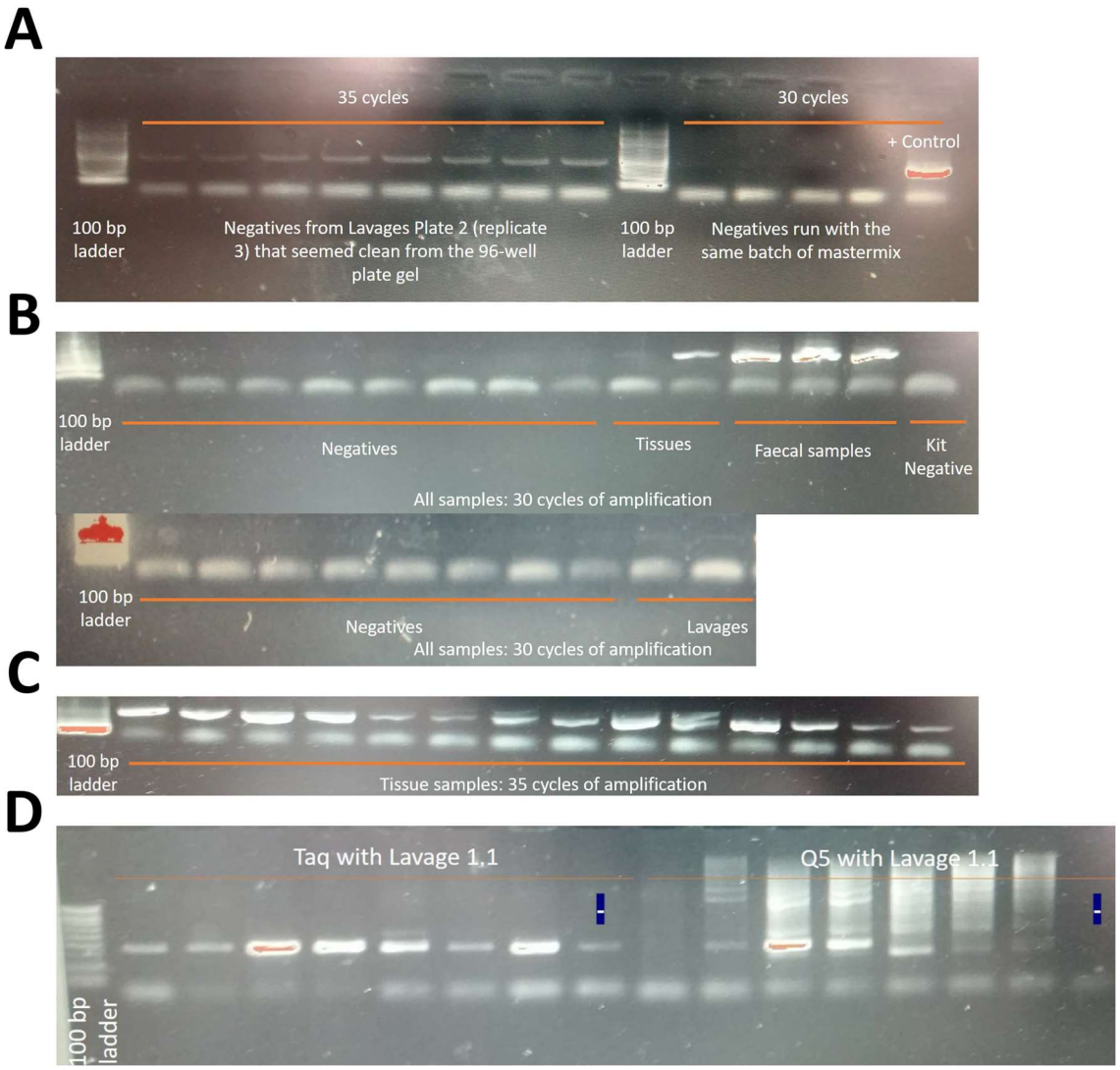
Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

Appendices

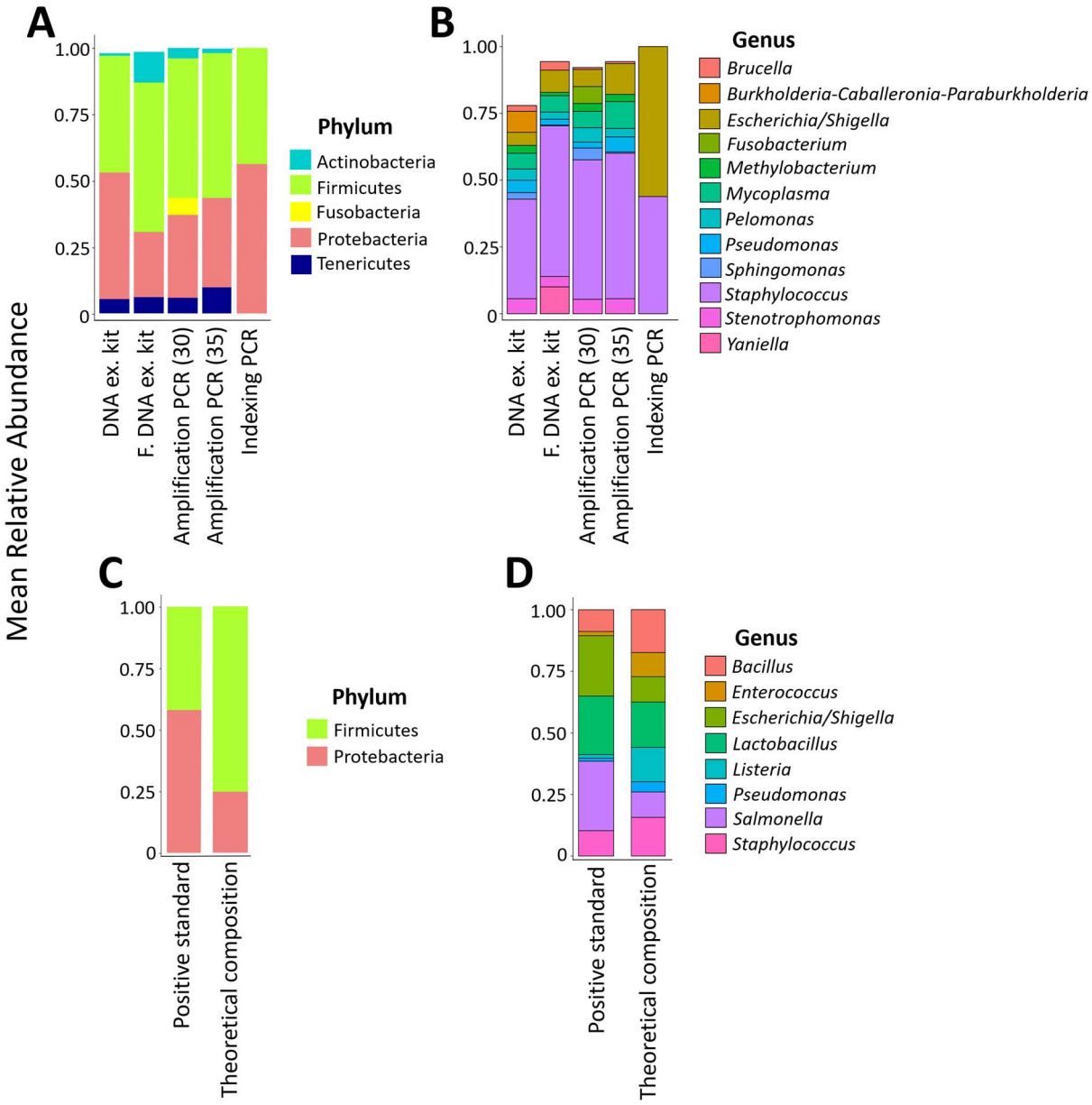
Supplementary Table 1. Primer sequences (5'–3' orientation) used in the amplification and indexing PCRs. For clarity, the functional regions of each primer have been separated by a "+". The locus-specific sequence is based on the Earth Microbiome Project (Thompson et al., 2017). The forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) cover the V4 hypervariable region (~254 base pairs) of the bacterial 16S SSU rRNA gene. The adapter used for trimming is indicated in bold. The paired-end index combinations were based on Kozich et al. (2013).

Amplification PCR primers	
	Illumina Nextera-compatible overhang + locus-specific sequence
515F	TCGTCGGCAGCGTC**AGATGTGTATAAGAGACAG** + GTGYCAGCMGCCGCGGTAA
806R	GTCTCGTGGGCTCGG**AGATGTGTATAAGAGACAG** + GGACTACNVGGGTWTCTAAT

Indexing PCR primers	
	Flow cell adapter + index + Illumina Nextera-compatible overhang
SA501	AATGATACGGCGACCACCGAGATCTACAC + ATCGTACG + TCGTCGGCAGCGTC
SA502	AATGATACGGCGACCACCGAGATCTACAC + ACTATCTG + TCGTCGGCAGCGTC
SA503	AATGATACGGCGACCACCGAGATCTACAC + TAGCGAGT + TCGTCGGCAGCGTC
SA504	AATGATACGGCGACCACCGAGATCTACAC + CTGCGTGT + TCGTCGGCAGCGTC
SA505	AATGATACGGCGACCACCGAGATCTACAC + TCATCGAG + TCGTCGGCAGCGTC
SA506	AATGATACGGCGACCACCGAGATCTACAC + CGTGAGTG + TCGTCGGCAGCGTC
SA507	AATGATACGGCGACCACCGAGATCTACAC + GGATATCT + TCGTCGGCAGCGTC
SA508	AATGATACGGCGACCACCGAGATCTACAC + GACACCGT + TCGTCGGCAGCGTC
SB501	AATGATACGGCGACCACCGAGATCTACAC + CTACTATA + TCGTCGGCAGCGTC
SB502	AATGATACGGCGACCACCGAGATCTACAC + CGTTACTA + TCGTCGGCAGCGTC
SB503	AATGATACGGCGACCACCGAGATCTACAC + AGAGTCAC + TCGTCGGCAGCGTC
SB504	AATGATACGGCGACCACCGAGATCTACAC + TACGAGAC + TCGTCGGCAGCGTC
SB505	AATGATACGGCGACCACCGAGATCTACAC + ACGTCTCG + TCGTCGGCAGCGTC
SB506	AATGATACGGCGACCACCGAGATCTACAC + TCGACGAG + TCGTCGGCAGCGTC
SB507	AATGATACGGCGACCACCGAGATCTACAC + GATCGTGT + TCGTCGGCAGCGTC
SB508	AATGATACGGCGACCACCGAGATCTACAC + GTCAGATA + TCGTCGGCAGCGTC
SB701	CAAGCAGAAGACGGCATACGAGAT + AAGTCGAG + GTCTCGTGGGCTCGG
SB702	CAAGCAGAAGACGGCATACGAGAT + ATACTTCG + GTCTCGTGGGCTCGG
SB703	CAAGCAGAAGACGGCATACGAGAT + AGCTGCTA + GTCTCGTGGGCTCGG
SB704	CAAGCAGAAGACGGCATACGAGAT + CATAGAGA + GTCTCGTGGGCTCGG
SB705	CAAGCAGAAGACGGCATACGAGAT + CGTAGATC + GTCTCGTGGGCTCGG
SB706	CAAGCAGAAGACGGCATACGAGAT + CTCGTTAC + GTCTCGTGGGCTCGG
SB707	CAAGCAGAAGACGGCATACGAGAT + GCGCACGT + GTCTCGTGGGCTCGG
SB708	CAAGCAGAAGACGGCATACGAGAT + GGTACTAT + GTCTCGTGGGCTCGG
SB709	CAAGCAGAAGACGGCATACGAGAT + GTATACGC + GTCTCGTGGGCTCGG
SB710	CAAGCAGAAGACGGCATACGAGAT + TACGAGCA + GTCTCGTGGGCTCGG
SB711	CAAGCAGAAGACGGCATACGAGAT + TCAGCGTT + GTCTCGTGGGCTCGG
SB712	CAAGCAGAAGACGGCATACGAGAT + TCGCTACG + GTCTCGTGGGCTCGG



Supplementary Gel Images 1. Gel electrophoresis (1% agarose gel stained with GelRed™ in a 1×TBE buffer at 70 volts for 45 minutes) imaged under a UV-lamp. Using the exact same PCR solution, 35 cycles of amplification produced a faint product band in the amplification PCR negative controls, while 30 cycles did not (A & B). 30 cycles of amplification were sufficient to produce a product band in faecal samples, but not in lavage and tissue samples harvested across fowl FRT, cloaca, and digestive tract (B). 35 cycles of amplification were enough to produce a product band in the most complex tissue samples, such as the upper FRT sections (C). These product bands were brighter than the ones in 35-cycle PCR negative controls (A), suggesting that the biological samples did contain bacterial DNA in addition to contaminant DNA. Finally, we were able to pinpoint the source of contamination by amplifying the exact same biological samples using the same pipeline and PCR solution, changing only the DNA Polymerase that was added to the PCR solution. The source of contamination was the *Taq* DNA Polymerase from *Thermus aquaticus* (D). Using the Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA) did not produce a product band in the PCR negative control (D), confirming our results. However, the Q5® High-Fidelity DNA Polymerase was not suitable for our biological samples, as it amplified a lot of non-specific product (D). We therefore decided to proceed with using the *Taq* DNA Polymerase from *Thermus aquaticus* and 35 cycles to ensure amplification in the biological samples.



Supplementary Figure 1. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A & C) and genera (B & D) detected in negative controls (A & B) and positive standards (C & D). Only taxa that contributed >1% are presented. Mean relative abundances per negative control type (A & B) were calculated using the post-*decontam* untrimmed contaminants-only data set. Mean relative abundances in the positive standards (C & D) were calculated using the pre-*decontam* untrimmed data set. Negative controls (A & B) are samples of the QIAGEN DNeasy® Blood & Tissue DNA extraction kit (DNA ex. kit; n=6), MO BIO PowerLyzer PowerSoil faecal DNA extraction kit (F. DNA ex. kit; n=1), amplification PCR negatives that were amplified for 30 cycles (n=2), amplification PCR negatives that were amplified for 35 cycles (n=12), and indexing PCR negatives (n=4). The positive standards (C & D) are the ZymoBIOMICS® Microbial Community Standards (n=2). For comparison purposes, the theoretical composition of the ZymoBIOMICS® Microbial Community Standard expected by the manufacturer is presented together with the positive standards (C & D). No correction was made to the data set based on the observed and expected positive standard mean relative abundances. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity.

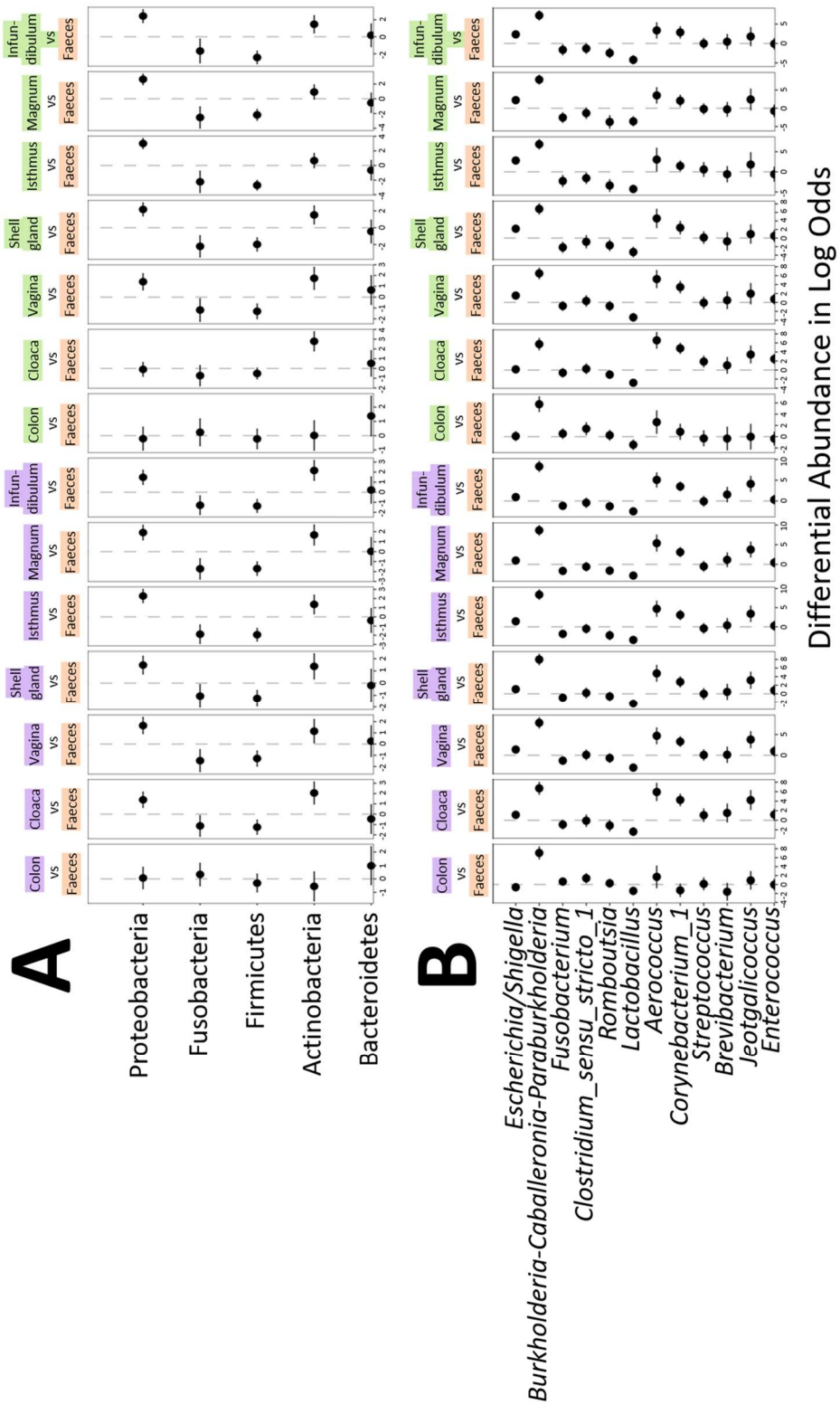
Supplementary Table 2. [ANOSIM] Microbiota composition variation between host ecosystems ($n_{reproductive\ tract}$=230, n_{cloaca}=46, or $n_{digestive\ tract}$=66; A) and sampling methods ($n_{lavage\ sample}$=182, $n_{tissue\ sample}$=140, or $n_{faecal\ sample}$=20; B) at bacterial phylum and genus level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=27). R=0 indicates high variability both within and between groups, R>0 indicates higher variability between groups than within groups, and R<0 indicates higher variability within groups than between groups. Significant p-values are in bold.

A

Host ecosystem	R	p-value
Weighted UniFrac		
Phylum-level	**0.27**	**0.001**
Genus-level	**0.34**	**<0.001**
Unweighted UniFrac		
Phylum-level	**0.08**	**0.004**
Genus-level	0.03	0.080
Bray-Curtis DS		
Phylum-level	**0.46**	**0.001**
Genus-level	**0.47**	**0.001**
Jaccard Index		
Phylum-level	**0.09**	**0.003**
Genus-level	0.03	0.104

B

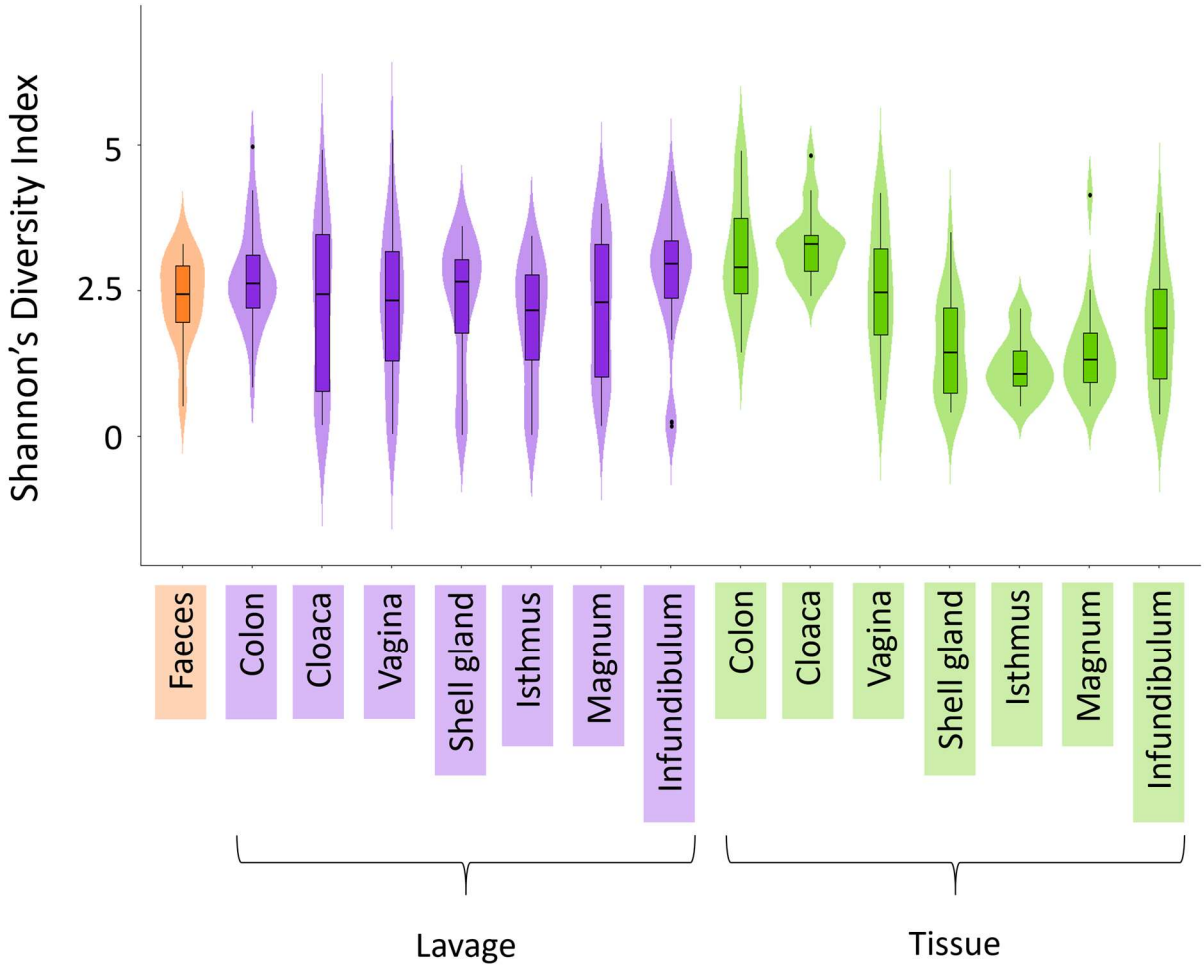
Sampling method	R	p-value
Weighted UniFrac		
Phylum-level	**0.04**	**0.006**
Genus-level	**0.05**	**0.001**
Unweighted UniFrac		
Phylum-level	**0.05**	**0.001**
Genus-level	**0.08**	**0.001**
Bray-Curtis DS		
Phylum-level	**0.06**	**0.001**
Genus-level	**0.11**	**0.001**
Jaccard Index		
Phylum-level	**0.05**	**0.001**
Genus-level	**0.11**	**0.001**



Supplementary Figure 2. Differential abundance (in log odds) of bacterial phyla (A) and genera (B) across sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20), controlling for female fowl health status, reproductive status, and age class. The grey dashed line represents the point of comparison, i.e. faeces. Data points with standard error bars depict the log odds estimates of the specific taxa. Positive log odds indicate significant enrichment, while negative log odds indicate the significant under-representation of the bacterial taxon. Lavage samples are indicated by lilac, tissue samples by lime green, and faecal samples by peach.

Supplementary Table 3. [GLMM, family=Gaussian, link="log"] Shannon's diversity index estimates per sample type ($n_{vagina\ lavage}$=26, $n_{shell\ gland\ lavage}$=26, $n_{isthmus\ lavage}$=26, $n_{magnum\ lavage}$=26, $n_{infundibulum\ lavage}$=26, $n_{cloaca\ lavage}$=26, $n_{colon\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, $n_{cloaca\ tissue}$=20, $n_{colon\ tissue}$=20, or n_{faeces}=20), health status ($n_{healthy}$=103 or $n_{symptomatic}$=239), reproductive status ($n_{developed\ ovary}$=54 or $n_{regressed\ ovary}$=288), and age class (n_{young}=224 or n_{old}=118). The *intercept* is the Shannon's diversity index estimate for faeces collected from young, healthy birds with a developed ovary. Functionally-distinct sections of the FRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold. The random effect *bird ID* (n=27) explained a relatively small amount of variation (S.D.=0.08).

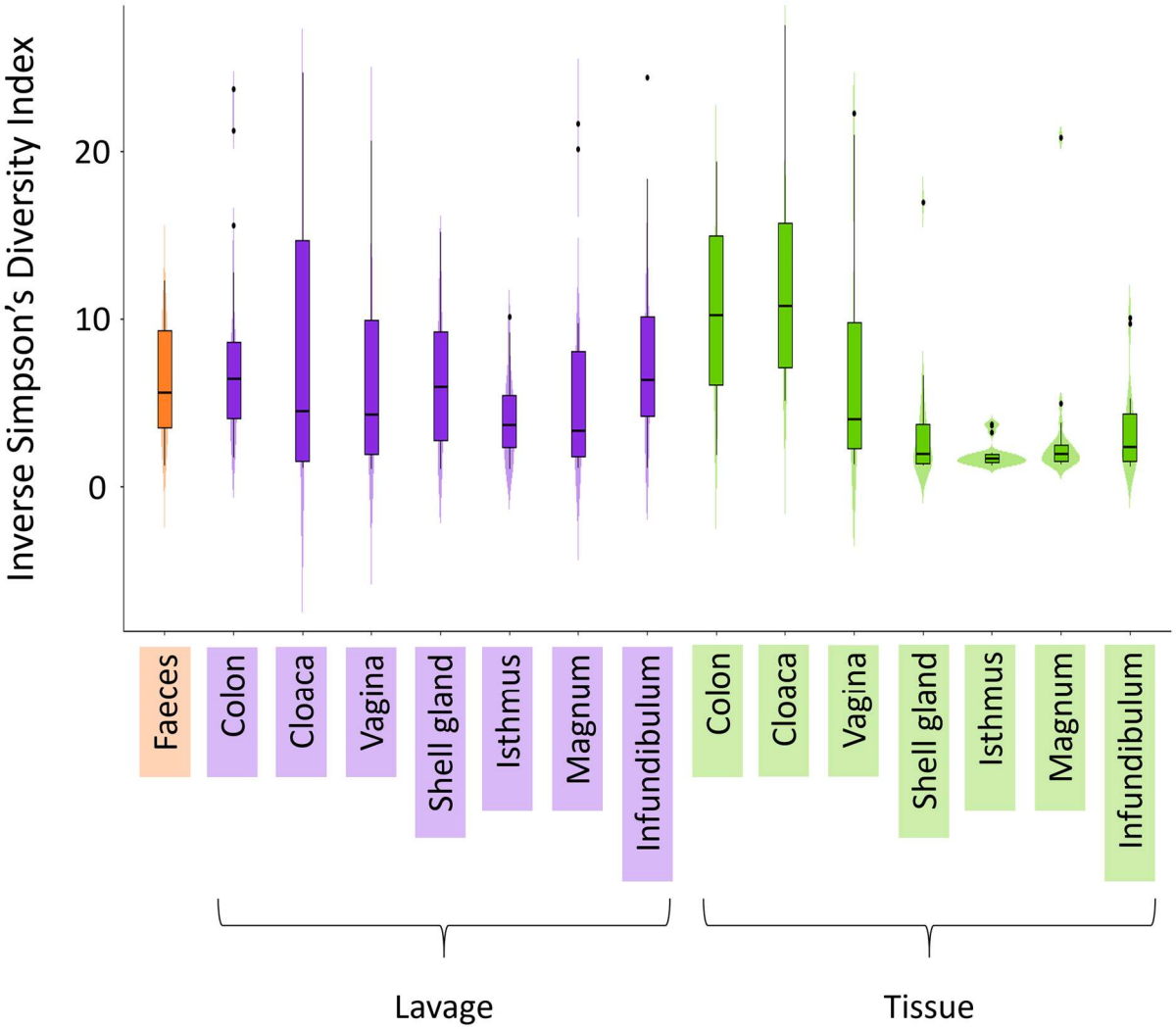
	Shannon's Diversity	Estimate	S.E.	t-value	p-value
	Intercept	**1.0**	**0.12**	**8.709**	**<0.001**
Lavage	Colon	0.15	0.12	1.323	0.186
Lavage	Cloaca	-0.03	0.12	-0.217	0.828
Lavage	Vagina	-0.01	0.12	-0.084	0.933
Lavage	Shell gland	0.01	0.12	0.059	0.953
Lavage	Isthmus	-0.16	0.13	-1.208	0.227
Lavage	Magnum	-0.03	0.12	-0.245	0.806
Lavage	Infundibulum	0.18	0.11	1.536	0.125
Tissue	Colon	**0.32**	**0.12**	**2.686**	**0.007**
Tissue	Cloaca	**0.39**	**0.12**	**3.369**	**<0.001**
Tissue	Vagina	0.11	0.13	0.880	0.380
Tissue	Shell gland	**-0.37**	**0.17**	**-2.215**	**0.027**
Tissue	Isthmus	**-0.60**	**0.20**	**-3.025**	**0.002**
Tissue	Magnum	**-0.40**	**0.17**	**-2.328**	**0.02**
Tissue	Infundibulum	-0.19	0.15	-1.289	0.197
	Symptomatic	-0.06	0.07	-0.869	0.385
	Regressed ovary	**-0.21**	**0.07**	**-2.922**	**0.003**
	Old	-0.02	0.06	-0.244	0.808



Supplementary Figure 3. Violin plots of Shannon's diversity index estimates per sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Functionally-distinct sections of the FRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

Supplementary Table 4. [GLMM, family=gamma, link="log"] Inverse Simpson's diversity index estimates per sample type ($n_{vagina\ lavage}$=26, $n_{shell\ gland\ lavage}$=26, $n_{isthmus\ lavage}$=26, $n_{magnum\ lavage}$=26, $n_{infundibulum\ lavage}$=26, $n_{cloaca\ lavage}$=26, $n_{colon\ lavage}$=26, $n_{vagina\ tissue}$=20, $n_{shell\ gland\ tissue}$=20, $n_{isthmus\ tissue}$=20, $n_{magnum\ tissue}$=20, $n_{infundibulum\ tissue}$=20, $n_{cloaca\ tissue}$=20, $n_{colon\ tissue}$=20, or n_{faeces}=20), fowl health status ($n_{healthy}$=103 or $n_{symptomatic}$=239), reproductive status ($n_{developed\ ovary}$=54 or $n_{regressed\ ovary}$=288), and age class (n_{young}=224 or n_{old}=118). The *intercept* is the inverse Simpson's diversity index estimate for faeces collected from young, healthy birds with a developed ovary. Functionally-distinct sections of the FRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold. The random effect *bird ID* (n=27) explained a considerable amount of variation (S.D.=0.86).

	Inverse Simpson's Diversity	Estimate	S.E.	t-value	p-value
	Intercept	**2.01**	**0.25**	**8.210**	**<0.001**
Lavage	Colon	**0.44**	**0.22**	**1.983**	**0.047**
	Cloaca	0.22	0.22	0.987	0.324
	Vagina	0.15	0.22	0.646	0.518
	Shell gland	-0.04	0.22	-0.180	0.857
	Isthmus	**-0.47**	**0.22**	**-2.107**	**0.035**
	Magnum	-0.16	0.22	-0.720	0.471
	Infundibulum	0.17	0.22	0.758	0.448
Tissue	Colon	**0.93**	**0.24**	**3.944**	**<0.001**
	Cloaca	**0.74**	**0.23**	**3.162**	**0.002**
	Vagina	0.11	0.24	0.449	0.654
	Shell gland	**-0.66**	**0.24**	**-2.767**	**0.006**
	Isthmus	**-1.21**	**0.23**	**-5.155**	**<0.001**
	Magnum	**-0.86**	**0.24**	**-3.648**	**<0.001**
	Infundibulum	**-0.64**	**0.23**	**-2.709**	**0.007**
	Symptomatic	-0.07	0.15	-0.448	0.654
	Regressed ovary	-0.28	0.17	-1.671	0.095
	Old	0.09	0.14	-0.619	0.536

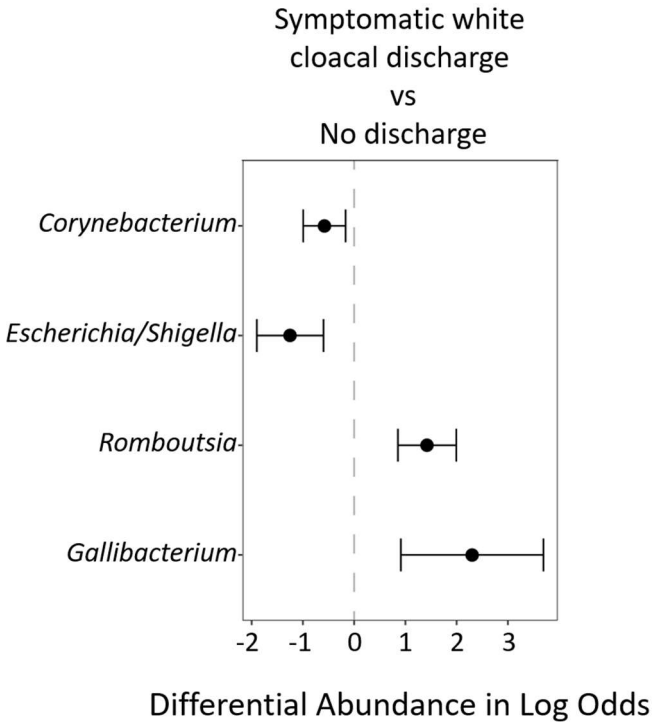


Supplementary Figure 4. Violin plots of inverse Simpson's diversity index estimates per sample type ($n_{per\ faeces}$=20, $n_{per\ lavage\ sample\ type}$=26, $n_{per\ tissue\ sample\ type}$=20). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Functionally-distinct sections of the FRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

Supplementary Methods 1. Statistical analysis of the differentially-abundant bacterial genera between the cloacal wipe samples collected from female red junglefowl with and without the symptomatic white cloacal discharge.

We used the data set of cloacal wipe samples that is analysed in **Chapter V** (see methods in **Chapter V**) for this comparison. During the collection of cloacal wipe samples, the experimenter made a note of whether the female bird had the symptomatic white cloacal discharge or not. 39 out of the 112 cloacal wipe samples collected from females had the white discharge.

The female cloacal wipe samples were tested for differentially-abundant bacterial genera with *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was agglomerated to genus level, filtered to include only the genera that contributed >1% to the mean relative bacterial abundances across the "cloacal discharge groups" ($n_{cloacal\ discharge}$=39 or $n_{no\ cloacal\ discharge}$=73), and tested with differentialTest(test="Wald", fdr_cutoff=0.05), while controlling for bacterial abundance across fowl age class (n_{young}=80 or n_{old}=32), and variance across the cloacal discharge groups and fowl age class. Results were visualised with *corncob* (Supplementary Figure 5).



Supplementary Figure 5. Differential abundance (in log odds) of bacterial genera on the cloacae of female red junglefowl with the symptomatic cloacal white discharge (n=39) in comparison with healthy fowl (n=73), controlling for fowl age class (n_{young}=80 or n_{old}=32). The grey dashed line represents the point of comparison, i.e. female fowl without the white cloacal discharge. Data points with standard error bars depict the log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds indicate the significant under-representation of the bacterial genus in symptomatic females compared to healthy females. Of note, *Gallibacterium anatis* (phylum Proteobacteria) was more abundant in symptomatic birds (β±S.E.=2.30±0.71, t=3.25, p=0.002).

Chapter III. The Male Reproductive Tract Microbiota of the Polygynandrous Red Junglefowl (*Gallus gallus*) is Ecologically Homogenous and Spatially Uniform

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Microbiota of the male reproductive tract (MRT) can have important consequences for male health and fitness. For example, pathogenic bacteria cause MRT inflammation, and may lower sperm motility and number. Considering that bacteria can affect sperm performance, MRT microbiota may also play a role in male reproductive fitness. Bacteria-induced fitness consequences can be particularly pronounced in polygynandrous species, where males and females mate and exchange bacteria with multiple partners, and sperm from different males compete to fertilise the egg. Despite increasing interest in the role of bacteria in male reproductive success, little is currently known about MRT bacterial ecology. Here, we studied the MRT microbiota of the polygynandrous red junglefowl, *Gallus gallus*—the main wild ancestor of the domestic chicken, *Gallus domesticus*—by collecting tissue samples from functionally-distinct MRT sections, characterising their bacterial communities, and comparing them to controls from the digestive tract. We found that fowl MRT microbiota is homogenous in its composition, total ASV richness, and diversity across the tract, while colon and in particular faeces harbour a divergent bacterial community. High diversity bacterial assemblages across the fowl MRT suggest that the presence of bacteria in the MRT of this particular host species is likely to be common. We also detected an individual phenotype effect on fowl microbiota, with old (4–6 years) and in particular very old (7–9 years) birds harbouring lower overall total ASV richness. Our study characterises red junglefowl MRT microbiota in its entirety, and lays the foundation for hypotheses about the role of reproductive tract bacteria in polygamous male host health and fitness.

Introduction

Multicellular organisms live in bacteria-dominated ecosystems. Not only are bacteria present in the environment, they are also on and within animal hosts, inhabiting their skin, oral and nasal cavities, gut, vagina, and other organs (Lozupone et al., 2013). Considerable effort has gone into characterising these host-associated bacteria—also known as microbiota—to understand their potential function. For example, skin microbiota forms a layer that protects the host from invasive bacteria (Grice & Segre, 2011), gut microbiota plays a functional role in host metabolism (Nicholson et al., 2012), and a "healthy", *Lactobacillus*-dominated vaginal microbiota reduces the risk of preterm birth, which is the main cause of newborn fatality (DiGiulio et al., 2015). Surprisingly little, however, is known about male reproductive tract (MRT) microbiota, although it could have significant health and fitness implications for the male host.

Due to medical interest in human health, most of the current research focus has been on the pathogenic bacteria present in the MRT. Pathogen invasions in human MRT may lead to urogenital infections, such as urethritis, bacterial prostatitis, and epididymitis (Rusz et al., 2012). Detrimental bacteria do not only cause tissue inflammation, but also come in contact with sperm cells and seminal fluid in the MRT. Studies of sexually-transmitted infections (STIs) present in human semen have found that *Chlamydia trachomatis* (phylum Chlamydiae), *Neisseria gonorrhoeae* (phylum Proteobacteria), *Ureaplasma urealyticum* (phylum Firmicutes), *Mycoplasma genitalium* (phylum Tenericutes), and *Gardnerella vaginalis* (phylum Actinobacteria) among others, compromise sperm function and semen quality, resulting in male infertility in severe cases (Weng et al., 2014; Damke et al., 2018).

Whether the MRT harbours beneficial bacterial strains remains largely unknown. Nevertheless, considering that MRT bacteria are found in the ejaculates of both healthy and symptomatic men (symptomatic men had at least one abnormal spermiogram parameter, e.g.

low total sperm count or altered sperm motility; Baud et al., 2019), it is possible that not all reproductive bacteria are detrimental for the male host. For example, the genus *Lactobacillus* (phylum Firmicutes) could be mutualistic in male humans. Indeed, lactobacilli were detected in healthy, fertile human seminal fluid, and were hypothesised to buffer the negative effects of pathogenic bacteria (Weng et al., 2014). However, a more recent paper reported that lactobacilli can attach to sperm cells, slowing them down, thus negatively affecting male reproductive fitness (Wang et al., 2020a).

Considering that bacteria can have a considerable impact on male health and sperm quality, male hosts are expected to invest into maintaining their MRT microbiota to increase their fitness. Failing to maintain a "healthy" MRT microbiota may be detrimental, and bacteria-induced reproductive fitness costs are expected to be particularly pronounced in polygynandrous species (Ashby & Gupta, 2013), where both females and males mate with multiple partners, and males have the opportunity to fertilise the eggs of more than one female. The sperm of different males in a polyandrous system compete to fertilise the egg—a process known as sperm competition (Parker, 1970)—and the number of sperm in the ejaculate, and their motility and velocity are some of the key determinants of sperm competition outcome (Simmons, 2001; Snook, 2005; Pizzari & Parker, 2009).

Considering that MRT bacteria can negatively affect sperm by reducing their motility (Diemer et al., 1996) or even killing them (Hosseinzadeh et al., 2001), studying the potential bacteria-induced fitness consequences in males of polygynandrous systems would allow several hypotheses to be made about the role of MRT microbiota in host sexual selection and sexual conflict (Rowe et al., 2020a). Here, we attempted to do that by characterising the bacterial communities across the entire MRT of the polygynandrous red junglefowl, *Gallus gallus*, including patterns of variation within and between males, and discussing the results in the context of host evolutionary ecology.

Our model system, the red junglefowl—the main wild ancestor of the domestic chicken (*Gallus domesticus*; Kanginakudru et al., 2008; Lawal et al., 2020; Wang et al., 2020b)—lives in socially-structured polygynandrous groups where males are under strong pre- and post-copulatory sexual selection (Pizzari & McDonald, 2019). During the breeding season, males have the opportunity to fertilise the eggs of multiple females. Sperm competition outcome in fowl is largely determined by sperm number, but also sperm swimming velocity (Birkhead et al., 1999; Pizzari et al., 2008). Previous work in red junglefowl has found culturable bacteria in the ejaculates of this species (Eccleston, 2017), while bacteria residing across the avian MRT have not been studied in detail to our knowledge.

We had three specific aims. First, we characterised the MRT microbiota of the polygynandrous red junglefowl, and compared it with male digestive tract and cloacal bacterial communities. Cloaca is a shared outlet for the avian reproductive, digestive, and urinary tracts (Figure 1). This anatomical overlap could facilitate bacterial crossovers between the different tracts. Whether bacterial translocation is common in avian species is currently unknown, and thus, we decided to collect comparative samples from the colon and faeces. Second, we investigated patterns of spatial structure within MRT microbiota. Previous research in male humans has found that bacteria are present across the MRT, however, there is a significant decrease in bacterial diversity in the upper tract (Mändar, 2013). Similarly, a study in male donkeys (*Equus asinus*) was able to detect a diverse microbiota of aerobic and microaerophilic bacterial genera on their external genitalia (Carleton et al., 2015). We collected tissue samples across the MRT to test whether comparable patterns are present in male red junglefowl. Third, we explored the variation in MRT microbiota associated with individual phenotype, in particular male age class. Previous research suggest that the health status and age amongst others may affect the MRT microbiota in humans (Altmäe, Franasiak & Mändar, 2019; Mändar

et al., 2017). Moreover, cloacal (van Dongen et al., 2013) and faecal microbiota (Kreisinger et al., 2017) have been shown to vary with age, and the same could hold true for MRT microbiota.

We hypothesised that functional differences between the MRT and digestive tract lead to a distinct divergence of their microbiota, with the shared outlet cloaca harbouring an intermediate bacterial assemblage. We also expected to detect bacteria across the MRT, as a previous study in house sparrows (*Passer domesticus*) found culturable bacteria in male testes and cloacae (Stewart & Rambo, 2000). However, we predicted a decrease in bacterial richness and diversity in the upper sections of the MRT in comparison with the lower tract. Finally, we hypothesised that fowl age class may have a significant effect on MRT microbiota, considering that ageing has been found to significantly affect the reproductive output of male fowl (Noguera et al., 2012), which may in turn influence the MRT microbiota. In particular, we expected older males to have lower bacterial diversity in their MRT as sperm and seminal fluid characteristics change with fowl age (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016), and if bacteria are dependent on sperm cells and seminal-compounds as an energy source (Dávila et al., 2018), their abundance may decrease.

Materials and Methods

Study Population

The captive red junglefowl population at the John Krebs Field Station of the University of Oxford in Wytham was used for MRT dissections (Figure 1). Prior to humane culling by Schedule 1 (Home Office, 2014), the experimental birds (n=12) were kept in single-sex flocks, and housed in covered outdoor aviaries where they were given food and water *ad libitum*.

All male fowl appeared healthy and were sexually-mature. Individual birds wore unique Darvic rings that were used for identification and determining bird age class. The fowl were categorised into three age classes, including “young” (1–3 years), “old” (4–6 years), and “very old birds” (7–9 years), based on previous literature that suggests that reproductive senescence

starts in 4–6-year-old male feral fowl and junglefowl (Dean et al., 2010; McDonald et al., 2017). Our study included four male birds per age class. All experimental work was conducted under the regulation of a Home Office project license (PIL: P50402706).

Dissections in the Field

In total, 12 male red junglefowl were sacrificed in the breeding season of 2018 (experimental period: 22 June–12 July). The humanely culled males were immediately dissected, practising sterile laboratory etiquette to minimise contamination risk (Salter et al., 2014). All dissections were carried out by a single experimenter who wore a clean laboratory coat and changed nitrile gloves between carcasses. A new set of individually-packaged, sterile dissection equipment was used per carcass. The gloves and equipment were sterilised with 70% ethanol and 1% Virkon^{TM} (DuPont, Wilmington, Delaware, USA) during individual bird dissections. All dissections were carried out on an ethanol-sterilised board that was covered with a sterile film, which was changed between carcasses.

MRTs were removed in their entirety and sectioned into five functionally-distinct sections (Figure 1). Tissue samples were collected from the cloaca (n=12), papillae (n=12), lower vasa deferentia (n=24), upper vasa differentia (n=24), and testes (n=24). The lower and upper vasa deferentia, and testes are duplicate organs—the two MRT sides reach their respective papilla and are joined by the cloaca (Figure 1). Thus, tissue samples were collected from both sides of the upper MRT. A colon tissue sample (n=12) and a faecal sample (n=12) were harvested as controls from the digestive tract. The weight of tissue samples was not standardised during collection due to time constraints and increased contamination risk in the field. However, the tissue collection site per functionally-distinct section was kept relatively constant across carcasses by eyeing the reproductive and digestive tracts. Collected samples (n=120) were immediately stored at -80°C in individual, sterile tubes.

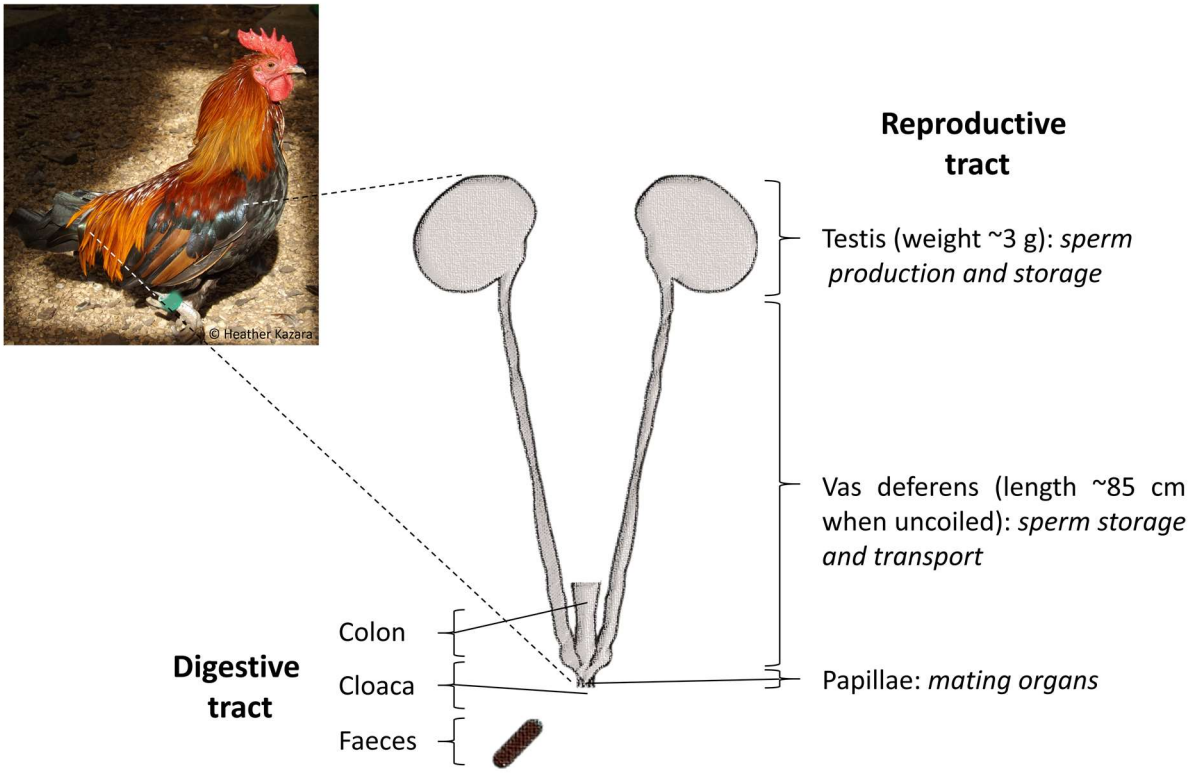

Figure 1. Male red junglefowl reproductive tract. Each functionally-distinct MRT section is indicated with its *function*. The cloaca was analysed separately from the reproductive and digestive tract samples, as it was expected to harbour an intermediate bacterial composition. Vasa deferentia, presented here as tightly coiled, were cut in half, producing lower and upper vasa deferentia samples. All presented sections (including faeces) were included in this study. The photo of a male red junglefowl in Wytham was taken by Dr Heather Kazara and is used with permission.

Tissue Sample Preparation in the Laboratory

Post-fieldwork laboratory processing took place in 2019–2020 at Silwood Park, Imperial College London. Additional tissue sample handling was needed to standardise sample weights across the data set and minimise inhibitors—in particular host DNA—in downstream laboratory processes. Preliminary experiments showed that including deeper layers of the epithelium resulted in non-specific and/or failed PCR amplification potentially due to the increased input of host cells, thus introducing inhibiting host DNA that we hypothesised caused the DNA amplification error. The secondary dissections were carried out on ice and in a biological safety cabinet, minimising sample degradation and contamination. Individual, sterile Petri dishes, scalpels, and forceps were used per dissection. The uppermost epithelial layer

inside each section was scraped off under a microscope, collecting a standardised ~10 mg tissue sample per functionally-distinct section.

The upper MRT was expected to harbour less bacterial biomass than the lower MRT, as has been reported in humans (Mändar, 2013), and thus, the left- and right-side lower and upper vasa deferentia, and testes were combined per individual bird to increase bacterial DNA extraction and amplification success ($n_{per\ section}$=12, n_{total}=84). In these cases, ~5 mg of tissue was collected from either side. There is currently no evidence suggesting that the duplicate sides of MRT harbour distinct bacterial communities.

DNA Extraction and Quantification

DNA from tissue samples was extracted with an unopened QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) in a pre-PCR biological safety cabinet that had previously been sterilised with DNAZap™ PCR DNA Degradation Solutions (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 70% ethanol.

Before lysis, the samples were handled on ice to avoid compromising their bacterial compositions. To increase yield from Gram-positive bacteria (Gill et al., 2016), the manufacturer's protocol for tissue samples was altered by adding lysozyme from chicken egg white (0.4 mg/ml; Sigma-Aldrich, St. Louis, Missouri, USA) to the ATL Buffer and allowing 30 minutes of incubation at 37°C, after which we proceeded with the Proteinase K step in the manufacturer's protocol. The second incubation step at 56°C lasted for an hour, after which all biological matter had disintegrated.

DNA from faecal samples was extracted with an opened *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, California, USA), following the manufacturer's protocol. We had to opt for another DNA extraction kit for the faecal samples as preliminary experiments revealed that the DNA concentration and PCR amplification were

low and not comparable with the rest of the biological samples when using the QIAGEN DNeasy® Blood & Tissue Kit with faeces.

Controls were collected from both DNA extraction kits for downstream contamination analysis (DNeasy® Blood & Tissue DNA extraction kit, n=1 (one per 96-well plate); and *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit, n=1). The samples were quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) with a Qubit® Fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol. All samples were standardised to a DNA concentration of 5 ng/µl and stored at -20°C.

Amplification Polymerase Chain Reactions (PCRs)

Before and after each set-up, the PCR hood was cleaned with DNAZap™ PCR DNA Degradation Solutions and 70% ethanol, followed by a 30-minute UV-sterilisation. Consumables were UV-sterilised separately for 30 minutes. PCRs were set up on ice with sterile, filtered pipette tips and reagents taken from unopened aliquots.

The V4 region (~254 base pairs) of the bacterial 16S SSU rRNA gene was amplified, following the guidelines of the Earth Microbiome Project (Thompson et al., 2017) and Walters et al. (2015). Reactions were prepared on 96-well plates, and in three replicates to minimise amplification bias and increase yield from rare bacterial strains. 5 ng or up to 5 µl (if the concentration was too low) of DNA template, was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus* (Sigma-Aldrich), 5 µl of *Taq* DNA Polymerase PCR Buffer 10X (Sigma-Aldrich), 0.5 µl of Bovine Serum Albumin (Sigma-Aldrich), 0.1 µl of 100 nM dNTP mix (Thermo Fisher Scientific), 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) to make up the rest of the reaction volume.

Two PCR negative controls per 96-well plate was prepared first and sealed before template DNA entered the PCR hood. A ZymoBIOMICS® Microbial Community Standard (Zymo Research) was used as a positive standard to assess amplification success.

Parameters for the amplification PCRs were as follows: initial three-minute denaturing at 94°C followed by 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all replicate plates. Amplification PCR products were tested on a 1% agarose gel, stained with GelRed™ (Biotium, Fremont, California, USA) according to the manufacturer's protocol, in a 1×TBE buffer at 80 V for 45 minutes.

Clean-Up, Indexing PCRs, and Normalisation

Replicate biological samples were pooled and cleaned of primer dimers with the ZR-96 DNA Clean-up Kit™ (Zymo Research), following the manufacturer's protocol.

The samples were dual-indexed with unique combinations proposed by Kozich et al. (2013). 20 ng of amplification PCR product was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus*, 5 µl of *Taq* DNA Polymerase PCR Buffer 10X, 0.1 µl of 100 nM dNTP mix, 0.0625 µM (final concentration) of forward and reverse index primer (Supplementary Table 1) each, and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the reaction volume.

An indexing PCR negative control was processed (n=1). DNA extraction kit controls (DNeasy® Blood & Tissue DNA extraction kit, n=1; and *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit, n=1), amplification PCR negative controls (n=6), and a single positive standard (n=1) were indexed as biological samples.

Parameters for the indexing PCRs were as follows: initial two-minute denaturing at 95°C followed by 15 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at

72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all indexing PCRs. Indexing PCR products were tested on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes.

The dual-indexed samples were quantified using the Qubit™ methodology. 5 ng of each sample was pooled into a DNA LoBind Tube (Eppendorf, Hamburg, Germany) and cleaned with the DNeasy® PowerClean Pro Cleanup Kit (QIAGEN).

The library was tested for size and quality on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes. Library purity was assessed from the 260/280 nm ratio, calculated using the Take3™ Micro-Volume Plate (BioTek Instruments, Inc., Winooski, Vermont, USA) in a BioTek microplate reader (BioTek Instruments, Inc.) and the Gen5 programme (BioTek Instruments, Inc.). The quality-checked library was stored at -20°C prior to sequencing. Immediately before sequencing, the library was quantified using the Qubit™ methodology and diluted to 4 nM.

Next-Generation Sequencing (NGS)

NGS was carried out at the Silwood Park sequencing facility (Imperial College London), following Illumina's proposed protocol for 16S SSU rRNA gene metagenomic sequencing (Illumina, 2013). A 15 pM library with a 5% PhiX control (Illumina, Inc., San Diego, California, USA) was loaded into the V3 chemistry MiSeq cartridge (Illumina, Inc.) to achieve 2*300 base pair (bp) sequencing reads across 84 biological and 10 control samples.

Bioinformatic Data Processing

Post-sequencing sample demultiplexing was carried out automatically by BaseSpace (Illumina, Inc.). Paired-end FASTQ files were processed with *dada2* (Callahan et al., 2016a; Version 1.12.1) in R (R Core Team, 2019; Version 3.6.1). Reads were quality-checked and

filtered using standard parameters (maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2) with additional truncLen=c(200, 200), resulting in a ~10% sequencing read loss. The error rates were calculated based on a random subset of samples (randomize=TRUE) and inference was carried out via pseudo-pooling (pool="pseudo"). Non-specific amplicons were removed, retaining only merged, denoised reads of 250–255 bp. Chimera check removed ~67% of the merged sequence variants, which accounted for ~17% of the total reads. SILVA reference database (Yilmaz et al., 2014; Version 132) was used for assigning taxonomies (100% identity) to the species level, when possible.

The processed data set with controls contained 94 samples, 4130 amplicon sequence variants or ASVs (203±13 S.E. per sample) and 2,278,465 reads (24,239±1376 per sample). *decontam* (Davis et al., 2017) was used for further filtering of potential contaminants, which were detected based on ASV prevalence in control versus biological samples, using a stringency threshold of 0.55 that split ASVs into contaminants (141 ASVs) and non-contaminants (3989 ASVs).

There were no biological samples with fewer than 100 sequencing reads. ASVs that were present in the taxonomy, but not in the data set due to data filtering (n=145) were excluded. Finally, ASVs that failed to classify at the phylum level (n=56), or that classified as chloroplasts (n=25) or mitochondria (n=9; de la Cuesta-Zuluaga & Escobar, 2016) were removed.

The final data set without controls contained 84 samples, 3754 ASVs (205±12 per sample), and 1,867,468 reads (22,232±1159 per sample). Mean relative bacterial abundances found in negative controls and the positive standard were visualised with *ggplot2* (Wickham, 2016) and are presented in the appendices (Supplementary Figure 1).

Statistical Analysis and Data Visualisation

Statistical analysis and data visualisation were carried out in R (Version 3.6.1). Significance threshold was set to $p<0.05$ in all tests.

Hereafter, we define male red junglefowl reproductive tract (tissue samples harvested across the MRT; n=48), cloaca (cloaca tissue samples; n=12), and digestive tract (colon tissue samples and faecal samples; n=24) as "host ecosystems"; we define tissue samples (all tissue samples harvested across MRT, cloaca, and digestive tract; n=72) and faecal samples (n=12) as "sampling method"; and we define papillae tissue, lower vasa deferentia tissue, upper vasa deferentia tissue, testes tissue, cloaca tissue, and colon tissue (n=12 per sample type), and faeces (n=12) as "sample type". We first tested higher-level variation across host ecosystems and sampling methods. Then, we compared the variation across specific sections and faeces by using sample type as a variable to even out sample sizes per group and avoid rank deficiency in statistical modelling.

(1) *Characterising bacterial compositions of functionally-distinct MRT sections*. MRT, cloacal, and digestive tract microbiota composition variation was analysed using β-diversity modelling, which allowed us to compare local bacterial diversity to regional bacterial diversity (Whittaker, 1960). *DECIPHER* (Wright, 2016) and *phangorn* (Schliep, 2011; Version 2.5.5) were used to align and create a phylogenetic tree respectively, as proposed by Callahan et al. (2016b). Weighted (wUF) and unweighted UniFrac (uwUF) distances (Lozupone & Knight, 2005) were calculated with *phyloseq* (McMurdie & Holmes, 2013), using the trimmed data set (singletons—defined as ASVs present only once across all samples—were removed: 9 ASVs, i.e. 0.24% of the ASVs) that was first agglomerated to either bacterial phylum or genus level, and then normalised via proportions (McMurdie & Holmes, 2014; Callahan et al., 2016b; McKnight et al., 2019). Using the same normalised-by-proportions data set, we also calculated

Bray-Curtis dissimilarities and Jaccard (dissimilarity) indices at both the phylum and genus level with *vegan* (Oksanen et al., 2019) for comparative β-diversity analysis.

We did not study bacterial strain-level variation as genus was the finest phylogenetic level that allowed us to assign a taxonomic identity to the majority of our sequencing reads (2547 out of 3754 ASVs, i.e. 67.8%, were assigned a genus identity). This was due to the current bacterial strain databases having limited information about non-human host microbiota, and because of the poor species- and strain-assignment potential of 16S SSU rRNA gene hypervariable region V4 (Gohl et al., 2016; Johnson et al., 2019b). Preliminary statistical tests revealed that strain-level β-diversity analyses with both ANOSIM and PERMANOVA had near-identical results with the genus-level β-diversity analyses, with the strain-level analyses showing slightly lower p-values that did not affect the significance patterns that we were able to observe at the genus-level. This assured us to proceed with our approach, knowing that our results at the phylum and genus level are biologically relevant, and allowing us to pinpoint specific key taxa that contribute to the variation.

Microbiota composition variation between host ecosystems ($n_{\text{reproductive tract}}$=48, n_{cloaca}=12, or $n_{\text{digestive tract}}$=24) and sampling methods ($n_{\text{tissue sample}}$=72 or $n_{\text{faecal sample}}$=12) was tested with separate ANOSIM models (anosim(); permutations=1000) from *vegan*, constraining the permutations within strata=bird ID (n=12).

To compare microbiota composition variation between sample type, PERMANOVA (adonis(); permutations=1000) from *vegan* was performed, setting sample type ($n_{\text{papillae tissue}}$=12, $n_{\text{lower vasa deferentia tissue}}$=12, $n_{\text{upper vasa deferentia tissue}}$=12, $n_{\text{testes tissue}}$=12, $n_{\text{cloaca tissue}}$=12, $n_{\text{colon tissue}}$=12, or n_{faeces}=12) and age class (n_{young}=28, n_{old}=28, or $n_{\text{very old}}$=28) as fixed effects, constraining the permutations within strata=bird ID (n=12). Significant PERMANOVA results were followed up with beta dispersion tests (betadisper() followed by permutest(); permutations=1000) from *vegan* to control for significant dispersion differences.

Stacked bar plots of mean relative bacterial abundances were created with *ggplot2*. Bacterial phyla and genera that contributed <1% to the mean relative abundances across sample type were considered rare and thus excluded from the figure. To visualise pairwise differences between sample type, a heatmap with average linkage hierarchical clustering was created with *Heatplus* (Ploner, 2019) based on genus-level Bray-Curtis dissimilarities, which were preferred over other distances as they explained the largest amount of microbiota β-diversity variation at the genus level (Table 1B).

Differential bacterial abundance across sample type was tested using *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was agglomerated to phylum or genus level, filtered to include only taxa that contributed >1% to the mean relative bacterial abundances across sample type, and tested with differentialTest(test="Wald", fdr_cutoff=0.05), while controlling for bacterial abundance across fowl age class, and variance across sample type and age class. Results were visualised with *corncob*.

(2) *Characterising bacterial richness and diversity of functionally-distinct MRT sections.* Spatial structure in fowl MRT microbiota was analysed with α-diversity modelling, which allowed us to explore and compare local bacterial diversity (Whittaker, 1960). Three different α-diversity measures were calculated using the untrimmed, i.e. including singletons, data set. Total ASV richness estimates were calculated with *breakaway* (Willis et al., 2018), and Shannon's and inverse Simpson's diversity index estimates with *phyloseq*. Normality of each α-diversity metric was assessed with a Shapiro-Wilk test, and suitable data distributions were determined from histograms, which were then confirmed with *fitdistrplus* (Delignette-Muller & Dutang, 2015), using Cullen and Frey graphs (bootstrap=500). Total ASV richness estimates followed a uniform distribution, Shannon's diversity index estimates followed a Gaussian distribution (link="log"), and inverse Simpson's diversity index estimates followed a gamma distribution (link="log").

α-diversity variation between host ecosystems ($n_{reproductive\ tract}$=48, n_{cloaca}=12, or $n_{digestive\ tract}$=24) and sampling methods ($n_{tissue\ sample}$=72 or $n_{faecal\ sample}$=12) was tested with separate models. Total ASV richness variation was compared using betta_random() from *breakaway*, with bird ID (n=12) as a random factor. Shannon's and inverse Simpson's diversity index estimates were compared with Kruskal-Wallis chi-squared tests. If Kruskal-Wallis chi-squared tests indicated significance, the groups were compared pairwise with Wilcoxon rank sum tests that were adjusted for false discovery rate (fdr).

Total ASV richness variation between sample type was statistically compared with betta_random() from *breakaway*, setting sample type ($n_{papillae\ tissue}$=12, $n_{lower\ vasa\ deferentia\ tissue}$=12, $n_{upper\ vasa\ deferentia\ tissue}$=12, $n_{testes\ tissue}$=12, $n_{cloaca\ tissue}$=12, $n_{colon\ tissue}$=12, or n_{faeces}=12) and age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28) as fixed effects, and bird ID (n=12) as a random effect.

Generalised linear mixed-effects models (GLMMs) with Laplace approximation were fitted to Shannon's and inverse Simpson's diversity index estimates with *lme4* (Bates et al., 2015), setting sample type and age class as fixed effects, and bird ID as a random effect. Goodness of fit was assessed from Q-Q and residual plots. Function Anova() from *car* (Fox & Weisberg, 2019) was used for deviance analyses (type II Wald chi-square tests). Specific trends and random effect statistics were obtained from model summaries. Total ASV richness was visualised with *breakaway*, and Shannon's and inverse Simpson's diversity index estimates with *ggpubr* (Kassambara, 2020).

(3) *Effect of individual variation in age class on male fowl microbiota*. We first inferred the overall effect of fowl age class on red junglefowl microbiota from β- and α-diversity modelling performed in aims (1) and (2) respectively, where fowl age class was included in models to control for its effect.

As we were additionally interested in the effect of age class solely on the MRT microbiota, we excluded the digestive tract controls and created a reduced data set of only the

MRT and cloaca samples ($n_{papillae\ tissue}$=12, $n_{lower\ vasa\ deferentia\ tissue}$=12, $n_{upper\ vasa\ deferentia\ tissue}$=12, $n_{testes\ tissue}$=12, $n_{cloaca\ tissue}$=12). We decided to include the cloaca as it is a part of the MRT. We explored differentially-abundant bacterial phyla and genera across male fowl age class using *corncob*, as in aim (1). The untrimmed data set, i.e. including singletons, was agglomerated to either phylum or genus level, filtered to include only taxa that contributed >1% to the mean relative bacterial abundances across age class (n_{young}=20, n_{old}=20, or $n_{very\ old}$=20), and tested with differentialTest(test="Wald", fdr_cutoff=0.05), while controlling for bacterial abundance across sample type, and variance across sample type and age class. Results were visualised with *corncob*.

Results

(1) *Characterising Bacterial Compositions of Functionally-Distinct MRT Sections*

Male red junglefowl reproductive tract, cloaca, and digestive tract harboured significantly distinct bacterial compositions (Figures 2A, 3A; Supplementary Table 2A). While the phylum Firmicutes dominated male fowl reproductive tract (47.6±3.4% S.E.), cloacal (51.9±5.9%), and digestive tract (61.6±6.3%) microbiota (Figure 2A), the reproductive tract harboured more Proteobacteria (16.4±2.7%), the cloaca more Actinobacteria (16.8±3.7%), and the digestive tract more Deferribacteres (4.8±3.3%; Figure 2A) in comparison with each other. *Fusobacterium* (phylum Fusobacteria) was more prevalent in fowl MRT (18.7±3.6%) and cloaca (21.5±8.1%), while *Lactobacillus* (phylum Firmicutes) was the most prevalent genus in the digestive tract (21.9%±6.5; Figure 3A).

Comparisons between sampling methods showed that tissue and faecal samples captured significantly different microbiota compositions (Figures 2B, 3B; Supplementary Table 2B). Tissues captured more *Fusobacterium* (20.5±3.1%), and faeces had a higher relative abundance of *Lactobacillus* (45.0±11.2%) and *Romboutsia* (phylum Firmicutes; 24.1±9.6%;

Figure 3B). Tissue samples also captured a large number of rare bacterial genera (29.4±0%) that contributed <1% each to the mean relative abundances (Figure 3B).

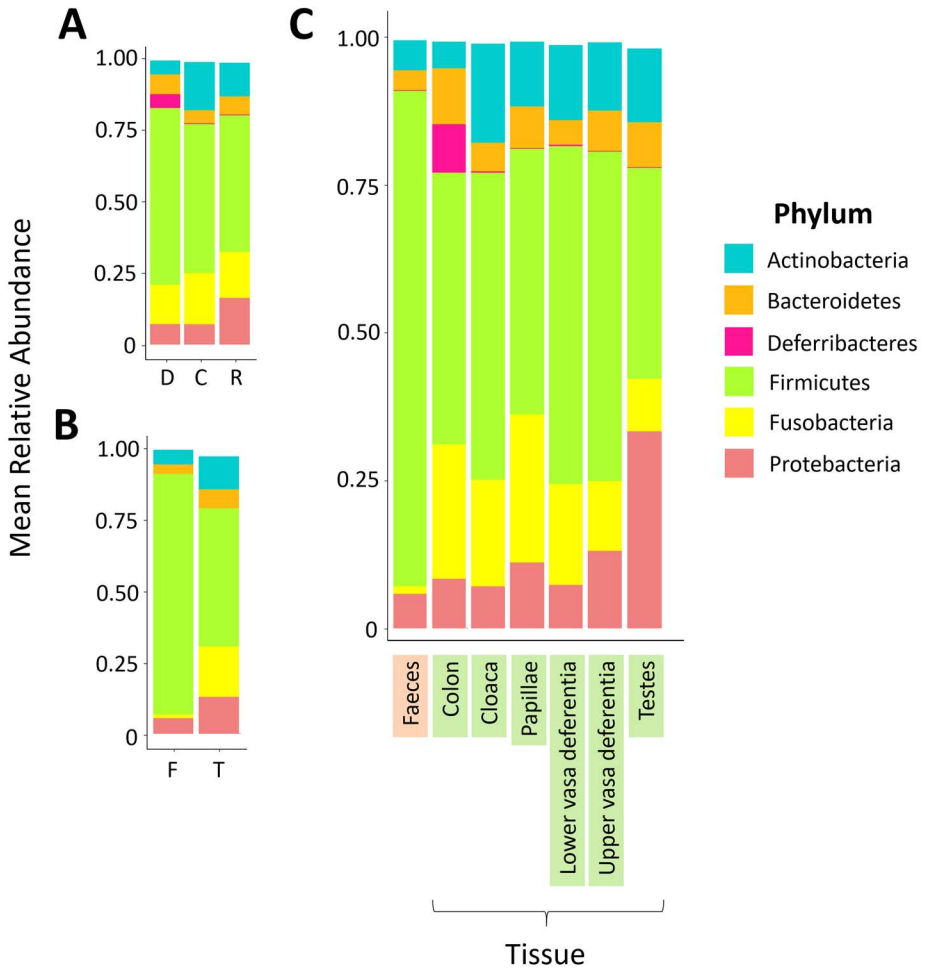

Figure 2. Stacked bar plots depicting mean relative bacterial phylum abundances per host ecosystem (D=digestive tract (n=24), C=cloaca (n=12), R=reproductive tract (n=48); A), sampling method (F=faecal sample (n=12), T=tissue sample (n=72); B), and sample type ($n_{\text{per sample type}}$=12; C). Functionally-distinct sections of the MRT (C) are presented from left to right in an anatomically-ascending order, starting with the cloaca. Each bacterial phylum is indicated by a unique colour. Phyla that contributed <1% to the mean relative abundances were excluded from the figures.

Sample types differed significantly from each other in their microbiota composition (Table 1), which was attributed to significant differential bacterial phylum and genus abundances (Figures 2C, 3C, 4). Beta dispersion was significantly different between sample types based on unweighted ($p<0.05$), but not weighted ($p>0.05$) UniFrac distances regardless of the phylogenetic level.

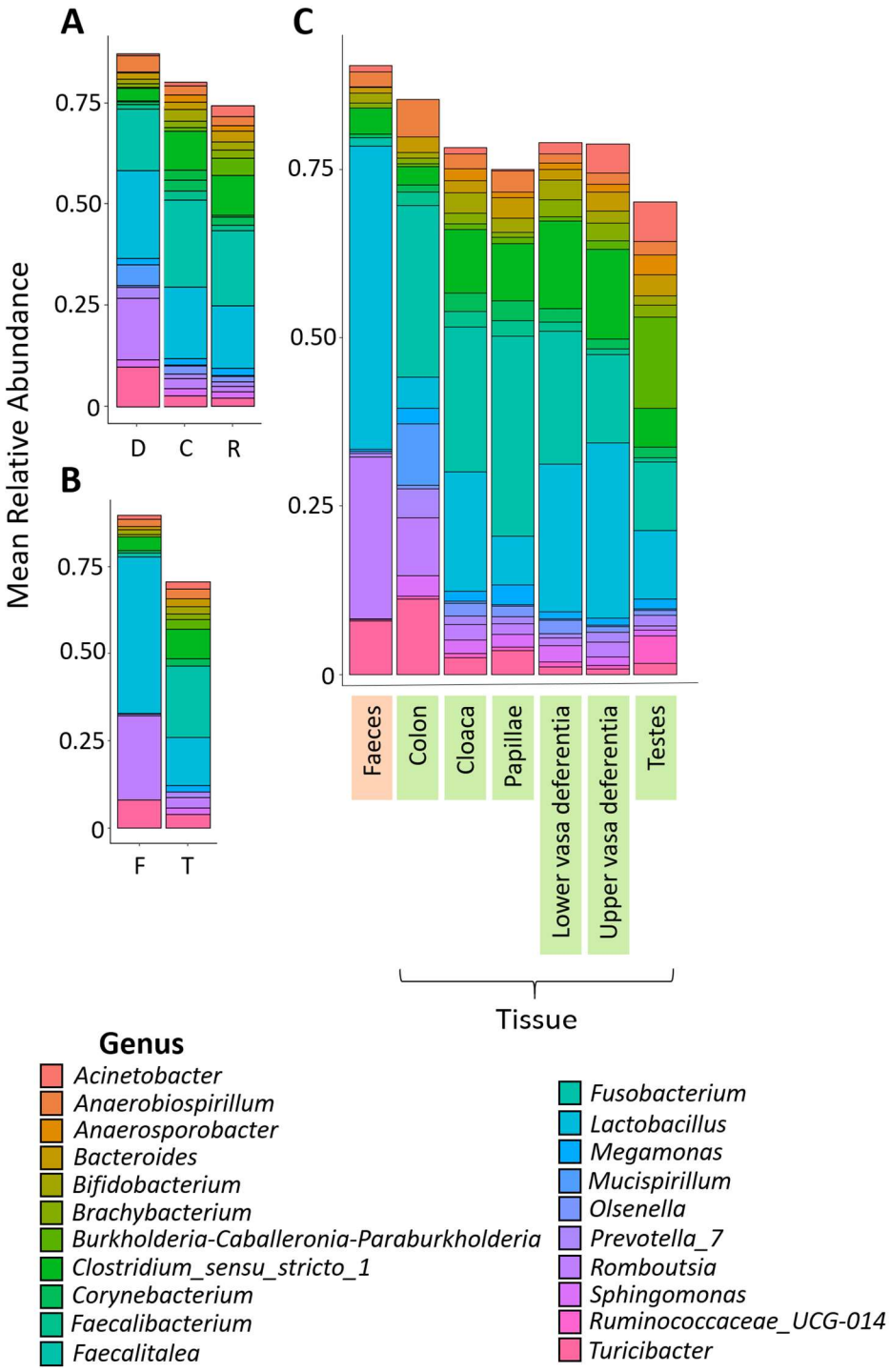

Figure 3. Stacked bar plots depicting mean relative bacterial genus abundances per host ecosystem (D=digestive tract (n=24), C=cloaca (n=12), R=reproductive tract (n=48); A), sampling method (F=faecal sample (n=12), T=tissue sample (n=72); B), and sample type ($n_{\text{per sample type}}$=12; C). Functionally-distinct sections of the MRT (C) are presented from left to right in an anatomically-ascending order, starting with the cloaca. Each bacterial genus is indicated by a unique colour and genera are separated by black lines for clarity. Genera that contributed <1% to the mean relative abundance were excluded from the figures.

Table 1. [PERMANOVA] Microbiota composition variation between sample type ($n_{per\ sample\ type}$=12) and age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28) at bacterial phylum (A) and genus level (B), using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=12). Significant p-values are in bold.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**3.44**	**6**	**<0.001**	**14.1%**
Age class	**14.96**	**2**	**<0.001**	**20.4%**
Bird ID	**3.37**	**9**	**<0.001**	**20.7%**
Residuals		66		44.8%
Unweighted UniFrac				
Sample type	**2.95**	**6**	**<0.001**	**14.8%**
Age class	**3.52**	**2**	**<0.001**	**5.9%**
Bird ID	**3.20**	**9**	**<0.001**	**24.1%**
Residuals		66		55.2%
Bray-Curtis DS				
Sample type	**3.72**	**6**	**<0.001**	**14.7%**
Age class	**16.26**	**2**	**<0.001**	**21.4%**
Bird ID	**3.43**	**9**	**<0.001**	**20.3%**
Residuals		66		43.6%
Jaccard Index				
Sample type	**2.44**	**6**	**<0.001**	**12.3%**
Age class	**4.06**	**2**	**<0.001**	**6.8%**
Bird ID	**3.39**	**9**	**<0.001**	**25.6%**
Residuals		66		55.3%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**2.56**	**6**	**<0.001**	**11.1%**
Age class	**11.29**	**2**	**<0.001**	**16.3%**
Bird ID	**3.82**	**9**	**<0.001**	**24.9%**
Residuals		66		47.7%
Unweighted UniFrac				
Sample type	**2.72**	**6**	**<0.001**	**14.4%**
Age class	**3.28**	**2**	**<0.001**	**5.8%**
Bird ID	**2.71**	**9**	**<0.001**	**21.5%**
Residuals		66		58.3%
Bray-Curtis DS				
Sample type	**2.81**	**6**	**<0.001**	**11.7%**
Age class	**11.70**	**2**	**<0.001**	**16.3%**
Bird ID	**4.18**	**9**	**<0.001**	**26.1%**
Residuals		66		45.9%
Jaccard Index				
Sample type	**2.09**	**6**	**<0.001**	**11.8%**
Age class	**2.98**	**2**	**<0.001**	**5.6%**
Bird ID	**2.36**	**9**	**<0.001**	**20.1%**
Residuals		66		62.5%

Beta dispersion was not significantly different between sample types based on Bray-Curtis dissimilarities ($p>0.05$) nor Jaccard indices ($p>0.05$), except for the genus-level Jaccard indices that showed significant dispersion variation between sample types ($p<0.05$). While the MRT and colon tissue were relatively similar to each other (Figures 2C, 3C), the significant microbiota composition differences were mostly driven by faeces harbouring a distinct bacterial composition (Figure 5).

Three bacterial phyla and 11 genera were significantly differentially-abundant across sample type (Figure 4). Of note, colon tissue harboured a significantly increased abundance of

the phylum Deferribacteres (β±S.E.=2.60±1.06, t=2.46, p=0.016) in comparison with other sample types (Figures 2C, 4A). *Clostridium_sensu_stricto_1* (phylum Firmicutes) and *Burkholderia-Caballeronia-Paraburkholderia* (phylum Proteobacteria) were predominantly abundant in MRT and colon tissue, but absent in faeces (Figures 3C, 4B). *Corynebacterium* (phylum Actinobacteria) was more prevalent in MRT tissue, while faeces were enriched with *Lactobacillus* (Figures 3C, 4B).

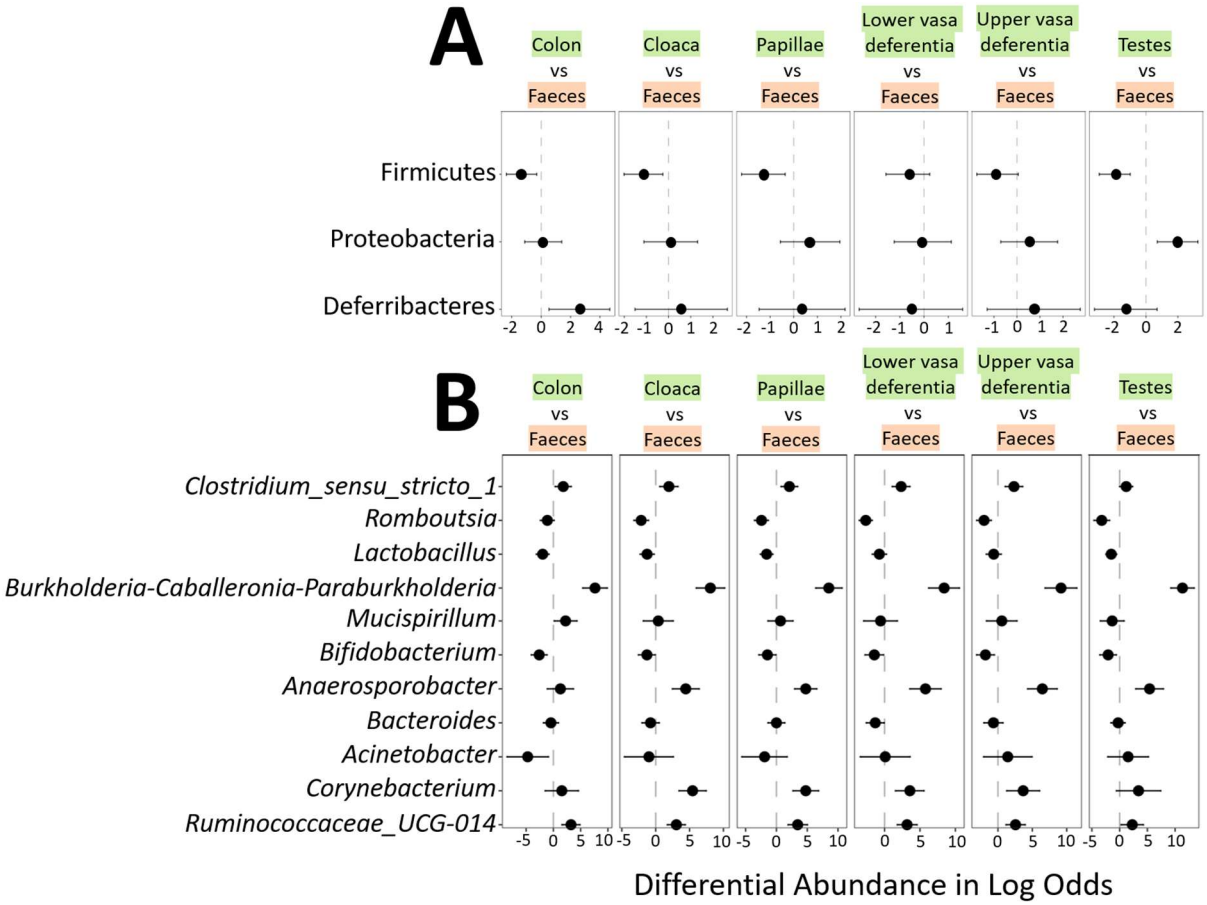

Figure 4. Differential abundance (in log odds) of bacterial phyla (A) and genera (B) between sample type ($n_{per\ sample\ type}$=12), controlling for fowl age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28). The grey dashed line represents the point of comparison, i.e. the faeces. Data points with standard error bars depict log odds estimates of the specific bacterial taxa. Positive log odds indicate significant enrichment, while negative log odds indicated the significant under-representation of the bacterial taxon. Tissue samples are indicated by lime green, faecal samples by peach.

(2) *Characterising Bacterial Richness and Diversity of Functionally-Distinct MRT Sections*

Host ecosystems revealed significant differences in total ASV richness estimates. Male fowl cloaca harboured the highest total ASV richness (intercept; β±S.E.=240.7±10.3, p<0.001), whereas MRT (β=-38.5±13.7, p=0.005) and digestive tract (β=-62.2±19.4, p=0.001) had significantly decreased richness estimates.

Shannon's diversity differed significantly between host ecosystems (χ^2=8.69, df=2, p=0.01), with the MRT and cloaca harbouring similar diversity (p=0.96), while the digestive tract had significantly lower diversity than either of them (p=0.02).

Inverse Simpson's diversity estimates revealed similar significant differences between host ecosystems (χ^2=9.23, df=2, p=0.01). The MRT and cloacal bacterial diversities were similar to each other (p=0.83), whereas fowl digestive tract harboured significantly lower bacterial diversity than either of the other two host ecosystems (p=0.02).

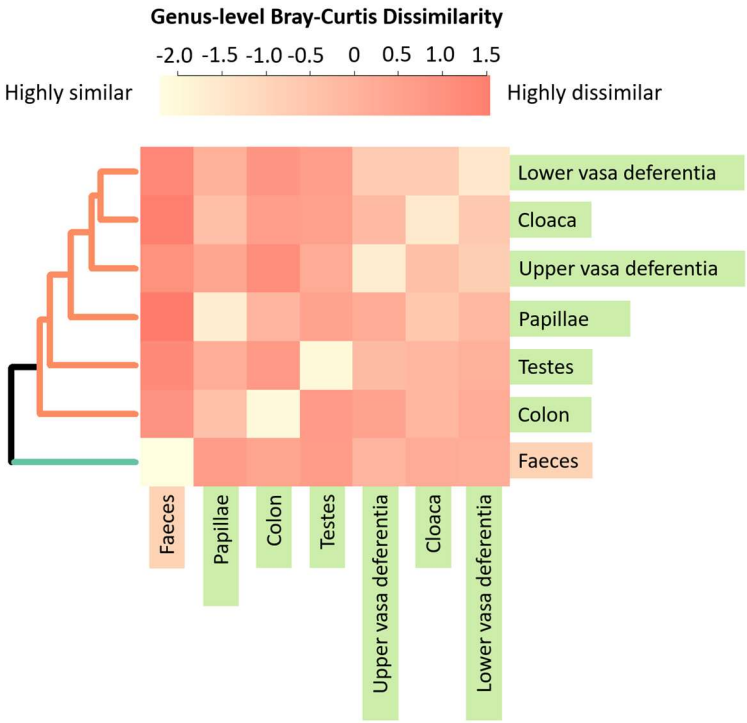

Figure 5. A heatmap with average linkage hierarchical clustering, depicting genus-level Bray-Curtis dissimilarities between sample type ($n_{\text{per sample type}}$=12). Tissue samples are indicated by lime green, and faeces by peach. Spectrum of similarity to dissimilarity is presented by a range from light yellow to light red. Two separate clusters (dendrogram cutting height=0.60) are highlighted by unique colours.

Sampling methods also significantly differed. Faecal samples (intercept; β=157.4±10.0, p<0.001) captured significantly lower total ASV richness than tissue samples (β=56.9±10.8, p<0.001). Shannon's (χ^2=7.14, df=1, p=0.008) and inverse Simpson's diversity (χ^2=8.35, df=1, p=0.004) were also significantly lower in faecal samples.

Total ASV richness estimates per sample type revealed that bacterial richness was higher in MRT tissue than in colon tissue and faeces (Table 2, Figure 6). Total ASV richness was high across the MRT, but showed an increase in vasa deferentia tissue, and a decrease in papillae and testes tissue (Table 2, Figure 5).

Table 2. [betta_random] Total ASV richness estimates per sample type ($n_{per\ sample\ type}$=12) and age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28). The *intercept* is the total ASV richness estimate for faeces collected from young birds. Functionally-distinct sections of the MRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold.

	Total ASV Richness	Estimate	S.E.	p-value
	Intercept	**194.9**	**9.3**	**<0.001**
Tissue	Colon	**67.3**	**24.4**	**0.006**
	Cloaca	**87.0**	**24.2**	**<0.001**
	Papillae	46.2	24.1	0.060
	Lower vasa deferentia	**107.8**	**24.2**	**<0.001**
	Upper vasa deferentia	**97.9**	**26.7**	**<0.001**
	Testes	3.7	24.2	0.880
	Old	**-56.1**	**18.0**	**0.002**
	Very old	**-91.3**	**13.1**	**<0.001**

Shannon's diversity was also significantly affected by sample type (χ^2=18.85, df=6, p=0.004). In particular, MRT tissue harboured significantly higher diversity than colon tissue and faeces (Supplementary Table 3; Supplementary Figure 2).

Similarly, inverse Simpson's diversity estimates differed between sample type (χ^2=26.5, df=6, p<0.001), with MRT and colon tissue harbouring more bacterial diversity than faeces (Supplementary Table 4; Supplementary Figure 3).

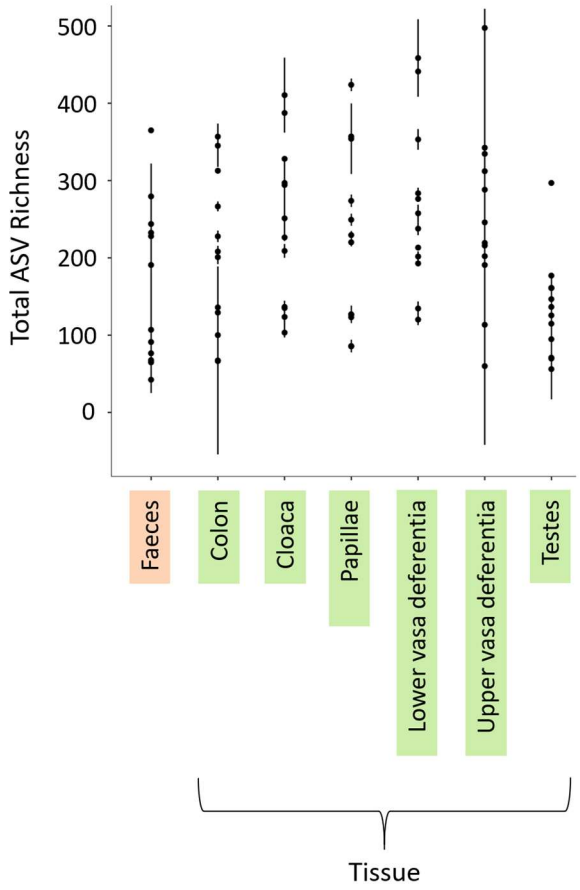

Figure 6. Total ASV richness estimates per sample type ($n_{per\ sample\ type}$=12). Dots with standard errors are individual samples (n=84). Functionally-distinct sections of the MRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

(3) *Effect of Individual Variation in Age Class on Male Fowl Microbiota*

Fowl age class had a significant overall effect on male fowl microbiota composition (Table 1), and these differences were not due to significant beta dispersion differences (p>0.05 for each β-diversity model; Table 1). A considerable amount of microbiota composition variation was explained by bird ID (mean±S.E.=22.8±1.01%; Table 1).

Both old (4–6 years) and very old birds (7–9 years) harboured significantly lower overall total ASV richness, with the effect stronger in very old males (Table 2). Shannon's (χ^2=2.62, df=2, p=0.27) and inverse Simpson's (χ^2=3.07, df=2, p=0.216) diversity estimates were not significantly affected by fowl age class, but showed a similar pattern of decreased bacterial diversity in old and in particular very old birds (Supplementary Tables 3, 4).

Five bacterial phyla and 15 bacterial genera were significantly differentially-abundant in MRT and cloaca across age class (Figure 7). Of note, *Fusobacterium* was significantly enriched in very old males (β±S.E.=5.99±0.49, t=12.14, p<0.001; Figure 7).

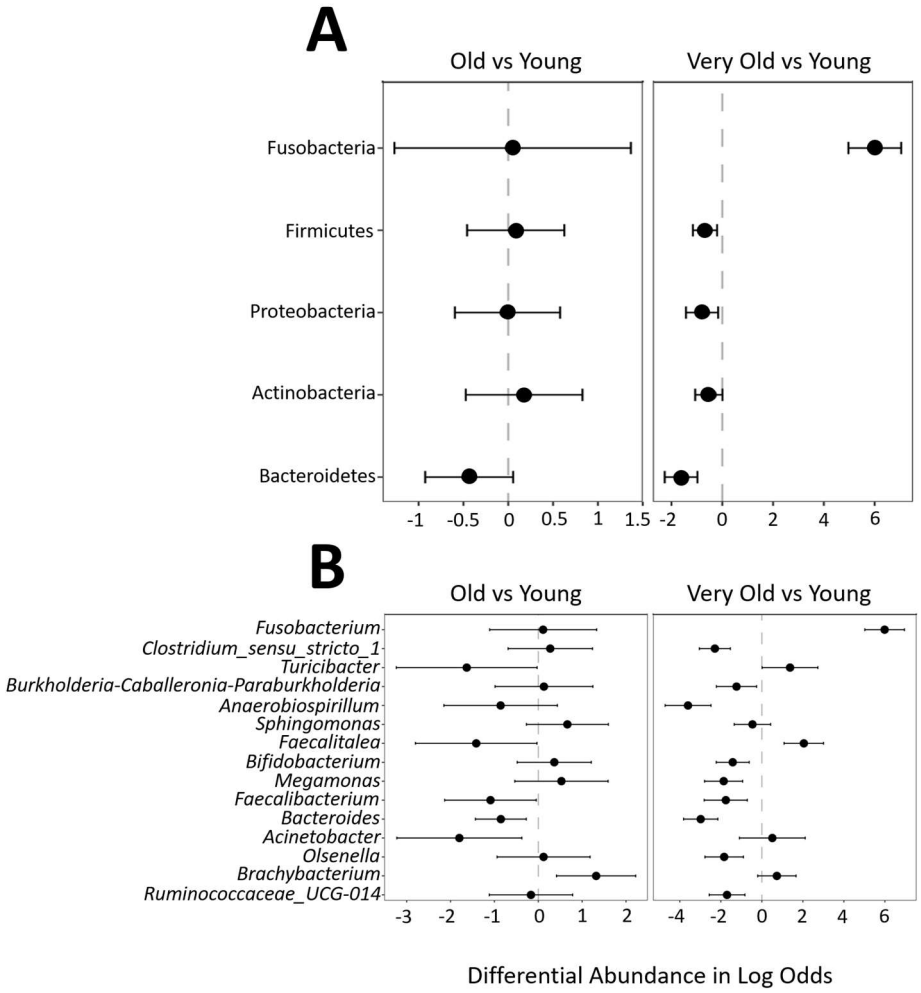

Figure 7. Differential abundance (in log odds) of bacterial phyla (A) and genera (B) between age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28), controlling for sample type. The grey dashed line represents the point of comparison, i.e. young male fowl. Data points with standard error bars depict log odds estimates of the specific bacterial taxa. Positive log odds indicate significant enrichment, while negative log odds the significant under-representation of the bacterial taxon across age class.

Discussion

Pathogenic bacteria present in the MRT may lower sperm motility and number, suggesting that detrimental bacteria could negatively affect male reproductive fitness. These costs are expected to be particularly pronounced for polygynandrous species, where males and females mate with multiple partners, and sperm from different males compete with each other to fertilise the egg (Parker, 1970; Møller & Birkhead, 1989; Parker, 1990). Despite increasing interest in the relationship between bacteria and male reproductive success, little is currently known about MRT bacterial ecology. We investigated the microbiota present in the MRT of the polygynandrous red junglefowl to characterise this previously understudied bacterial ecosystem.

We found that the microbiota of the functionally-distinct MRT sections were ecologically-homogenous, while the digestive tract microbiota composition diverged from the reproductive tract. The MRT and cloaca tissue samples indicated uniformly high bacterial richness and diversity across the tract, regardless of the functionally-distinct section. On the other hand, comparative samples from fowl colon and in particular faeces harboured significantly lower bacterial richness and diversity. However, the differences detected in faecal samples may have been due methodological processing differences, rather than biological reasons (Supplementary Table 2B). Red junglefowl microbiota composition was also significantly affected by male age class, with old and in particular very old birds harbouring significantly lower overall total ASV richness. Fowl age class also had a significant effect on the MRT and cloacal microbiota compositions, with the differences driven by differently-abundant bacteria.

We found bacteria across the MRT of red junglefowl. Our results are congruent with studies in male humans that have found bacteria across the genital tract (Mändar, 2013), and a study in male house sparrows that reported culturable bacteria both on the cloacae and in the

testes of experimental birds (Stewart & Rambo, 2000). To our knowledge, this study is one of the first attempts to characterise the microbiota along the male avian reproductive tract continuum in detail. Functionally-distinct MRT sections, cloaca, colon, and faeces were dominated by the phylum Firmicutes in red junglefowl. Some members of Firmicutes produce short chain fatty acids (SCFAs), which have been proposed to buffer the negative effects of pathogenic bacteria in the caeca of domestic chickens, but also provide an energy source for colonocytes (Polansky et al., 2016). The MRT also harboured Proteobacteria, Fusobacteria, Actinobacteria, Bacteroidetes and Deferribacteres. Firmicutes, Proteobacteria, and Actinobacteria are often found on wild bird cloacae as the most prevalent bacterial phyla (Kreisinger et al., 2015; Escallón, Belden, & Moore, 2019).

Male fowl digestive tract harboured more Bacteroidetes and Deferribacteres in comparison with the MRT and cloaca, and was similar to the domestic chicken gut microbiota (Yeoman et al., 2012; Oakley et al., 2014; Shaufi et al., 2015). Members of Bacteroidetes also produce SCFAs of which propionate in particular has been found to buffer *Salmonella* (phylum Proteobacteria) invasions in the guts of mice (*Mus musculus*; Jacobson et al., 2018). Deferribacteres—including the genus *Mucispirillum* detected in our study—are found in the domestic chicken caecum, but their function is currently unknown (Polansky et al., 2016).

At the genus level, *Clostridium_sensu_stricto_1* and *Burkholderia-Caballeronia-Paraburkholderia* were significantly more abundant in MRT and colon tissue, but absent in faeces. Members of the genus *Clostridium* have been reported as opportunistic bird pathogens, with some of the strains causing lethal avian botulism (Benskin et al., 2009). *Burkholderia* has been identified as a laboratory contaminant (Salter et al., 2014), however, our rigorous negative control system and bioinformatic data filtering did not categorise *Burkholderia* as a contaminant (Supplementary Figure 1B). As we practised sterile laboratory etiquette, *Burkholderia* is likely to be naturally present in male fowl, yet its function remains unknown.

Previously, *Burkholderia* has been found in the faeces of probiotic-treated domestic chickens (Baldwin et al., 2018). Fowl faeces harboured a high abundance of *Lactobacillus*. *Lactobacillus* has been recognised as a commensal genus in the domestic chicken, protecting the host gut against *Campylobacter* (phylum Proteobacteria) invasions (Messaoudi et al., 2011). Among others, we found *Lactobacillus kitasatonis* and *Lactobacillus aviarius* in male red junglefowl. Of final note on bacterial genera, *Corynebacterium* was more prevalent in MRT tissue in comparison with male digestive tract. A strain of pathogenic *Corynebacterium* has been reported to be sexually-transmitted in black-legged kittiwakes (*Rissa tridactyla*) and cause reproductive failure in female hosts (van Dongen et al., 2019). Further research is necessary, however, to understand the ecology and function of specific MRT bacteria in red junglefowl.

Despite bacterial abundance differences, we found that the functionally-distinct MRT sections and cloaca were ecologically-homogenous, with even colon tissue bacteria surprisingly similar to the microbiota found in fowl MRT. Unlike our results, longitudinal studies of avian gastrointestinal microbiota have reported bacterial community variation across the digestive tract (Zhang et al., 2017; Drovetski et al., 2018). Relative homogeneity observed between MRT, cloacal, and male digestive tract bacteria could be due to the shared organ cloaca (Figure 1). Indeed, bacterial crossovers between the tracts may serve an important role in microbiota maintenance. For example, the avian gut could serve as a source of commensal Firmicutes and Bacteroidetes that translocate to the MRT via the cloaca. On the other hand, bacterial crossovers may also be detrimental, if the bacteria are pathogenic. For example, *E. coli* is an established gut commensal, but may cause female and male infertility in the human reproductive tract (Cools, 2017). Further research is needed to explore the possibility and significance of bacterial crossovers between the MRT and digestive tract in species with the shared outlet cloaca.

Contrary to what has been reported in humans (Mändar, 2013), our results suggest that bacterial richness and diversity do not decrease in the upper MRT of the red junglefowl. Uniformly high bacterial diversity across the functionally-distinct sections suggests that bacteria ascend and descend across the MRT with ease. Whilst lower bacterial diversity in the upper MRT has been linked to higher male fertility in humans (Mändar, 2013), our results allow us to speculate that the high bacterial diversity present in the MRT may impose negligible fitness costs for male fowl. In fact, there are a few explanations for how and why high bacterial diversity may persist in the MRT of red junglefowl.

First, the experimental males were healthy and sexually-active. It is therefore possible that they were all harbouring a well-maintained MRT microbiota of commensals and mutualists. As such, female fowl may benefit from incorporating male reproductive bacterial diversity into their own reproductive microbiota (Lombardo, Thorpe & Power, 1999; Smith & Mueller, 2015). Second, while these bacteria may serve a functional role in males, their effect may be negligible in the female fowl reproductive tract, putting minimal pressure on male hosts to maintain their reproductive microbiota. Finally, reproductive microbiota over-maintenance may be detrimental. Host bacterial control is often carried out with the help of AMPs. For example, lysozyme has been proposed as a potential bactericide in bird semen (Rowe et al., 2013). However, some AMPs have been shown to possess cytotoxic effects that could kill sperm cells (Boman, 2003). Thus, male fowl may have a trade-off between sperm number and maintaining a "healthy" MRT microbiota.

Finally, we report that fowl age class significantly affected the overall microbiota composition in red junglefowl. The differences were particularly evident from β-diversity modelling with weighted UniFrac distances and Bray-Curtis dissimilarities, suggesting that the significant differences were driven by differentially-abundant bacteria in young (1–3 years), old (4–6 years), and very old (7–9 years) birds (Lozupone et al., 2007). Old and in particular

very old males harboured significantly lower overall total ASV richness. Reproductive senescence starts in 4–6-year-old male feral fowl and red junglefowl (Dean et al., 2010; McDonald et al., 2017), and leads to altered host physiology, lower mating rates, and different ejaculate and sperm cell characteristics (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016). Age-related changes could in turn affect fowl MRT microbiota. For example, bacteria have been hypothesised to use sperm cells and/or seminal compounds as an energy source (Dávila et al., 2018). If reproductive senescence causes a reduction in sperm number, the bacterial diversity and number may also change in the MRT. Lastly, old and very old birds harboured a significantly higher abundance of *Fusobacterium mortiferum* in their MRT and cloaca. *F. mortiferum* inhibits the pathogenic *Salmonella enteritidis* in poultry gut (Portrait, Cottenceau & Pons, 2000), and its function in fowl MRT should be investigated further.

Takeaways

- Red junglefowl MRT and cloacal microbiota are ecologically homogenous, while colon and in particular faeces harbour a divergent bacterial composition
- Despite compositional differences, a large number of bacterial genera are shared between the MRT and digestive tract, suggesting that bacteria may translocate between the tracts via the shared outlet cloaca
- Contrary to the patterns of variation detected in human MRT, bacterial richness and diversity are uniformly high across the fowl tract
- Old and very old males harbour lower bacterial richness, which may be due to lower mating rates and/or physiological changes in ageing males

References

Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

Appendices

Supplementary Table 1. Primer sequences (5'–3' orientation) used in the amplification and indexing PCRs. For clarity, the functional regions of each primer have been separated by a "+". The locus-specific sequence is based on the Earth Microbiome Project (Thompson et al., 2017). The forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) cover the V4 hypervariable region (~254 base pairs) of the bacterial 16S SSU rRNA gene. The adapter used for trimming is indicated in bold. The paired-end index combinations were based on Kozich et al. (2013).

Amplification PCR primers	
	Illumina Nextera-compatible overhang + locus-specific sequence
515F	TCGTCGGCAGCGTC**AGATGTGTATAAGAGACAG** + GTGYCAGCMGCCGCGGTAA
806R	GTCTCGTGGGCTCGG**AGATGTGTATAAGAGACAG** + GGACTACNVGGGTWTCTAAT

Indexing PCR primers	
	Flow cell adapter + index + Illumina Nextera-compatible overhang
SA501	AATGATACGGCGACCACCGAGATCTACAC + ATCGTACG + TCGTCGGCAGCGTC
SA502	AATGATACGGCGACCACCGAGATCTACAC + ACTATCTG + TCGTCGGCAGCGTC
SA503	AATGATACGGCGACCACCGAGATCTACAC + TAGCGAGT + TCGTCGGCAGCGTC
SA504	AATGATACGGCGACCACCGAGATCTACAC + CTGCGTGT + TCGTCGGCAGCGTC
SA505	AATGATACGGCGACCACCGAGATCTACAC + TCATCGAG + TCGTCGGCAGCGTC
SA506	AATGATACGGCGACCACCGAGATCTACAC + CGTGAGTG + TCGTCGGCAGCGTC
SA507	AATGATACGGCGACCACCGAGATCTACAC + GGATATCT + TCGTCGGCAGCGTC
SA508	AATGATACGGCGACCACCGAGATCTACAC + GACACCGT + TCGTCGGCAGCGTC
SB501	AATGATACGGCGACCACCGAGATCTACAC + CTACTATA + TCGTCGGCAGCGTC
SB502	AATGATACGGCGACCACCGAGATCTACAC + CGTTACTA + TCGTCGGCAGCGTC
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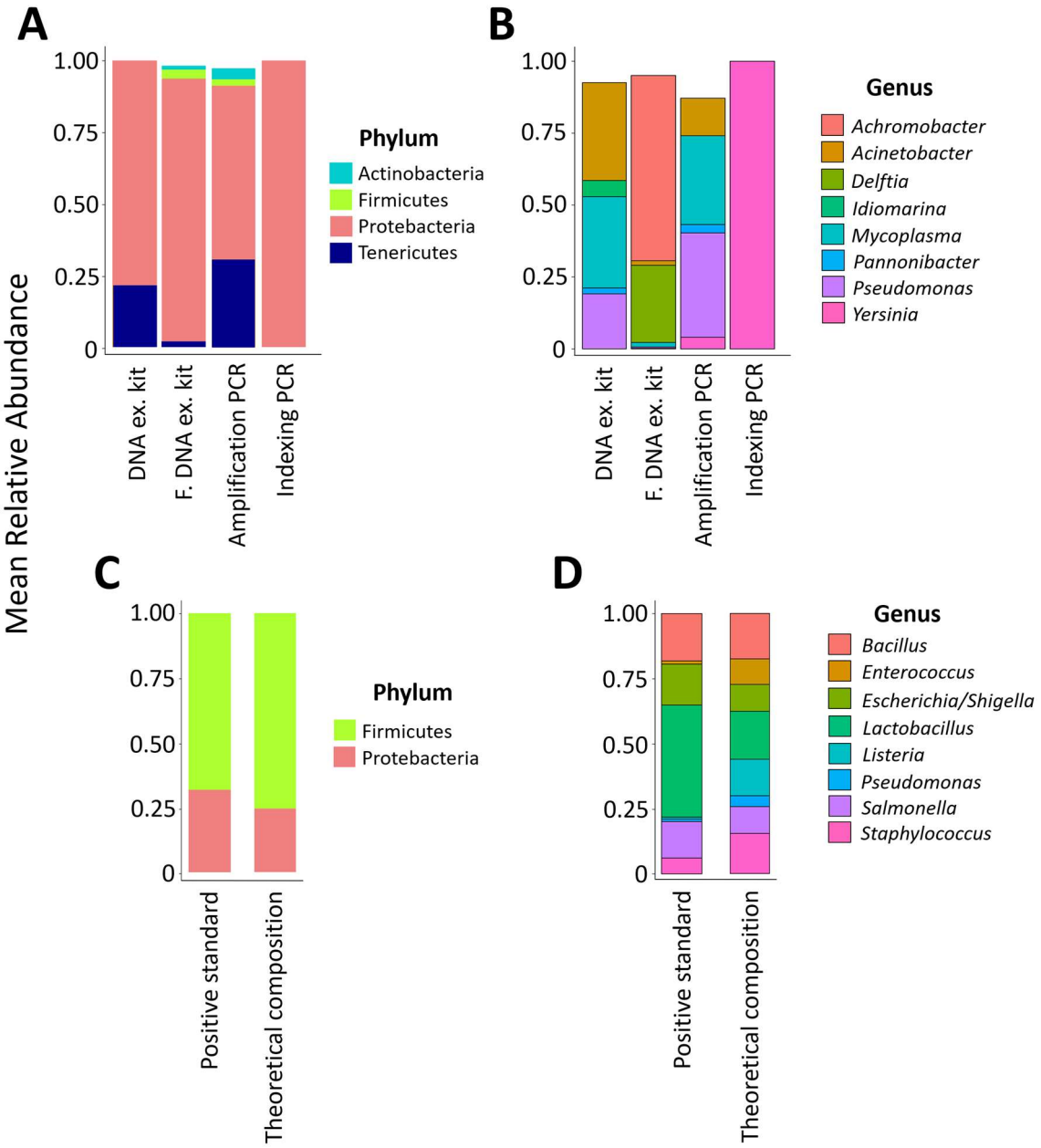
SB504	AATGATACGGCGACCACCGAGATCTACAC + TACGAGAC + TCGTCGGCAGCGTC
SB505	AATGATACGGCGACCACCGAGATCTACAC + ACGTCTCG + TCGTCGGCAGCGTC
SB506	AATGATACGGCGACCACCGAGATCTACAC + TCGACGAG + TCGTCGGCAGCGTC
SB508	AATGATACGGCGACCACCGAGATCTACAC + GTCAGATA + TCGTCGGCAGCGTC
SA712	CAAGCAGAAGACGGCATACGAGAT + TCGCTATA + GTCTCGTGGGCTCGG
SB701	CAAGCAGAAGACGGCATACGAGAT + AAGTCGAG + GTCTCGTGGGCTCGG
SB702	CAAGCAGAAGACGGCATACGAGAT + ATACTTCG + GTCTCGTGGGCTCGG
SB703	CAAGCAGAAGACGGCATACGAGAT + AGCTGCTA + GTCTCGTGGGCTCGG
SB704	CAAGCAGAAGACGGCATACGAGAT + CATAGAGA + GTCTCGTGGGCTCGG
SB705	CAAGCAGAAGACGGCATACGAGAT + CGTAGATC + GTCTCGTGGGCTCGG
SB706	CAAGCAGAAGACGGCATACGAGAT + CTCGTTAC + GTCTCGTGGGCTCGG
SB707	CAAGCAGAAGACGGCATACGAGAT + GCGCACGT + GTCTCGTGGGCTCGG
SB708	CAAGCAGAAGACGGCATACGAGAT + GGTACTAT + GTCTCGTGGGCTCGG
SB709	CAAGCAGAAGACGGCATACGAGAT + GTATACGC + GTCTCGTGGGCTCGG
SB710	CAAGCAGAAGACGGCATACGAGAT + TACGAGCA + GTCTCGTGGGCTCGG
SB711	CAAGCAGAAGACGGCATACGAGAT + TCAGCGTT + GTCTCGTGGGCTCGG
SB712	CAAGCAGAAGACGGCATACGAGAT + TCGCTACG + GTCTCGTGGGCTCGG



Supplementary Figure 1. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A & C) and genera (B & D) detected in negative controls (A & B) and the positive standard (C & D). Only taxa that contributed >1% are presented. Mean relative abundances per negative control type (A & B) were calculated using the post-*decontam* untrimmed contaminants-only data set. Relative abundances in the positive standard (C & D) were calculated using the pre-*decontam* untrimmed data set. Lowering the inclusion threshold to 0.1% was necessary to detect the genera *Listeria* and *Pseudomonas* in the positive standard (D). Negative controls (A & B) are a sample of the DNeasy® Blood & Tissue DNA extraction kit (DNA ex. kit; n=1), a sample of the *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (F. DNA ex. kit; n=1), amplification PCR negatives (n=6), and an indexing PCR negative (n=1). The positive standard (C & D) is a single ZymoBIOMICS® Microbial Community Standard (n=1). For comparison purposes, the theoretical composition of the ZymoBIOMICS® Microbial Community Standard expected by the manufacturer is presented together with the positive standard (C & D). No correction was made to the data set based on the observed and expected positive standard relative abundances. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity.

Supplementary Table 2. [ANOSIM] Microbiota composition variation between host ecosystems ($n_{reproductive\ tract}$=48, n_{cloaca}=12, or $n_{digestive\ tract}$=24; A) and sampling methods ($n_{tissue\ sample}$=72 or $n_{faecal\ sample}$=12; B) at bacterial phylum and genus level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=12). R=0 indicates high variability both within and between groups, R>0 indicates higher variability between groups than within groups, and R<0 indicates higher variability within groups than between groups. Significant p-values are in bold.

A

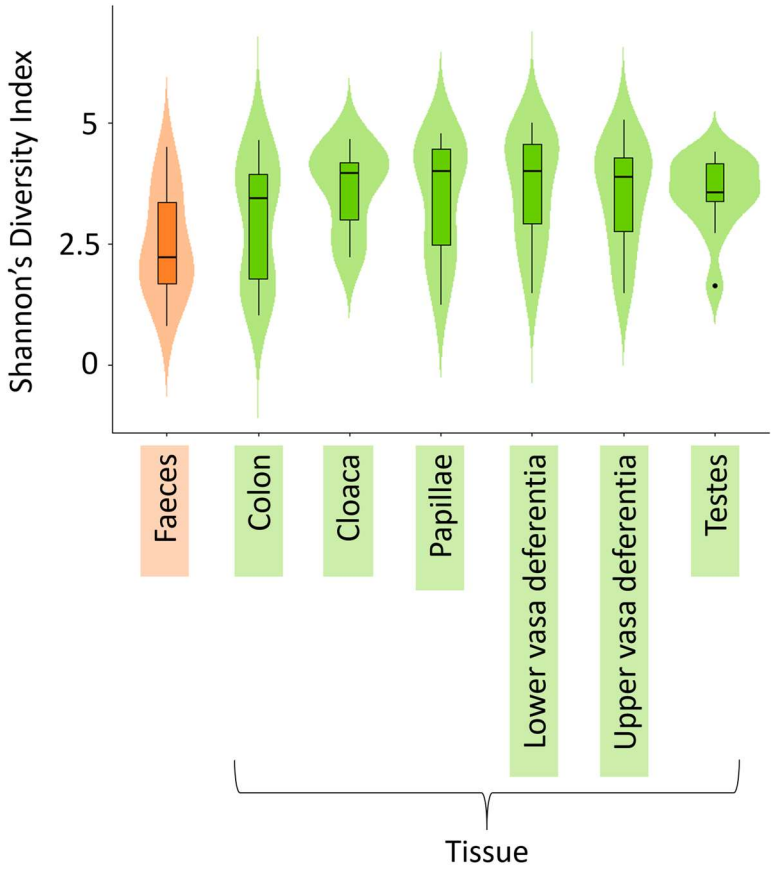
Host ecosystem	R	p-value
Weighted UniFrac		
Phylum-level	**0.07**	**0.039**
Genus-level	**0.13**	**0.002**
Unweighted UniFrac		
Phylum-level	**0.13**	**0.008**
Genus-level	**0.12**	**0.005**
Bray-Curtis DS		
Phylum-level	**0.13**	**0.002**
Genus-level	**0.13**	**<0.001**
Jaccard Index		
Phylum-level	**0.07**	**0.047**
Genus-level	**0.11**	**0.024**

B

Sampling method	R	p-value
Weighted UniFrac		
Phylum-level	**0.22**	**<0.001**
Genus-level	**0.30**	**<0.001**
Unweighted UniFrac		
Phylum-level	**0.40**	**<0.001**
Genus-level	**0.50**	**<0.001**
Bray-Curtis DS		
Phylum-level	**0.37**	**<0.001**
Genus-level	**0.29**	**<0.001**
Jaccard Index		
Phylum-level	**0.36**	**<0.001**
Genus-level	**0.49**	**<0.001**

Supplementary Table 3. [GLMM, family=Gaussian, link="log"] Shannon's diversity index estimates per sample type ($n_{per\ sample\ type}$=12) and age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28). The *intercept* is the Shannon's diversity index estimate for faeces collected from young birds. Functionally-distinct sections of the MRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold. The random effect *bird ID* (n=12) explained a moderate amount of variation (S.D.=0.17).

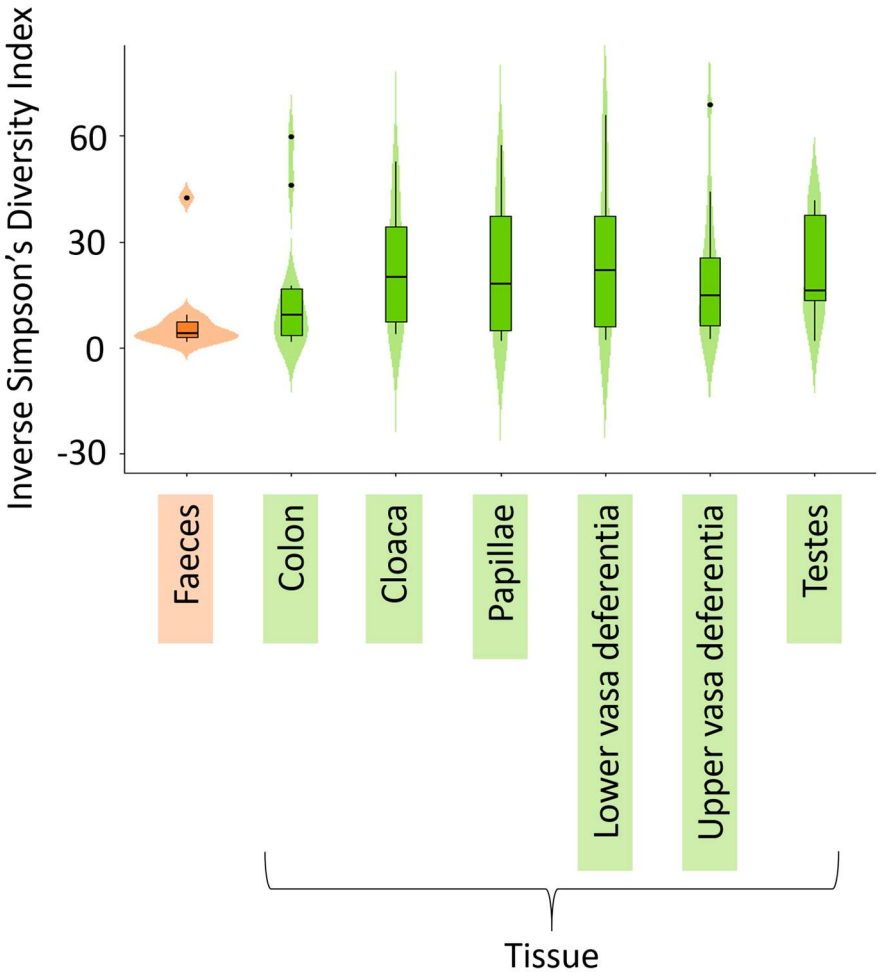
	Shannon's Diversity	Estimate	S.E.	t-value	p-value
	Intercept	**0.99**	**0.14**	**7.31**	**<0.001**
Tissue	Colon	0.18	0.11	1.58	0.115
	Cloaca	**0.38**	**0.11**	**3.56**	**<0.001**
	Papillae	**0.32**	**0.11**	**2.95**	**0.003**
	Lower vasa deferentia	**0.38**	**0.11**	**3.61**	**<0.001**
	Upper vasa deferentia	**0.35**	**0.11**	**3.24**	**0.001**
	Testes	**0.33**	**0.15**	**3.03**	**0.002**
	Old	-0.04	0.15	-0.26	0.795
	Very old	-0.23	0.15	-1.52	0.129



Supplementary Figure 2. Violin plots of Shannon's diversity index estimates per sample type ($n_{\text{per sample type}}$=12). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Functionally-distinct sections of the MRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

Supplementary Table 4. [GLMM, family=gamma, link="log"] Inverse Simpson's diversity index estimates per sample type ($n_{per\ sample\ type}$=12) and age class (n_{young}=28, n_{old}=28, or $n_{very\ old}$=28). The *intercept* is the inverse Simpson's diversity index estimate for faeces collected from young birds. Functionally-distinct sections of the MRT are presented in an anatomically-ascending order, starting with the cloaca. Significant values in comparison to the intercept are in bold. The random effect *bird ID* (n=12) explained a considerable amount of variation (S.D.=0.48).

	Inverse Simpson's Diversity	Estimate	S.E.	t-value	p-value
	Intercept	**2.10**	**0.40**	**5.27**	**<0.001**
Tissue	Colon	**0.62**	**0.29**	**2.16**	**0.031**
	Cloaca	**1.05**	**0.29**	**3.65**	**<0.001**
	Papillae	**1.17**	**0.29**	**3.97**	**<0.001**
	Lower vasa deferentia	**1.15**	**0.29**	**3.99**	**<0.001**
	Upper vasa deferentia	**0.97**	**0.29**	**3.38**	**<0.001**
	Testes	**1.17**	**0.29**	**4.07**	**<0.001**
	Old	-0.07	0.48	-0.15	0.883
	Very old	-0.77	0.48	-1.58	0.113



Supplementary Figure 3. Violin plots of inverse Simpson's diversity index estimates per sample type ($n_{\text{per sample type}}$=12). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Functionally-distinct sections of the MRT are presented from left to right in an anatomically-ascending order, starting with the cloaca.

Chapter IV. The Ejaculate Microbiota of the Polygynandrous Red Junglefowl (*Gallus gallus*) is Consistently Diverse

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Ejaculate-associated bacteria can affect sperm motility and number, and infect inseminated females. These fitness consequences are expected to be particularly prominent in polygynandrous species, where males and females mate with multiple partners, and the ejaculates of different males compete for fertilisation. Despite increasing evidence that the male reproductive tract (MRT) harbours a microbiota, little is known about the bacteria and patterns of variation present in non-human ejaculates. Polygynandrous males are known to strategically modulate sperm and seminal fluid investment in their ejaculates based on socio-sexual context. Ejaculate characteristics also often vary with male age, during a breeding season, and across successive mating opportunities. It is thus likely that ejaculate microbiota may also exhibit similarly dynamic patterns of variation. To study this, we characterised variation in microbiota composition and sperm investment in the ejaculates of the polygynandrous red junglefowl, *Gallus gallus*, comparing young and old males over a breeding season, and across an experimental mating sequence. We report that the red junglefowl ejaculate microbiota is distinct from bacterial communities found across the MRT, and is dominated by Actinobacteria, in particular the genera *Janibacter* and *Brachybacterium*. Ejaculates from old males differed from young birds in their ejaculate microbiota. However, contrary to predictions, patterns of ejaculate bacterial richness, diversity, composition, and load were consistent over the course of breeding season and across a sequence of matings. Ejaculate total ASV richness did not significantly covary with sperm velocity (VAP) nor concentration. Ejaculate bacterial load, on the other hand, showed patterns suggestive of modest, non-significant covariation with sperm VAP and concentration, with ejaculates that harboured a higher bacterial load also having a higher sperm velocity and concentration. Combined, our results indicate that the polygynandrous red junglefowl ejaculates harbour a distinct microbiota that varies across male age class, but appears independent of short-term variation in ejaculate characteristics.

Introduction

Animals live in environments dominated by bacteria where they act as hosts to diverse bacterial communities (microbiota). These host-associated bacteria have inspired research into the complex host-microbiota eco-evolutionary dynamics. For example, gut microbiota has been found to play a considerable role in host metabolism (Nicholson et al., 2012), immunity (Dominguez-Bello et al., 2019), and even cognition and behaviour (Cryan & Dinan, 2012; Münger et al., 2018). Recent studies have also found bacteria in host-associated ecosystems that were previously considered sterile, including the male reproductive tract (MRT; Mändar, 2013) and seminal fluid (Altmäe, Franasiak & Mändar, 2019). Consequently, a new area of interest has emerged that attempts to resolve the role of bacteria in male reproductive fitness.

Pathogenic bacteria have been proposed to be costly in animal reproduction (Lockhart, Thrall & Antonovics, 1996). Studies in humans have shown that some bacteria can induce severe inflammation in the MRT (Rusz et al., 2012), and increasing evidence has linked specific bacterial strains to decreased sperm quality. For example, *Escherichia coli* (phylum Proteobacteria) has been reported to attach to human sperm cells, clumping them together and reducing sperm motility (Diemer et al., 1996). *Chlamydia trachomatis* serovar E (phylum Chlamydiae) has been found to increase tyrosine phosphorylation of human sperm-associated proteins, which leads to sperm cell death (Hosseinzadeh et al., 2001). Additionally, bacteria are hypothesised to consume compounds necessary for sperm cell survival or even use sperm cells themselves as an energy source (Dávila et al., 2018). Pathogenic bacteria may also cause indirect harm by triggering a host immune response. Presence of white blood cells in human ejaculate, or leukocytospermia, leads to an increase in reactive oxygen species (ROS) that further damage sperm cell membranes (Fraczek et al., 2007).

Alternatively, ejaculate microbiota may exert no costs or even provide benefits for the male host. A study in humans found bacteria in the ejaculates of both “healthy” and

symptomatic (at least one abnormal sperm parameter, e.g. low total sperm count) men (Baud et al., 2019). Indeed, harbouring $\geq 10^3$ colony forming units (cfu) per ml of ejaculate, known as bacteriospermia (World Health Organisation, 1999), is not always associated with an increase in leukocytes in human ejaculates (Domes et al., 2012; Vilvanathan et al., 2016), suggesting that some bacterial strains may not be as costly for male reproductive fitness. In fact, bacteria could also benefit an ejaculate. For example, the genus *Lactobacillus* (phylum Firmicutes) has been detected in human ejaculates and proposed to exhibit probiotic traits that may buffer the detrimental effects of pathogenic bacterial genera, such as *Prevotella* (phylum Bacteroidetes) and *Pseudomonas* (phylum Proteobacteria; Weng et al., 2014). However, some *Lactobacillus* strains have also been found to attach to human sperm cells and negatively affect their motility (Wang et al., 2020a).

Bacteria-induced costs and benefits for male reproductive fitness are expected to be more pronounced in polygynandrous species (Ashby & Gupta, 2013), where females and males mate with multiple partners, and males have the opportunity to fertilise the eggs of multiple females. First, female polyandry forces the ejaculates of different males to compete for fertilisation—a process known as sperm competition (Parker, 1970; Møller & Birkhead, 1989; Parker, 1990). The number of sperm inseminated and their motility, such as swimming velocity, are some of the key predictors of the outcome of sperm competition (Simmons, 2001; Snook, 2005; Pizzari & Parker, 2009). Bacterial strains that reduce sperm number (Hosseinzadeh et al., 2001) and motility (Diemer et al., 1996) may thus severely interfere with sperm competition. Second, polyandrous females may exert sexual selection during and/or after mating by biasing the outcome of sperm competition in favour of the ejaculates of certain males and against those of other males—a mechanism known as cryptic female choice (Eberhard, 1996; Firman et al. 2017). If the sexually-transmitted ejaculate bacteria provided, for example, an advantageous function in the inseminated female, such as female and offspring health

benefits, certain bacterial strains could also make the ejaculate more favourable in cryptic female choice (Lombardo, Thorpe & Power, 1999; Smith & Mueller, 2015; Rowe at al., 2020).

Relatively little, however, is known about the microbiota present in the ejaculates of polygynandrous non-human species, the way in which this microbiota relates to male and female reproductive tract microbiota, and crucially, the patterns of variation in ejaculate microbiota between and within males. We address these gaps in knowledge by characterising the patterns of variation in the ejaculate microbiota of a polygynandrous bird, the red junglefowl, *Gallus gallus*.

The red junglefowl lives in fission-fusion polygynandrous groups where males face intense pre- and post-copulatory sexual selection (Pizzari & McDonald, 2019). The outcome of sperm competition in fowl is determined by the relative number of inseminated sperm and to some degree by sperm swimming velocity (Birkhead et al., 1999; Pizzari et al., 2008). Males that mate repeatedly with the same female tend to fertilise a larger share of the eggs by maintaining a high number of their sperm in the sperm storage tubules of a female (Collet et al., 2012; McDonald et al., 2017; Pizzari & McDonald, 2019). Successive matings deplete the number of sperm that a male can allocate to an ejaculate, and males tend to reduce their sperm investment in recently mated females, while preferentially investing sperm in sexually novel females (Pizzari et al., 2003; Alvarez-Fernandez et al., 2019). The seminal fluid proteome of an ejaculate also varies over a succession of mating opportunities with familiar and novel partners (Alvarez-Fernandez et al., 2019). Thus, strategic response to partner novelty may also modulate the abundance of individual proteins present in the seminal fluid, which have also been hypothesised to serve as an energy source for bacteria (Dávila et al., 2018). Indirect evidence suggests that males may also differentially allocate seminal fluid to modulate their sperm swimming velocity (Cornwallis & O'Connor, 2009). In addition to these rapid changes in ejaculate composition detected over successive matings, substantial ejaculate variation

occurs over the course of breeding season (Rakha et al., 2017; Wang, 2020c) and across age cohorts, with older male fowl typically producing fewer sperm of lower motility, and a different seminal fluid proteome compared with younger males (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016). Under the assumption that ejaculate microbiota can be influenced by ejaculate characteristics, such as sperm number and seminal fluid composition, and can in turn affect fertilisation success in polygynandrous species, it is plausible that the composition and number of ejaculate-associated bacteria may display patterns of variation at different temporal scales, e.g. across male age, over a breeding season, and more rapidly, over a succession of mating opportunities.

We had five specific objectives in this chapter. First, we characterised the red junglefowl ejaculate microbiota, and performed a preliminary comparison between the bacterial assemblages in the ejaculates, and the bacterial compositions present in the male and female fowl reproductive tracts, previously described in **Chapter III** and **Chapter II** respectively. Second, we investigated variation in ejaculate microbiota across male fowl age class. Third, we explored variation in ejaculate bacterial communities over the course of breeding season. Fourth, we tested for rapid changes in ejaculate microbiota across experimental mating sequences. And finally, we investigated whether ejaculate bacterial load and/or diversity covary with sperm velocity and/or concentration.

Our predictions were that the red junglefowl ejaculate microbiota shares bacterial strains with the previously characterised male and female reproductive tract bacterial compositions, in particular with MRT microbiota. Male ageing reduces both sperm motility and number in fowl (Dean et al., 2010) and humans (Kidd, Eskenazi & Wyrobek, 2001), which could in turn affect ejaculate bacterial assemblages. Moreover, cloacal microbiota has been found to vary between adult and chick black-legged kittiwakes (*Rissa tridactyla*; van Dongen et al., 2013), while faecal microbiota differs between parent and offspring barn swallows

(*Hirundo rustica*; Kreisinger et al., 2017), suggesting age-related microbiota variation in avian species that could also be evident in red junglefowl ejaculates. A study in wool rams (*Ovis aries*) found that old males were significantly more likely to have bacteria-induced reproductive tissue inflammation, potentially due to a lifelong accumulation of pathogens (Wiemer & Ruttle, 1987). We also expected old fowl to harbour more pathogenic bacteria than young birds. Repeated ejaculation leads to avian ejaculates harbouring fewer sperm (Bakst & Cecil, 1981). We hypothesised that intense mating over the course of breeding season will also lower fowl ejaculate bacterial diversity. In support of this, the cloacal microbiota of male rufous-collared sparrows (*Zonotrichia capensis*) was reported to vary between the breeding and non-breeding season (Escallón, Belden & Moore, 2019). Similarly, we expected different bacteria in varying numbers to be incorporated into male fowl ejaculates across a sequence of matings with sexually familiar and novel females. In particular, we predicted a decline in bacterial diversity and load over two successive matings with the same female, but less so between two matings with two different females, as has been reported with sperm number allocation in male fowl (Pizzari et al., 2003). Finally, we hypothesised that ejaculates with higher bacterial load and diversity have lower sperm velocity and concentration, as ejaculate microbiota may have an overall negative effect on sperm motility and viability.

Materials and Methods

Study Population

We studied the captive red junglefowl population at the John Krebs Field Station of the University of Oxford in Wytham. 16 healthy, sexually-active males were sampled for ejaculate microbiota during the breeding season (~April–September). Prior to the experiments, the birds were kept in single-sex flocks and housed in covered outdoor aviaries, where they were given food and water *ad libitum*.

Individual birds wore unique Darvic rings that were used for identification and determining bird age class. Males were categorised into "young" (3–4 years) and "old" (5–6 years) based on previous literature, that suggests that fowl sperm swimming velocity peaks around the age of 4 (Dean et al., 2010; Cornwallis, Dean & Pizzari, 2014; Rakha et al., 2017). Our study included eight young and eight old birds.

Ejaculate collection was carried out via controlled copulations, i.e. the male bird mounted and mated with a female kept in soliciting position by the experimenter, under the regulation of a Home Office project license (PIL: P50402706).

Experimental Protocol

Ejaculate collection was carried out across two consecutive breeding seasons (experimental period: July–October in 2017, and April–June in 2018).

Experimental trials lasted for 30 days, hereafter referred to as "the experimental month". Males were housed individually in 3.08 × 6.66 m pens with gravel grounding that they were familiar with. The pen was divided in half on the long edge using a metal net that was familiar to the birds. Pens that were adjacent to the experimental pen were visually isolated with forest green tarpaulin that the experimental birds were familiar with. Experimental fowl were given food and water *ad libitum* during the experimental month.

Experiments were carried out in the evenings, three hours before sunset. Evening trials allowed us to capture one of the daily socio-sexual behavioural peaks in the red junglefowl that engage in inter-individual interactions at the start and end of the day, while foraging and sleeping in the daytime (Løvlie & Pizzari, 2007).

Each male had an experimental trial every other day, with the rest days allowing full replenishment of the males' sperm reserves (Etches, 1996). In each 60-minute experimental assay, the focal male was given an opportunity to mate up to six times. Some of the

experimental male fowl were presented with a sexually novel female after the third mating opportunity, while the rest of the focal fowl had the opportunity to mate with the same female for all six mating opportunities. The trial was terminated, if the male did not mate for 15 minutes. If the focal males that were allowed to mate with two females had mated with the first female at least once, but then stopped for 15 minutes, they were presented with the second female and given three opportunities to mate with the new partner. The combs of the experimental females were measured, and only females that had an intermediate comb size were used in the experiment to standardise the males' response to their female partners.

Ejaculate collection was carried out via controlled copulations. The female bird was placed on the ground on a board that was covered with a plastic film. The plastic cover was cleaned with 70% ethanol before and after each mating attempt, and when the female or male accidentally contaminated the board by moving around. The focal female was kept in a soliciting position in front of the focal male by the experimenter. The female bird wore an ethanol-sterilised harness that was cleaned with 70% ethanol after each copulation attempt, and in cases of accidental contact by the birds or the experimenter. The harness was used to prevent cloacal contact between mating partners and facilitate cleaner ejaculate collection. Each ejaculate was immediately transferred with a sterile pipette into a sterile tube. Only males that had previously been trained to mate routinely using this technique were used in the experiment.

To investigate red junglefowl ejaculate microbiota variation over the course of breeding season (objective 3), we collected 24 ejaculates in total from eight different males at the start, middle, and end of the experimental month (Figure 1). Each of these ejaculates was the first ejaculate of the day. The three ejaculates per male were collected at least 10 days, but no more than 20 days apart from another.

To investigate ejaculate microbiota changes across an experimental mating sequence (objective 4), we collected 34 ejaculates in total from 14 males. These ejaculates were the first

and second ejaculate of a male in a given trial, with a maximum of 30 minutes between the two ejaculations. A distinction was made between ejaculates that were collected during an experimental mating sequence with the same female or with two different females (Figure 1). These samples were harvested more opportunistically due to variation in red junglefowl mating behaviour and the set-up of the experimental protocol, and thus were collected on any given day during the experimental month, when the opportunity presented itself.

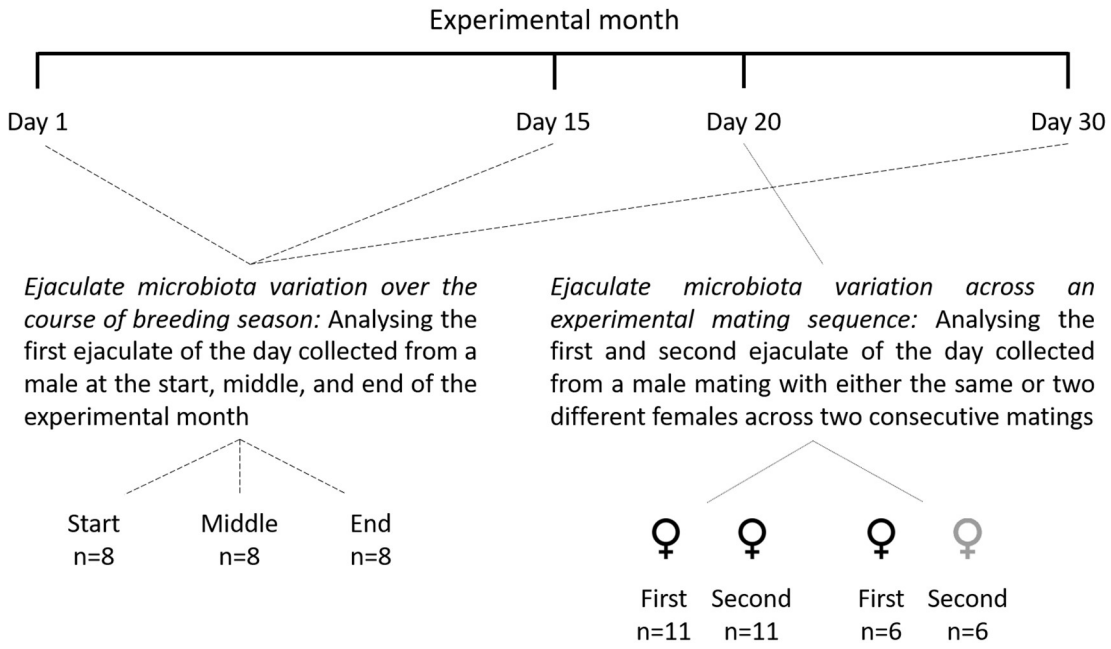


Figure 1. Illustrative schematic of ejaculate collection during the experimental month. First ejaculates of the day at the start, middle, and end of the experimental month (illustrated here as Day 1, 15, and 30) were used to explore ejaculate microbiota variation over the course of breeding season (objective 3). The first and second ejaculates of the day (illustrated here as Day 20) from an experimental mating sequence with either the same female (indicated by black) or two different females (indicated by black and grey) were used to investigate rapid ejaculate microbiota variation (objective 4). Ejaculate collection across the two breeding seasons of 2017–2018 resulted in 58 ejaculate samples collected from eight young and eight old red junglefowl.

In total, we collected 58 ejaculates from 16 different males, with some males providing ejaculates for both objective (3) and (4). Each ejaculate was classed into a single category, hereafter referred to as "experimental group", as either an ejaculate collected at the start (n=8), middle (n=8), or end (n=8) of the experimental month, or the first (n=11) or second (n=11)

ejaculate across two matings with the same female, or the first (n=6) or second (n=6) ejaculate across two matings with two different females (Figure 1).

The entire data set (n=58) was used to characterise the red junglefowl ejaculate microbiota, and compare it to the bacterial assemblages from the male and female fowl reproductive tracts (objective 1).

To investigate ejaculate microbiota variation across male age class (objective 2), we used the whole ejaculate data set (n=58), which consisted of ejaculates collected from eight young (n=34) and eight old birds (n=24).

Ejaculate Processing in the Field

While one of the experimenters was following the experimental protocol in the pen, the other experimenter handled and processed the ejaculate samples immediately upon ejaculation. 5 µl of each ejaculate was transferred into an individual, sterile tube within a minute after ejaculation, and stored at -80°C for downstream laboratory microbiota processing.

For a subset of samples, we were able to measure sperm average path velocity (VAP; µm/s), which was used as a proxy for sperm swimming velocity, and sperm concentration (number of sperm cells/µl).

VAP, which represents an accurate measure of ejaculate fertilising efficiency in fowl (Wishart, 2009), was measured within 10 minutes after ejaculation by mixing 1 µl of ejaculate with a previously prepared solution of 180 µl of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, Missouri, USA) and 20 µl of Standard Foetal Bovine Serum (Thermo Fisher Scientific, Waltham, Massachusetts, USA) that was kept in a 41˚C (body temperature of the red junglefowl) water bath. 2 µl of this mixture was then transferred to a 10-micron Leja microscope slide chamber (MICROPTIC S.L., Barcelona, Spain) that was fixed on a heated stage (41˚C) of a Nikon Eclipse E200 microscope (Nikon Corporation, Minato City, Tokyo,

Japan), which was connected to a Basler Area Scan A312fc camera (Basler AG, Ahrensburg, Germany). Red junglefowl sperm was tracked using the Sperm Class Analyzer® CASA System for semen analysis (MICROPTIC S.L.; Version SCA® v.6.2.0.15). Per ejaculate, a minimum of 200 individual sperm cells were tracked across at least three different image fields, using the following parameters: Ph- optics, 10× magnification, capture 25 images/second, and track objects of 50–200 microns in size. Based on the captures, a VAP measure was logged for each ejaculate.

Sperm concentration was measured within three hours after ejaculate collection. 2.5 µl of ejaculate was mixed with 197.5 µl of Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich). After calibrating with a PBS-blank, 70 µl of the mixture was transferred into a cuvette, which was then put in a WPA Biotech Photometer UV 1101 (Biochrom Ltd., Cambridge, UK) to measure ejaculate sperm concentration via light absorption (Donoghue et al., 1996). Two spectrophotometer measurements at 595 nm were collected per ejaculate. The average of the two measurements and a pre-established standard curve (R^2=0.98; Tan et al., 2017) were used to calculate sperm concentration per ejaculate (Bakst & Cecil, 1997). The observed sperm concentrations matched the previously published data for this species (Rakha et al., 2017; Tan et al., 2017; Alvarez-Fernandez et al., 2019).

In total, we were able to record the VAP for 24 ejaculates and sperm concentration for 42 ejaculates. These measures were analysed under objective (5) to test whether sperm quality covaries with ejaculate microbiota parameters.

DNA Extraction and Quantification

Post-fieldwork microbiota processing took place in 2019–2020 at Silwood Park, Imperial College London. DNA from ejaculate samples was extracted with a previously sealed QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany), processing the

ejaculates as blood samples according to the manufacturer's protocol. Sample handling was carried out in a pre-PCR biological safety cabinet that was sterilised with DNAZap™ PCR DNA Degradation Solutions (Thermo Fisher Scientific) and 70% ethanol. Prior to lysis, the ejaculates were handled on ice to minimise compromising their bacterial compositions. To increase yield from Gram-positive bacteria (Gill et al., 2016), the manufacturer's protocol was altered by adding lysozyme from chicken egg white (0.4 mg/ml; Sigma-Aldrich) to the ATL Buffer and allowing 30 minutes of incubation at 37°C, after which the manufacturer's protocol proceeded with the Proteinase K step. The second incubation step at 56°C was carried out for an hour.

A single DNA extraction kit control per 96-well plate was collected for downstream contamination assessment. The samples were quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) with a Qubit® Fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol. DNA extractions were diluted to a standardised concentration of 5 ng/µl and all samples were stored at -20°C.

Amplification Polymerase Chain Reactions (PCRs)

Before and after each set-up, the PCR hood was sterilised with DNAZap™ PCR DNA Degradation Solutions and 70% ethanol, followed by a 30-minute UV-sterilisation. Consumables were prepared with a separate 30-minute UV-sterilisation. PCRs were set up on ice with sterile, filtered pipette tips and reagents taken from unopened aliquots.

The V4 region (~254 base pairs) of the bacterial 16S SSU rRNA gene was amplified, following the guidelines of the Earth Microbiome Project (Thompson et al., 2017) and Walters et al., 2015. Reactions were prepared on 96-well plates and in three replicates to minimise amplification bias and ensure the amplification of rare bacterial strains. 5 ng or up to 5 µl (if the DNA template concentration was too low) of DNA was added to a 50 µl reaction volume,

consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus* (Sigma-Aldrich), 5 µl of *Taq* DNA Polymerase PCR Buffer 10X (Sigma-Aldrich), 0.5 µl of Bovine Serum Albumin (Sigma-Aldrich), 0.1 µl of 100 nM dNTP mix (Thermo Fisher Scientific), 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) to make up the rest of the reaction volume.

Two PCR negative controls per 96-well plate were prepared and sealed before template DNA entered the PCR hood. A positive standard, ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, California, USA) was added to each set-up to assess amplification success.

Parameters for the amplification PCRs were as follows: initial three-minute denaturing at 94°C followed by 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all replicate plates.

The PCR products were tested on a 1% agarose gel, stained with GelRed™ (Biotium, Fremont, California, USA) according to the manufacturer's protocol, in a 1×TBE buffer at 80 V for 45 minutes.

Quantitative Polymerase Chain Reactions (qPCRs)

DNA extractions were additionally amplified with qPCRs to quantify and explore bacterial load variation across ejaculates (n=58).

The PCR hood and consumables were sterilised as for the amplification PCRs. Reactions were prepared with the Thermo Scientific ABsolute qPCR SYBR® Green Mix (Thermo Fisher Scientific) following the manufacturer's protocol, adding 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer

(806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the 25 µl reaction volume. The amount of template DNA was kept congruent with the 16S SSU rRNA gene amplification PCR step for standardised downstream comparisons. Technical replicates were not included.

A three-point serial dilution of a standard (*Escherichia coli*; quantified using the Qubit™ methodology) and four negative controls were added to the 96-well format, which was then processed using the LightCycler® 96 Instrument (Roche Holding AG, Basel, Switzerland).

Parameters for the qPCRs were as follows: 15-minute enzyme activation at 95°C followed by 50 cycles of 45 seconds at 95°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step of at 72°C. This was followed by a melt curve: 10 seconds at 95°C, 60 seconds at 65°C, and one second at 97°C. A single set-up was sufficient to process all samples.

Standard curve (primer efficiency=129.2%, R^2=0.99) and concentration analysis (ng/µl) were carried out in the LightCycler® 96 Application (Roche Holding AG). Samples were omitted, if they failed to amplify or amplified less than five cycles before primer dimer formation in negative controls (C_q=30.00). This data filtering resulted in the loss of three ejaculate samples, which were the ejaculates collected from bird K40 at the middle and end of the experimental month, and the first ejaculate of K40 during a mating sequence with the same female fowl.

DNA concentrations in ng/µl were corrected for the collected ejaculate volume (5 µl), DNA elution buffer volume (200 µl), and template DNA volume that was processed with the qPCR (0.5 µl). The DNA concentrations were then converted into 16S SSU rRNA gene copy number per µl of ejaculate by setting the average weight of a base pair (bp) to 660 Daltons and using the following equation: $\frac{DNA\ (ng) * Avogadro\ number\ (molecule\ /mole)}{Template\ full\ length\ (bp) * 660\ (g/mole) * 1 * 10^9\ (ng/g)}$.

The first ejaculate of bird H28 during a mating sequence with two different females was considered an outlier ($2.72*10^9$ copy number/µl) in comparison with the rest of the data set ($1.55*10^8 \pm 2.24*10^7$ S.E. copy number/µl) and thus excluded from analysis. In total, we were able to measure bacterial load in 54 ejaculates.

Clean-Up, Indexing PCRs, and Normalisation

Replicate biological samples were pooled and cleaned of primer dimers using the ZR-96 DNA Clean-up Kit™ (Zymo Research), following the manufacturer's protocol.

Samples were dual-indexed with unique combinations proposed by Kozich et al., 2013. 20 ng of the amplification PCR product was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus*, 5 µl of *Taq* DNA Polymerase PCR Buffer 10X, 0.1 µl of 100 nM dNTP mix, 0.0625 µM (final concentration) of forward and reverse index primer (Supplementary Table 1) each, and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the reaction volume.

An indexing PCR negative control (n=1) was processed together with the biological samples. The DNA extraction kit control (QIAGEN DNeasy® Blood & Tissue DNA extraction kit, n=1), amplification PCR negative controls (n=6), and a single positive standard (n=1) were indexed as biological samples.

Parameters for the indexing PCRs were as follows: initial two-minute denaturing at 95°C followed by 15 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final 10-minute extension step at 72°C. PCR products were tested on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes.

Dual-indexed samples were quantified using the Qubit™ methodology. 5 ng of each sample was pooled into a DNA LoBind Tube (Eppendorf, Hamburg, Germany) and cleaned with the DNeasy® PowerClean Pro Cleanup Kit (QIAGEN).

The library was tested for size and quality on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes. Library purity was implied from the 260/280 nm ratio, calculated using the Take3™ Micro-Volume Plate (BioTek Instruments, Inc., Winooski, Vermont, USA) in a BioTek microplate reader (BioTek Instruments, Inc.), and the Gen5 programme (BioTek Instruments, Inc.). The quality-checked library was stored at -20°C prior to sequencing. Immediately before sequencing, the library was quantified using the Qubit™ methodology and diluted to 4 nM.

Next-Generation Sequencing (NGS)

NGS was carried out at the Silwood Park sequencing facility (Imperial College London), following Illumina's proposed protocol for 16S SSU rRNA gene metagenomic sequencing (Illumina, 2013). A 15 pM library with a 5% PhiX control (Illumina, Inc., San Diego, CA, USA) was loaded into the V3 chemistry MiSeq cartridge (Illumina, Inc.) to achieve 2*300 bp sequencing reads across 58 biological and nine control samples.

Bioinformatic Data Processing

Post-sequencing sample demultiplexing was carried out automatically by BaseSpace (Illumina, Inc.). Paired-end FASTQ files were processed with *dada2* (Callahan et al., 2016a; Version 1.12.1) in R (R Core Team, 2019; Version 3.6.1). Reads were quality-checked and filtered using standard parameters (maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2) with additional truncLen=c(200, 200), resulting in a ~10% sequencing read loss. The error rates were calculated based on a random subset of samples (randomize=TRUE) and inference was carried out via pseudo-pooling (pool="pseudo"). Non-specific amplicons were removed, retaining only merged, denoised reads of 250–256 bp. Chimera check removed ~46% of the merged sequence variants, which accounted for ~12% of the total reads. SILVA reference

database (Yilmaz et al., 2014; Version 132) was used for assigning taxonomies (100% identity) to the species level, when possible.

The processed data set including controls contained 67 samples, 3844 amplicon sequence variants or ASVs (250±19 S.E. per sample) and 1,165,408 reads (17,394±1292 per sample). *decontam* (Davis et al., 2017) was used for further filtering of potential contaminants, which were detected based on ASV prevalence in negative controls versus biological samples, using a stringency threshold of 0.55 that split ASVs into contaminants (145 ASVs) and non-contaminants (3699 ASVs).

There were no biological samples that had fewer than 100 sequencing reads. ASVs that were present in the taxonomy, but not in the data set due to data filtering (n=80) were excluded. Finally, ASVs that failed to classify at the phylum level (n=47), or that classified as chloroplasts (n=16) or mitochondria (n=13; de la Cuesta-Zuluaga & Escobar, 2016) were removed.

The final data set without controls contained 58 samples, 3543 ASVs (264±17 per sample), and 881,609 reads (15,200±1004 per sample). Mean relative bacterial abundances found in negative controls and the positive standard were visualised with *ggplot2* (Wickham, 2016), and are presented in the appendices (Supplementary Figure 1).

Statistical Analysis and Data Visualisation

Statistical analysis and data visualisation were carried out in R (Version 3.6.1). Significance threshold was set to $p<0.05$ in all tests.

(1) *Characterisation of the red junglefowl ejaculate microbiota composition, and comparisons with the male and female fowl reproductive tract microbiota*. We first characterised the red junglefowl ejaculate microbiota based on the mean relative abundances of bacterial phyla and genera found in the 58 collected ejaculate samples. Stacked bar plots

were created with *ggplot2*, and bacterial phyla and genera that were <1% abundant were considered rare, and thus excluded from the figure.

We then compared the ejaculate microbiota to male and female fowl reproductive tract (including cloacal samples) microbiota. A qualitative comparison of exclusive and shared ASVs between ejaculate bacterial communities, and male (**Chapter III**; n=60) and female reproductive tract bacterial assemblages (**Chapter II**; n=276) was visualised as a Venn diagram. Colon and faecal samples that were processed in the male (**Chapter III**; $n_{colon\ tissue}$=12, n_{faeces}=12) and female fowl data sets (**Chapter II**; $n_{colon\ lavage}$=26, $n_{colon\ tissue}$=20, n_{faeces}=20) were not included, and the ASVs that were solely associated with colon and faecal samples were excluded from the Venn diagram.

An additional comparison of interest was carried out between the microbiota compositions found in ejaculates and the functionally-distinct sections of the MRT (**Chapter III**; Supplementary Methods 1). In this case, samples from the male digestive tract (i.e. colon tissue and faeces) were also included in the analysis, as the ejaculates may come in contact with and contain bacteria associated with the digestive tract. It is important to note that the laboratory and bioinformatic processing differed slightly between the chapters, and the ejaculate samples did not come from the same individual males that were tested in **Chapter III**. However, the ejaculate, and MRT tissue and faecal samples (**Chapter III**) were processed in the laboratory at the same time, using the same reagents, and were combined in the same sequencing library and run.

Taking this into account, we decided to analyse the microbiota composition variation that may stem from differences between ejaculate samples (n=58), tissue samples (all tissue samples harvested across the MRT, cloaca, and digestive tract in **Chapter III**; n=72), and faecal samples (collected from the same males that were harvested for tissue samples in **Chapter III**; n=12), hereafter referred to as "sampling methods" (Supplementary Methods 1).

We further focused on the microbiota composition differences between ejaculates, and the functionally-distinct sections of the MRT ($n_{papillae\ tissue}$=12, $n_{lower\ vasa\ deferentia\ tissue}$=12, $n_{upper\ vasa\ deferentia\ tissue}$=12, $n_{testes\ tissue}$=12), cloaca tissue (n=12), colon tissue (n=12), and faeces (n=12), hereafter referred to as "sample type" (Supplementary Methods 1). We decided not to include the age class of male fowl in this part of the analysis, as this chapter and **Chapter III** categorised birds differently based on their age, and **Chapter III** also studied very old birds in addition to young and old fowl.

We also performed a differential bacterial abundance analysis to detect variation between ejaculate, and the MRT and cloacal microbiota, excluding colon tissue and faecal samples (Supplementary Methods 1).

(2) *Age-associated variation in red junglefowl ejaculate microbiota.* We used the entire data set (n=58) to compare the microbiota found in the ejaculates of eight young (n=34) and eight old birds (n=24).

Ejaculate microbiota composition variation was analysed with β-diversity modelling that allowed us to compare local bacterial diversity to regional bacterial diversity (Whittaker, 1960). *DECIPHER* (Wright, 2016) and *phangorn* (Schliep, 2011; Version 2.5.5) were used to align and create a phylogenetic tree, as proposed by Callahan et al. (2016b). Weighted (wUF) and unweighted UniFrac (uwUF) distances (Lozupone & Knight, 2005) were calculated with *phyloseq*, using the trimmed data set (singletons—ASVs present only once across all samples—were removed: 5 ASVs, i.e. 0.1% of all the ASVs) that was first agglomerated to either bacterial phylum or genus level, and then normalised via proportions (McMurdie & Holmes, 2014; Callahan et al., 2016b; McKnight et al., 2019). Using the same normalised-by-proportions data set, we also calculated Bray-Curtis dissimilarities and Jaccard (dissimilarity) indices at both the phylum and genus level with *vegan* (Oksanen et al., 2019) for comparative β-diversity analysis.

We did not study bacterial strain-level variation as genus was the finest phylogenetic level that allowed us to assign a taxonomic identity to the majority of our sequencing reads (2196 out of 3543 ASVs, i.e. 62.0%, were assigned a genus identity). This was due to the current bacterial strain databases having limited information about non-human host microbiota, and because of the poor species- and strain-assignment potential of 16S SSU rRNA gene hypervariable region V4 (Gohl et al., 2016; Johnson et al., 2019b). Preliminary statistical tests revealed that strain-level β-diversity analyses with PERMANOVA had near-identical results with the genus-level β-diversity analyses, with the strain-level analyses showing slightly lower p-values that did not affect the significance patterns that we were able to observe at the genus-level. This assured us to proceed with our approach, knowing that our results at the phylum and genus level are biologically relevant, and allowing us to pinpoint specific key taxa that contribute to the variation.

Ejaculate microbiota compositions were statistically compared with PERMANOVA (adonis(); permutations=1000) from *vegan*, setting experimental group (n_{start}=n_{middle}=n_{end}=8, two matings with the same female: n_{first}=n_{second}=11, two matings with two different females: n_{first}=n_{second}=6) and age class (n_{young}=34, n_{old}=24) as fixed effects, constraining the permutations within=bird ID (n=16). Significant PERMANOVA factors were followed up with beta dispersion tests (betadisper() followed by permutest(); permutations=1000) from *vegan* to control for significant dispersion differences.

Differential abundance of bacterial phyla and genera was tested using *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was agglomerated to either phylum or genus level, filtered to include only taxa that contributed >1% to the relative bacterial abundances across the data set, and analysed with differentialTest(test="Wald", fdr_cutoff=0.05). The effect of male age class was modelled while controlling for differential abundance across experimental group, and variance across experimental group and age class.

Any phylum or genus that contributed <1% to the relative bacterial abundances was considered rare and thus excluded from the differential abundance analysis.

We used α-diversity modelling to explore local bacterial diversity variation (Whittaker, 1960). Three different α-diversity metrics were calculated with the untrimmed data set. Total ASV richness was calculated with *breakaway* (Willis et al., 2018), and Shannon's and inverse Simpson's diversity index estimates with *phyloseq* (McMurdie & Holmes, 2013). Normality was assessed with a Shapiro-Wilk test, and suitable data distributions were determined from histograms, which were confirmed with *fitdistrplus* (Delignette-Muller & Dutang, 2015), using Cullen and Frey graphs (bootstrap=500). All three α-diversity metrics followed a gamma distribution (link="log"). A generalised linear mixed-effects model (GLMM) by Laplace approximation was fitted to each α-diversity metric with *lme4* (Bates et al., 2015), setting experimental group and age class as fixed effects, and bird ID as a random effect.

Models for Shannon's and inverse Simpson's diversity revealed a negligible effect of bird ID (S.D.≈0). In these cases, the effect of bird ID was tested via information-theoretic model selection, comparing a nested model without the random effect to the full model. For both α-diversity metric, the AIC_C score (Akaike's information criterion corrected for small sample size; k =2; Burnham & Anderson, 1998) calculated with *MuMIn* (Barton, 2020) was lower for the reduced model, and a generalised linear model (GLM) was preferred to the GLMM. In these instances, the effect of bird ID was assessed from the GLM ΔAIC_C scores and weights.

Preliminary data analysis revealed that including the time point (around the start vs middle vs end of the experimental month) and/or sampling year (2017 vs 2018) as random effects in the models led to the dropping of independent factor levels due to small sample size, and we therefore decided against nesting bird ID within the time point and/or sampling year in this and the following statistical analyses to minimise the risk of over-parametrisation.

Bacterial load data followed a gamma distribution (link="log"). A GLMM by Laplace approximation was fitted, setting experimental group and age class as fixed effects, and bird ID as a random effect.

Goodness of fit of each model was assessed from Q-Q and residual plots. Function Anova() from *car* (Fox & Weisberg, 2019) was used to carry out deviance analyses (type II Wald chi-square tests) of the main effects.

(3) *Ejaculate microbiota variation over the course of breeding season*. For this objective, we used the 24 ejaculates collected from eight males at the start, middle, and end of the experimental month (n_{start}=n_{middle}=n_{end}=8).

Ejaculate total ASV richness, Shannon's and inverse Simpson's diversity, microbiota composition, and bacterial load variation over the course of breeding season were statistically analysed using the same methods as described in objective (2). All three α-diversity metrics followed a Gaussian distribution (link="log"). A GLMM by Laplace approximation was fitted to each α-diversity metric with *lme4*, setting experimental group (n_{start}=n_{middle}=n_{end}=8) and age class (n_{young}=18 or n_{old}=6) as fixed effects, and bird ID (n=8) as a random effect. Models for total ASV richness and inverse Simpson's diversity indices revealed a negligible effect of bird ID and ΔAIC_C scores and weights were calculated. α-diversity metrics were visualised with *ggplot2*.

1384 singletons (36.7% of the ASVs) were removed for β-diversity modelling, and a PERMANOVA was performed, setting experimental group and male age class as fixed effects, constraining the permutations (=1000) within strata=bird ID. Stacked bar plots of relative bacterial abundances were created with *ggplot2*. Any phylum or genus that contributed <1% was considered rare, and was thus excluded from the figure.

Bacterial load data followed a gamma distribution (link="log"). A GLMM by Laplace approximation was fitted, setting experimental group and age class as fixed effects, and bird ID as a random effect. Bacterial load was visualised with *ggplot2*.

(4) *Ejaculate microbiota variation across mating sequences.* For this objective, we used the 34 ejaculates collected from 14 males as the first (n=11) or second (n=11) ejaculate across two consecutive matings with the same female, or the first (n=6) or second (n=6) ejaculate collected across two consecutive matings with two different females.

Ejaculate total ASV richness, Shannon's and inverse Simpson's diversity, microbiota composition, and bacterial load variation across experimental mating sequences were statistically analysed and visualised using the same methods as described in objectives (2–3).

Total ASV richness and Shannon's diversity index estimates followed a Gaussian distribution (link="log"), while inverse Simpson's diversity index estimates were normally distributed. Total ASV richness and Shannon's diversity were both fitted a GLMM by Laplace approximation, setting experimental group (two matings with the same female: n_{first} =11 or n_{second}=11, or two matings with two different females: n_{first}=6 or n_{second}=6) and age class (n_{young}=16 or n_{old}=18) as fixed effects, and bird ID (n=14) as a random effect. A linear mixed model (LMM) by restricted maximum likelihood (REML) was fitted to inverse Simpson's diversity and the main effects of the model were tested with a type II Wald F-test with Kenward-Roger degrees of freedom (Kenward & Roger, 2009), using Anova() from *car*.

1084 singletons (28.2% of the ASVs) were removed for β-diversity modelling, and a PERMANOVA was performed, setting experimental group and male age class as fixed effects, constraining the permutations (=1000) within strata=bird ID.

A GLMM by Laplace approximation was fitted to the bacterial load data that followed a gamma distribution (link="log"), setting experimental group and age class as fixed effects, and bird ID as a random effect.

(5) *Covariation between sperm velocity/concentration, and ejaculate bacterial load/richness.* For this objective, we analysed the 24 ejaculate samples that were measured for sperm VAP—a proxy for sperm swimming velocity—and the 42 ejaculate samples that were measured for sperm concentration.

Sperm VAP and concentration were normally distributed. An LMM by REML was fitted to sperm velocity data, setting experimental group (n_{start}=2, n_{middle}=2, n_{end}=4, two matings with the same female: n_{first} =5, n_{second}=4, or two matings with two different females: n_{first}=2, n_{second}=3), age class (n_{young}=10 or n_{old}=12), sperm concentration, bacterial load, and total ASV richness as fixed effects, and bird ID (n=10) as a random effect.

An LMM by REML was fitted to sperm concentration data, setting experimental group (n_{start}=5, n_{middle}=5, n_{end}=4, two matings with the same female: n_{first} =8, n_{second}=9, or two matings with two different females: n_{first}=4, n_{second}=5), age class (n_{young}=17 or n_{old}=23), bacterial load, and total ASV richness as fixed effects, and bird ID (n=13) as a random effect. The sperm concentration model revealed a negligible effect of bird ID (S.D.≈0). Thus, the effect of bird ID was tested via information-theoretic model selection, comparing the nested model without the random effect to the full model. The AIC_C score was lower for the full model (K=11, AIC_C=1028.75, ΔAIC_C=0, weight=1.00) and the LMM was preferred over a linear model (LM). Scatter plots of the linear relationships between ejaculate bacterial load (log-normalised for the figures), and sperm VAP and concentration were created with *ggplot2*.

We did not include Shannon's nor inverse Simpson's diversity in this part of the analysis due to small sample size and risk of over-parametrisation. We decided in favour of total ASV richness as it is an intuitive measure of unique bacterial strains present in ejaculates and because our analysis revealed that bird ID had a larger effect on total ASV richness, while there was less variation across the other two α-diversity metrics.

Results

(1) *Characterisation of the Red Junglefowl Ejaculate Microbiota Composition, and Comparisons with the Male and Female Fowl Reproductive Tract Microbiota*

Red junglefowl ejaculates were dominated by Actinobacteria (45.6%±2.5% S.E.), but also harboured Proteobacteria (21.0±2.1%) and Firmicutes (20.8±2.0%) in large relative abundances (Figure 2A). At the genus level, ejaculates had high numbers of *Janibacter* (phylum Actinobacteria; 17.1±1.7%) and *Brachybacterium* (phylum Actinobacteria; 16.7±1.9%; Figure 2B). Ejaculates also harboured a large relative abundance of rare genera (32.7±1.7%) that each had a <1% relative abundance across ejaculates (Figure 2B).

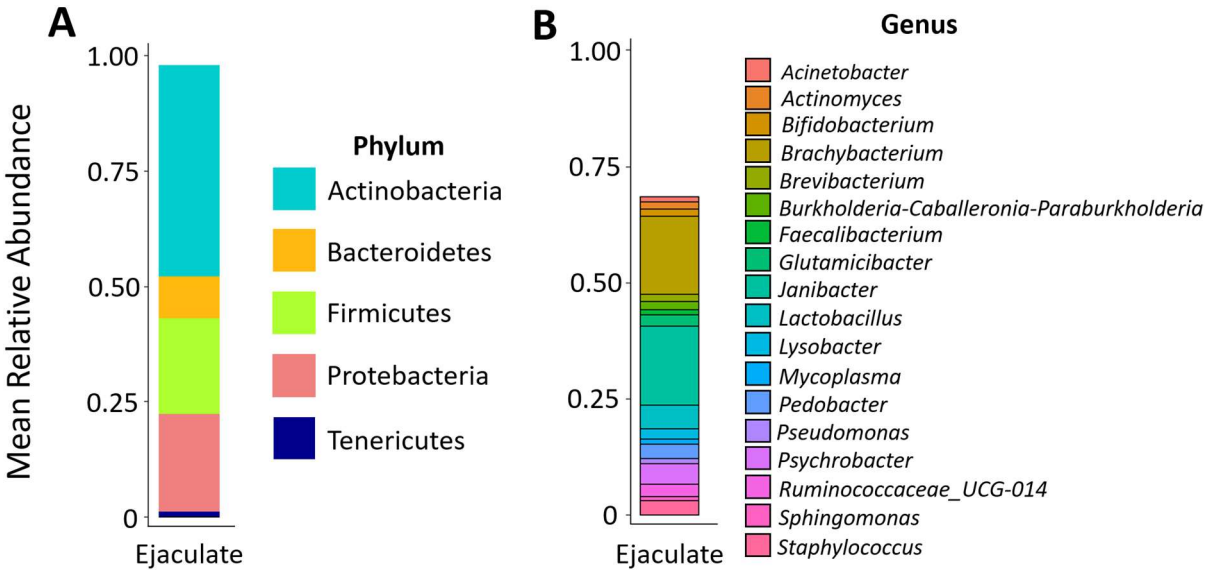


Figure 2. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A) and genera (B) present in red junglefowl ejaculates (n=58). Only taxa that contributed >1% to the mean relative abundances are visualised. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity (B).

Ejaculate microbiota was found to harbour 3543 ASVs, which we were able to assign to 35 bacterial phyla and 567 genera. A qualitative comparison between exclusive and shared ASVs between ejaculates, and samples collected across the functionally-distinct sections of the male and female fowl reproductive tracts revealed that each ecosystem harbours a high richness

of exclusive ASVs, while a small amount is also shared (Figure 3). The most prevalent shared genus between the three ecosystems was *Lactobacillus* (3.68% of the universally shared ASVs).

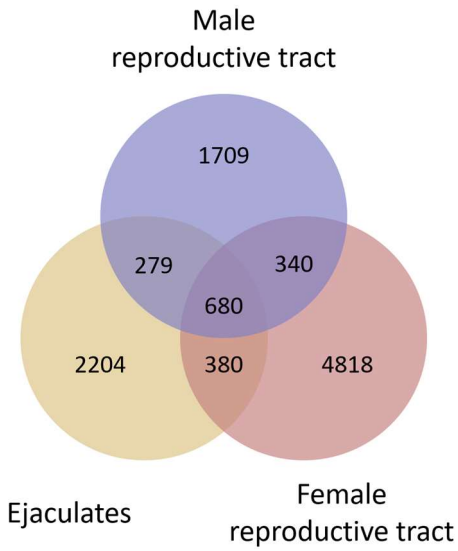


Figure 3. A Venn diagram indicating the exclusive and shared ASVs between red junglefowl ejaculates (n=58), male reproductive tract tissue samples (n=60), and female reproductive tract lavage and tissue samples (n=276). ASVs associated exclusively with male and female fowl digestive tract samples that were not found in any of the male and female fowl reproductive tract samples (**Chapters II–III**) were excluded from the figure.

We report that ejaculate samples captured the same bacterial phyla—except for Fusobacteria—but in significantly different abundances in comparison with MRT and digestive tract tissue samples, and faecal samples (Figure 4A; Supplementary Table 2). Comparing ejaculates to the functionally-distinct tissue sections of the MRT, cloaca tissue, colon tissue, and faeces revealed significant differences, with ejaculates significantly distinct from the rest of the sample types (Figure 4, 5; Supplementary Table 3).

Differential bacterial abundance analysis between ejaculates, and the MRT and cloacal microbiota revealed three bacterial phyla and 19 genera that were significantly differentially-abundant. Ejaculates harboured significantly more Actinobacteria ($\beta \pm S.E. = 1.67 \pm 0.17$, $t = 9.93$, $p < 0.001$), and significantly less Fusobacteria ($\beta = -5.22 \pm 0.41$, $t = -12.86$, $p < 0.001$) and Firmicutes ($\beta = -0.78 \pm 0.24$, $t = -3.24$, $p = 0.002$; Figure 4A). Of note, ejaculates were significantly

enriched with the genera *Janibacter* (β=3.23±0.25, t=12.85, p<0.001), *Brachybacterium* (β=2.25±0.22, t=10.26, p<0.001), and *Psychrobacter* (phylum Proteobacteria; β=5.01±0.65, t=7.73, p<0.001; Figure 4B; Supplementary Figure 3) in comparison with the other sample types.

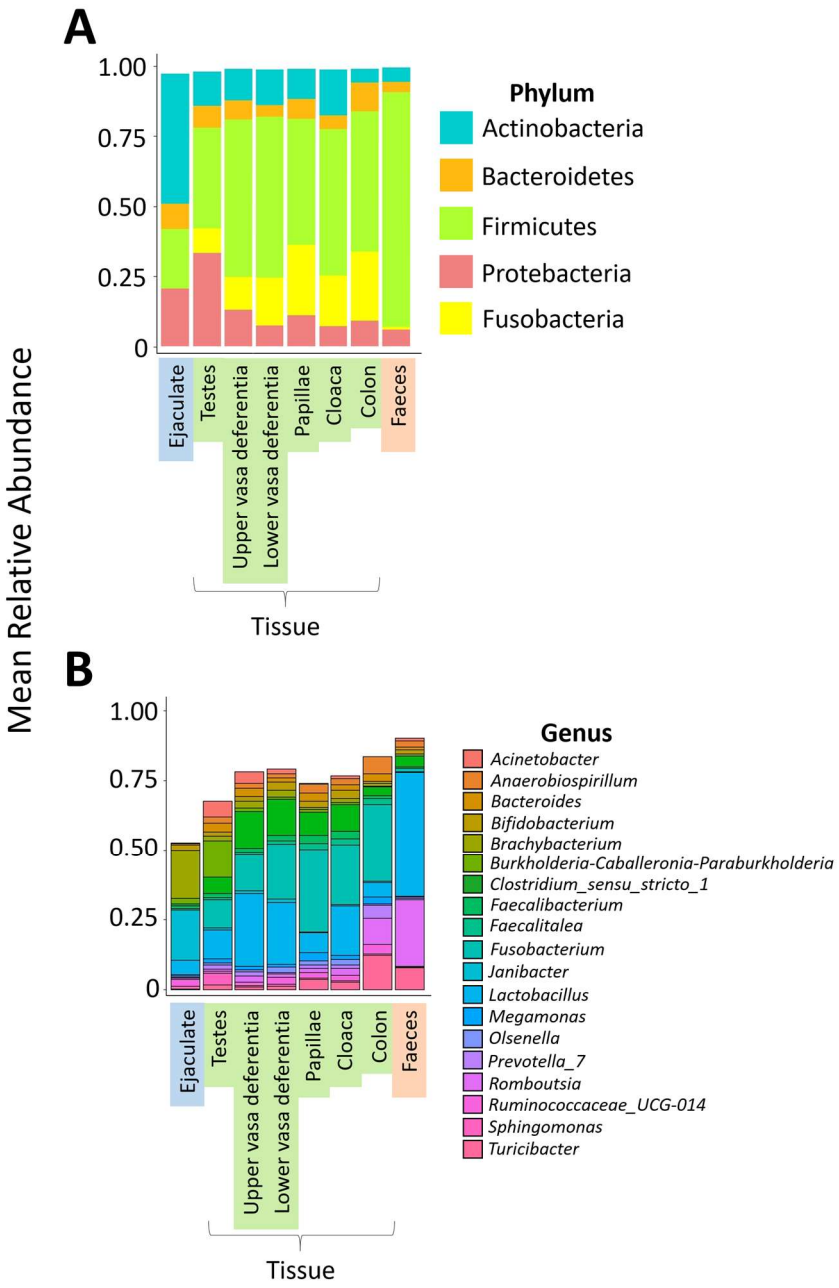


Figure 4. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A) and genera (B) present per sample type ($n_{ejaculate}$=58, n_{testes}=12, $n_{upper\ vasa\ deferentia}$=12, $n_{lower\ vasa\ deferentia}$=12, $n_{papillae}$=12, n_{cloaca}=12, n_{colon}=12, or n_{faeces}=12). Only taxa that contributed >1% to the mean relative abundances are visualised. The functionally-distinct MRT sections are presented from left to right in an anatomically-descending order, starting with the testes. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity (B).

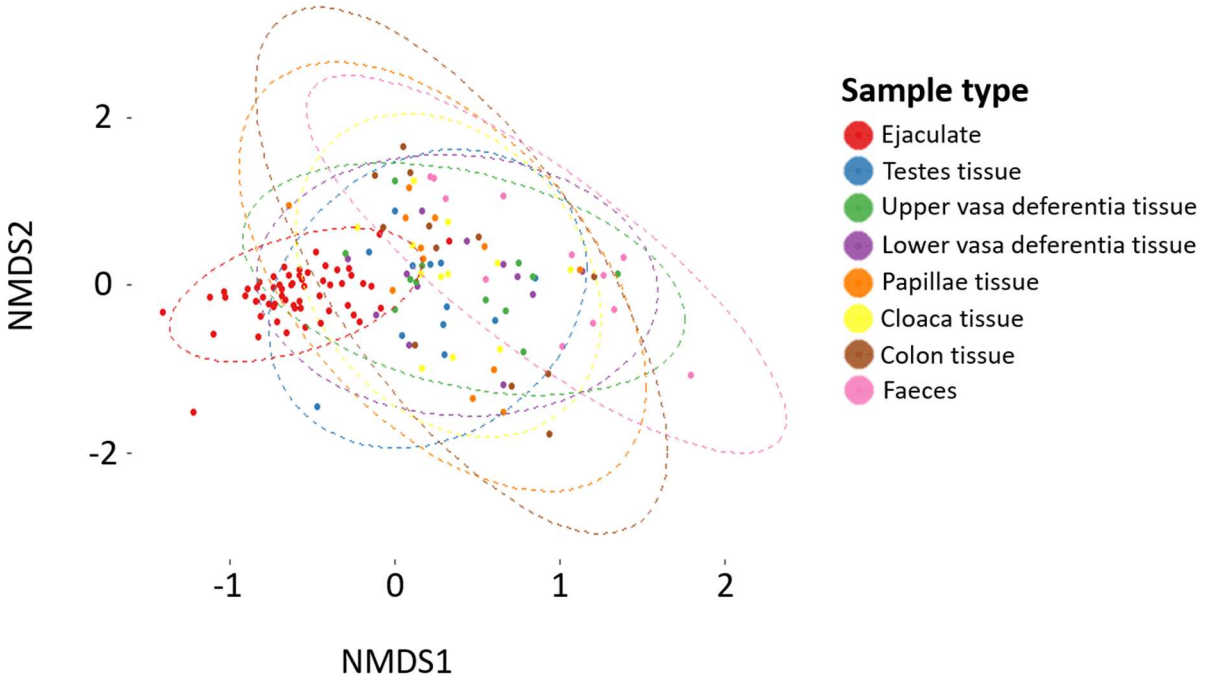


Figure 5. A non-metric multidimensional scaling (NMDS) ordination (stress=0.20) depicting compositional differences between sample types ($n_{ejaculate}$=58, n_{testes}=12, $n_{upper\ vasa\ deferentia}$=12, $n_{lower\ vasa\ deferentia}$=12, $n_{papillae}$=12, n_{cloaca}=12, n_{colon}=12, or n_{faeces}=12), each indicated by a unique colour. The ordination is based on genus-level Bray-Curtis dissimilarities. Each dot is an individual ejaculate, tissue, or faecal sample. The dashed ellipses indicate the respective multivariate normal distributions. The significant differences between ejaculates and the rest of the sample types was contributed to significant beta dispersion differences (pseudo-$F_{7, 134}$=3.72, p=0.002).

(2) *Age-Associated Variation in Red Junglefowl Ejaculate Microbiota*

Ejaculate microbiota composition variation was significantly associated with age class, even when controlling for the effect of experimental group first (Supplementary Table 4). Two bacterial phyla and two genera were significantly differentially-abundant across age class. Tenericutes were significantly more abundant (β=0.93±0.34, t=2.70, p=0.010), while Firmicutes significantly under-represented (β=-0.76±0.25, t=-3.02, p=0.004) in old males (Figures 6A, 9A). At the genus level, *Sphingomonas* (phylum Proteobacteria) was significantly more abundant (β=1.12±0.34, t=3.28, p=0.002) and *Lactobacillus* significantly less abundant (β=-0.70±0.24, t=-2.90, p=0.006) in old males (Figures 6B, 9B).

After controlling for the experimental group effect, male age class did not have a significant effect on red junglefowl ejaculate total ASV richness (GLMM: χ^2=2.16, df=1,

p=0.141), Shannon's diversity (GLM: χ^2=3.32, df=1, p=0.068), inverse Simpson's diversity (GLM: χ^2=0.44, df=1, p=0.507), nor bacterial load (GLMM: χ^2=0.24, df=1, p=0.624). Bird ID had a small effect on total ASV richness (S.D.=0.27) and bacterial load (S.D.=0.76), and a negligible effect on Shannon's (model without the random effect performed better: K=9, AIC_C=196.05, ΔAIC_C=0, weight=0.92) and inverse Simpson's diversity (model without the random effect performed better: K=9, AIC_C=480.16, ΔAIC_C=0, weight=0.92).

(3) *Ejaculate Microbiota Variation Over the Course of Breeding Season*

Controlling for male fowl age class, the total ASV richness of ejaculate microbiota did not significantly vary across ejaculates collected at the start, middle, and end of the experimental month (GLM: χ^2=2.52, df=2, p=0.283; Figure 5A). Bird ID did not affect ejaculate total ASV richness (model without the random effect performed better: K=5, AIC_C= 312.27, ΔAIC_C=0, weight=0.94) over the course of breeding season. Similarly, Shannon's (GLMM: χ^2=0.03, df=2, p=0.987) and inverse Simpson's diversity (GLM: χ^2=0.36, df=2, p=0.835) were also uniform across ejaculates collected over the experimental month, while controlling for age class (Figure 5B & C). Bird ID had a minimal effect on Shannon's (S.D.=0.04) and inverse Simpson's diversity (model without the random effect performed better: K=5, AIC_C= 199.91, ΔAIC_C=0, weight=0.94).

Controlling for male age class, ejaculate bacterial composition did not significantly differ between samples collected at the start, middle, and end of the experimental month (Table 1; Figure 7A & B). Age class and bird ID, however, had a significant effect on ejaculate microbiota composition variation over the course of breeding season (Table 1).

Bacterial load did not differ between ejaculates collected at the start, middle, and end of the experimental month (GLMM: χ^2=3.82, df=2, p=0.148; Figure 6), while controlling for male age class. Bird ID (S.D.=0.68) had a small effect on bacterial load.

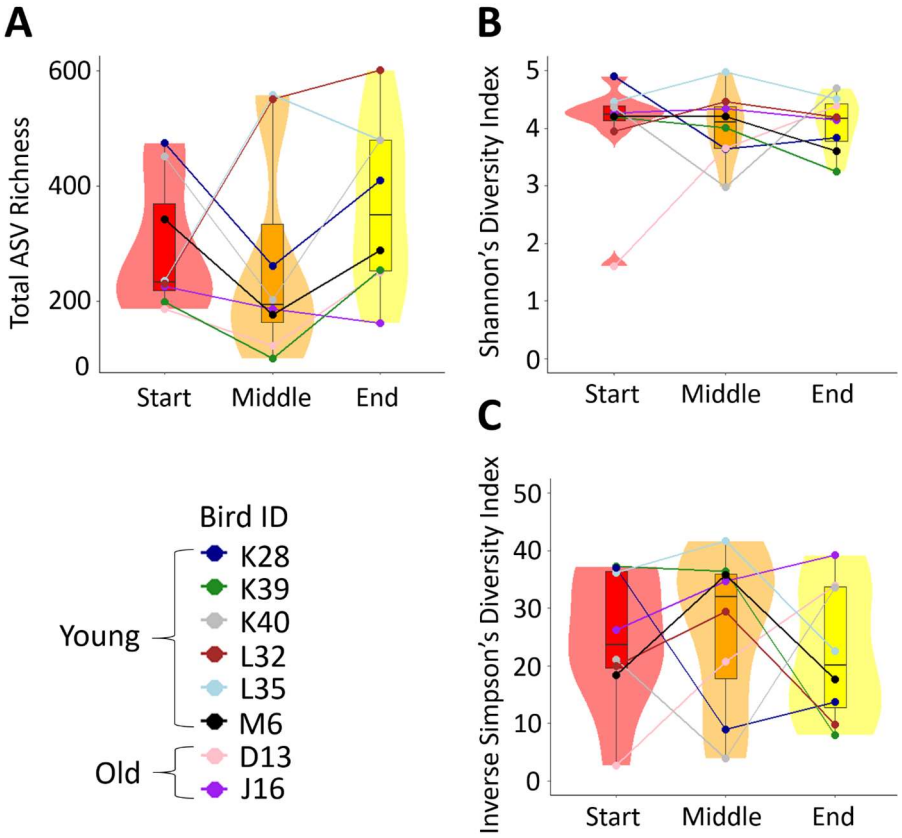


Figure 5. Ejaculate microbiota α-diversity across the experimental month. Ejaculates collected at the start (red, n=8), middle (orange, n=8), and end (yellow, n=8) of the experimental month were compared to one another using three α-diversity measures: total ASV richness (A), Shannon's diversity (B), and inverse Simpson's diversity (C). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Ejaculate samples of each individual are presented as uniquely coloured dots and are connected with lines.

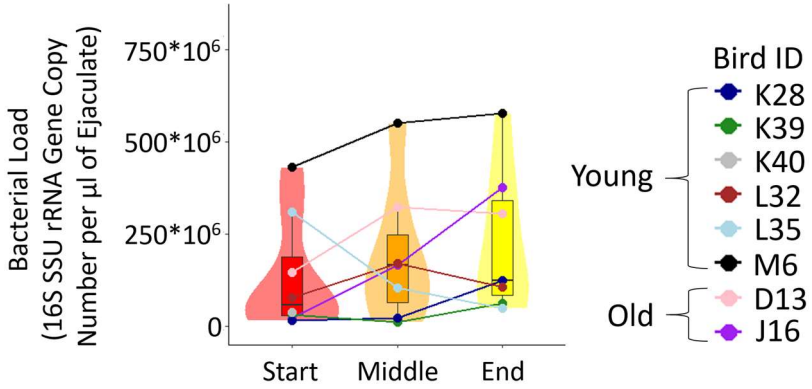


Figure 6. 16S SSU rRNA gene copy number per µl of ejaculate measured at the start (red, n=8), middle (orange, n=7), and end (yellow, n=7) of the experimental month. All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Ejaculate samples of each individual are presented as uniquely coloured dots and are connected with lines. qPCRs failed to amplify bacterial DNA from the ejaculates of bird K40 collected at the middle and end of the experimental month.

Table 1. [PERMANOVA] Ejaculate microbiota composition variation comparison between experimental group (n_{start}=8, n_{middle}=8, or n_{end}=8) and age class (n_{young}=18 or n_{old}=6) at bacterial phylum (A) and genus (B) level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID*. Significant p-values are in bold. Beta dispersion tests revealed no significant variation in genus-level uwUF distances (pseudo-$F_{1, 22}$=0.84, p=0.376) nor genus-level Jaccard indices (pseudo-$F_{1, 22}$=0.39, p=0.542) across age class.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	0.13	2	0.990	1.1%
Age class	0.93	1	0.441	4.0%
Bird ID	1.29	6	0.285	33.7%
Residuals		14		61.2%
Unweighted UniFrac				
Experimental group	1.08	2	0.398	7.2%
Age class	2.24	1	0.051	7.5%
Bird ID	**1.91**	**6**	**0.010**	**38.4%**
Residuals		14		46.9%
Bray-Curtis DS				
Experimental group	0.07	2	0.990	0.7%
Age class	0.64	1	0.605	3.1%
Bird ID	0.98	6	0.498	28.4%
Residuals		14		67.8%
Jaccard Index				
Experimental group	1.15	2	0.340	7.8%
Age class	2.02	1	0.056	6.9%
Bird ID	**1.82**	**6**	**0.007**	**37.4%**
Residuals		14		47.9%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	0.68	2	0.820	5.5%
Age class	0.88	1	0.495	3.5%
Bird ID	1.39	6	0.082	34.0%
Residuals		14		57.0%
Unweighted UniFrac				
Experimental group	0.90	2	0.640	7.1%
Age class	**1.66**	**1**	**0.035**	**6.5%**
Bird ID	**1.33**	**6**	**0.023**	**31.4%**
Residuals		14		55.0%
Bray-Curtis DS				
Experimental group	0.98	2	0.501	7.3%
Age class	1.25	1	0.217	4.7%
Bird ID	**1.59**	**6**	**0.009**	**35.7%**
Residuals		14		52.3%
Jaccard Index				
Experimental group	0.99	2	0.430	7.9%
Age class	**1.66**	**1**	**0.022**	**6.6%**
Bird ID	**1.25**	**6**	**0.034**	**30.0%**
Residuals		14		55.5%

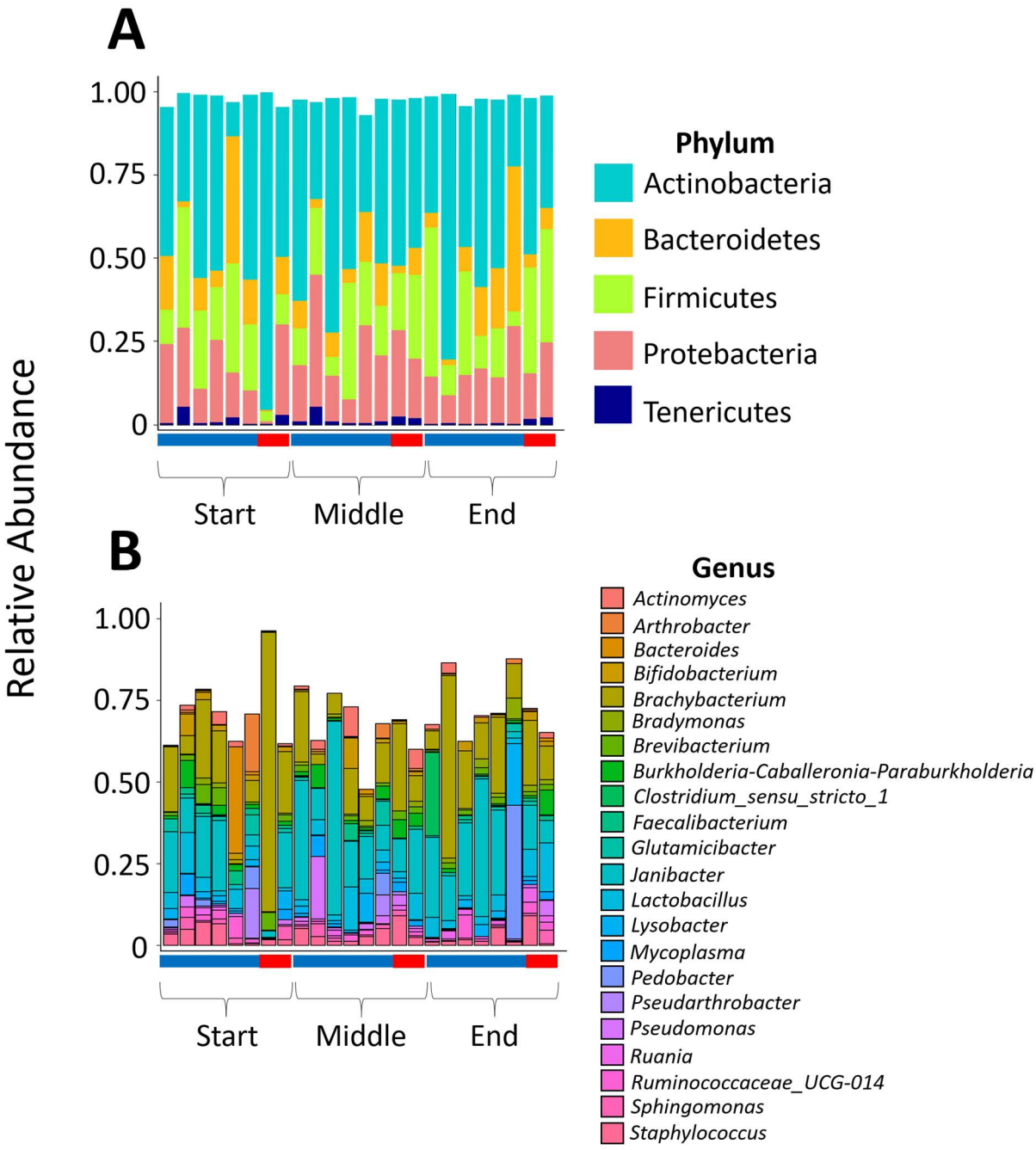


Figure 7. Stacked bar plots depicting the relative abundances of bacterial phyla (A) and genera (B) present in the ejaculates collected at the start, middle and end of the experimental month (n=8 for each collection time point) from six young and two old male red junglefowl. Only taxa that were >1% abundant are visualised. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity (B). Experimental groups are clustered together and the order of individual birds within each experimental group is kept consistent. Six young birds are indicated with a blue bar and two old birds with a red bar under the stacked bar plots.

(4) *Ejaculate Microbiota Variation Across Mating Sequences*

Controlling for male age class, total ASV richness was uniform in the first and second ejaculate collected across an experimental mating sequence (GLMM: χ^2=5.49, df=3, p=0.140), regardless of whether the second mating was with the same or a different female (Figure 8A). Bird ID, on the other hand, had a substantial effect on total ASV richness variation across short mating sequences (S.D.=26.67).

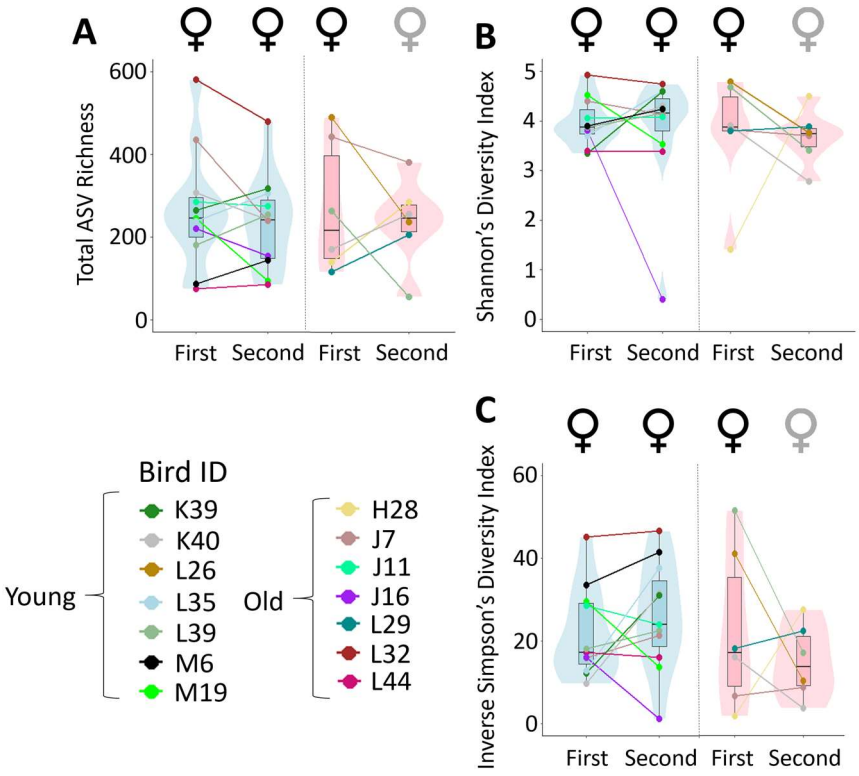


Figure 8. Ejaculate microbiota α-diversity in the first and second ejaculate of a mating sequence. Ejaculates collected from males mating with the same (light blue, n_{first}=n_{second}=11) or two different females (light pink, n_{first}=n_{second}=6) across two consecutive matings (first and second mating of the day) were compared to one another using three α-diversity measures: total ASV richness (A), Shannon's diversity (B), and inverse Simpson's diversity (C). All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Ejaculate samples of each individual are presented as uniquely coloured dots and are connected with lines.

Shannon's (GLMM: χ^2=0.32, df=3, p=0.957) and inverse Simpson's diversity (LMM: $F_{3,\ 21.8}$=0.77, p=0.522) were also uniform across the first and second ejaculates of mating sequences (Figure 8B & C). Bird ID had a minimal effect on Shannon's diversity (S.D.=0.06), while having a relatively large effect on inverse Simpson's diversity (S.D.=5.04).

Similarly, ejaculate microbiota composition did not differ across a sequence of matings, regardless of whether the second mating was with a familiar or novel female, except when comparing genus-level Bray-Curtis dissimilarities (Table 1B). This significant pattern was not due to significant beta dispersion variation (pseudo-$F_{3,\ 30}$=0.83, p=471). Fowl age class and bird ID had a significant effect on ejaculate microbiota composition after controlling for compositional variation across a sequence of matings (Table 2; Figure 9A & B).

The first and second ejaculate of a mating sequence did not differ in their bacterial load (GLMM: χ^2=1.96, df=3, p=0.581; Figure 10), while controlling for male age class. Bird ID had a small effect on ejaculate bacterial load across mating sequences (S.D.=0.75).

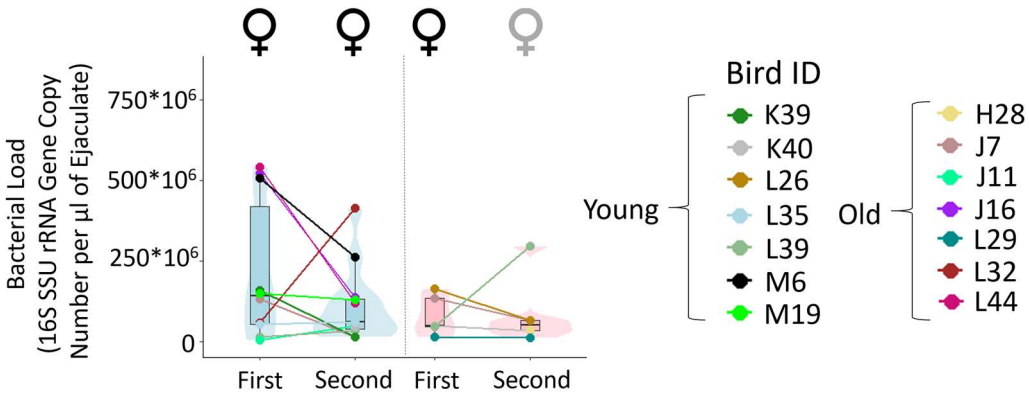


Figure 10. 16S SSU rRNA gene copy number per µl of ejaculate measured across two consecutive matings with either the same (light blue; n_{first}=10, n_{second}=11) or two different (light pink; n_{first}=5, n_{second}=6) female fowl. All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. The shape of the violin plot shows the spread of data. Ejaculate samples of each individual are presented as uniquely coloured dots and are connected with lines. qPCR failed to amplify bacterial DNA from the first ejaculate of bird K40 that mated with the same female in two consecutive matings. The first ejaculate of bird H28 that mated with two different females in two consecutive matings was considered an outlier ($2.72*10^9$ copy number/µl), and thus excluded.

Table 2. [PERMANOVA] Ejaculate microbiota composition variation between experimental group (two matings with the same female: n_{first} =11 or n_{second}=11, or two matings with two different females: n_{first}=6 or n_{second}=6) and age class (n_{young}=16 or n_{old}=18) at bacterial phylum (A) and genus (B) level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=14). Significant p-values are in bold. Significant factors were followed up by beta dispersion tests that revealed no significant dispersion variation (p>0.05) across age class nor experimental group regardless of the taxonomic level and β-diversity distance metric.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	0.75	3	0.701	6.3%
Age class	**3.99**	**1**	**0.011**	**11.2%**
Bird ID	1.03	12	0.400	34.8%
Residuals		17		47.7%
Unweighted UniFrac				
Experimental group	0.68	3	0.827	4.8%
Age class	1.71	1	0.111	4.0%
Bird ID	**1.85**	**12**	**0.007**	**51.7%**
Residuals		17		39.5%
Bray-Curtis DS				
Experimental group	0.82	3	0.596	6.6%
Age class	**4.98**	**1**	**0.002**	**13.2%**
Bird ID	1.10	12	0.351	35.1%
Residuals		17		45.1%
Jaccard Index				
Experimental group	0.85	3	0.682	5.5%
Age class	1.74	1	0.113	3.7%
Bird ID	**2.11**	**12**	**<0.001**	**54.3%**
Residuals		17		36.5%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	1.16	3	0.260	8.7%
Age class	**2.64**	**1**	**0.008**	**6.6%**
Bird ID	**1.39**	**12**	**0.048**	**41.9%**
Residuals		17		42.8%
Unweighted UniFrac				
Experimental group	0.89	3	0.704	6.6%
Age class	**1.76**	**1**	**0.028**	**4.3%**
Bird ID	**1.59**	**12**	**<0.001**	**47.1%**
Residuals		17		42.0%
Bray-Curtis DS				
Experimental group	**1.49**	**3**	**0.022**	**10.1%**
Age class	**2.22**	**1**	**0.012**	**5.0%**
Bird ID	**1.70**	**12**	**<0.001**	**46.3%**
Residuals		17		38.6%
Jaccard Index				
Experimental group	1.00	3	0.438	7.3%
Age class	**1.68**	**1**	**0.014**	**4.1%**
Bird ID	**1.59**	**12**	**<0.001**	**46.9%**
Residuals		17		41.7%

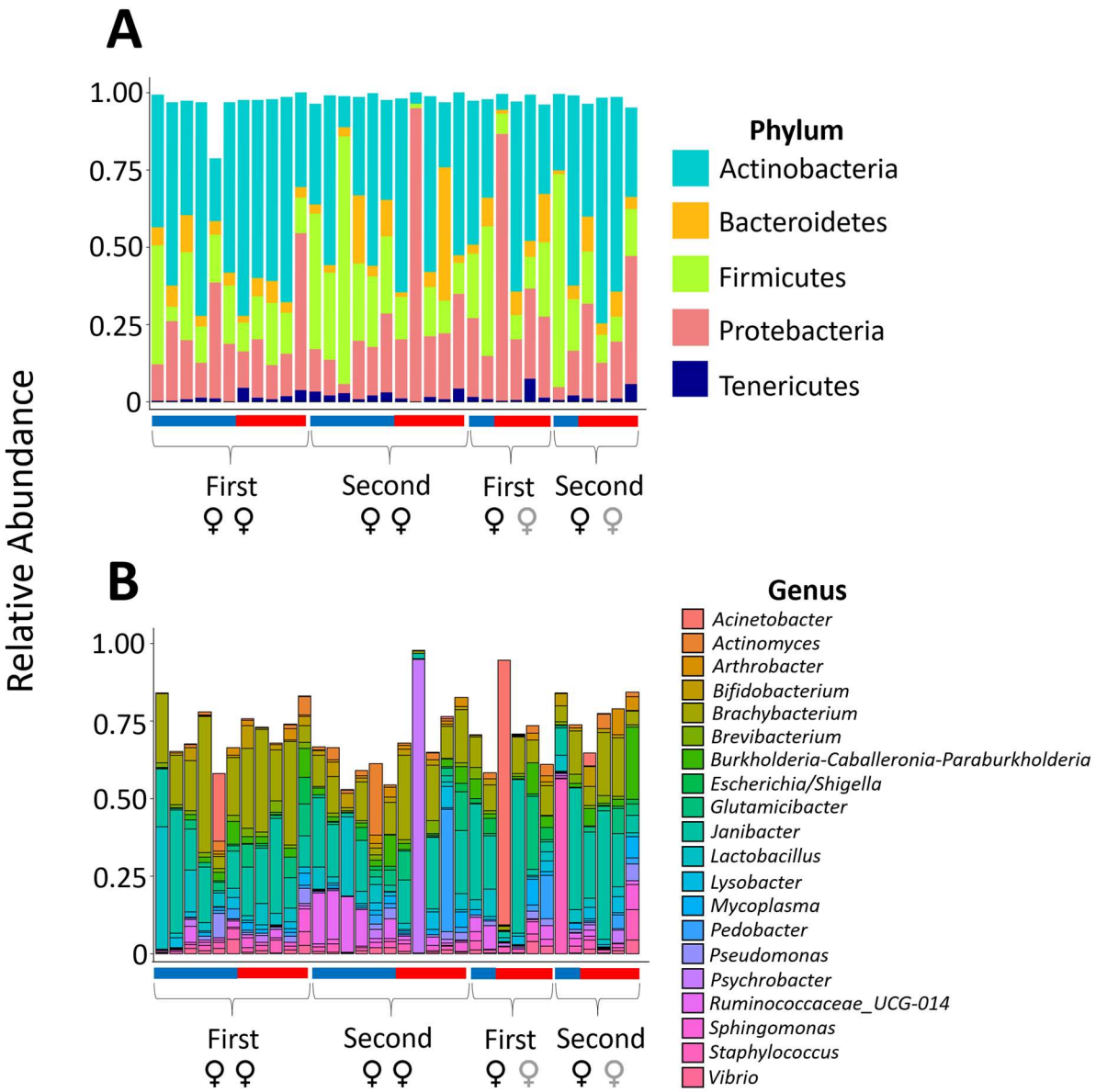


Figure 9. Stacked bar plots depicting the relative abundances of bacterial phyla (A) and genera (B) present in red junglefowl ejaculates collected across short mating sequences (two matings with the same female (indicated by black): $n_{first}=n_{second}=11$; two matings with two different females (indicated by black and grey): $n_{first}=n_{second}=6$). Only taxa that were >1% abundant are visualised. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity (B). Experimental groups are clustered together and the order of individual birds within experimental group is kept consistent. Six young and five old birds were sampled for ejaculates across two consecutive matings with the same female, and two young and four old males were sampled across two consecutive matings with two different females. Young birds are indicated with a blue bar and old birds with a red bar under the stacked bar plots.

(5) *Covariation Between Sperm Velocity/Concentration, and Ejaculate Bacterial Load/Richness*

After controlling for experimental group (LMM: $F_{6, 7.3}$=1.47, p=0.308), age class ($F_{1, 4.4}$=0.16, p=0.708), and sperm concentration ($F_{1, 8.5}$=0.04, p=0.842), VAP did not significantly covary with bacterial load ($F_{1, 9.1}$=4.87, p=0.054) nor total ASV richness ($F_{1, 7.7}$=0.08, p=0.786). Bird ID had a considerable effect on VAP (S.D.=9.65).

Similarly, after controlling for experimental group (LMM: $F_{6, 26.0}$=0.91, p=0.504) and age class ($F_{1, 5.4}$=2.65, p=0.106), bacterial load ($F_{1, 10.1}$=3.13, p=0.107) and total ASV richness ($F_{1, 22.4}$=0.32, p=0.575) did not significantly covary with sperm concentration. Bird ID had a negligible effect on sperm concentration (S.D.≈0). Combined, our results suggest that bacterial load may covary with sperm VAP and concentration (Figure 11), however the small sample size and non-significant results limit our speculations.

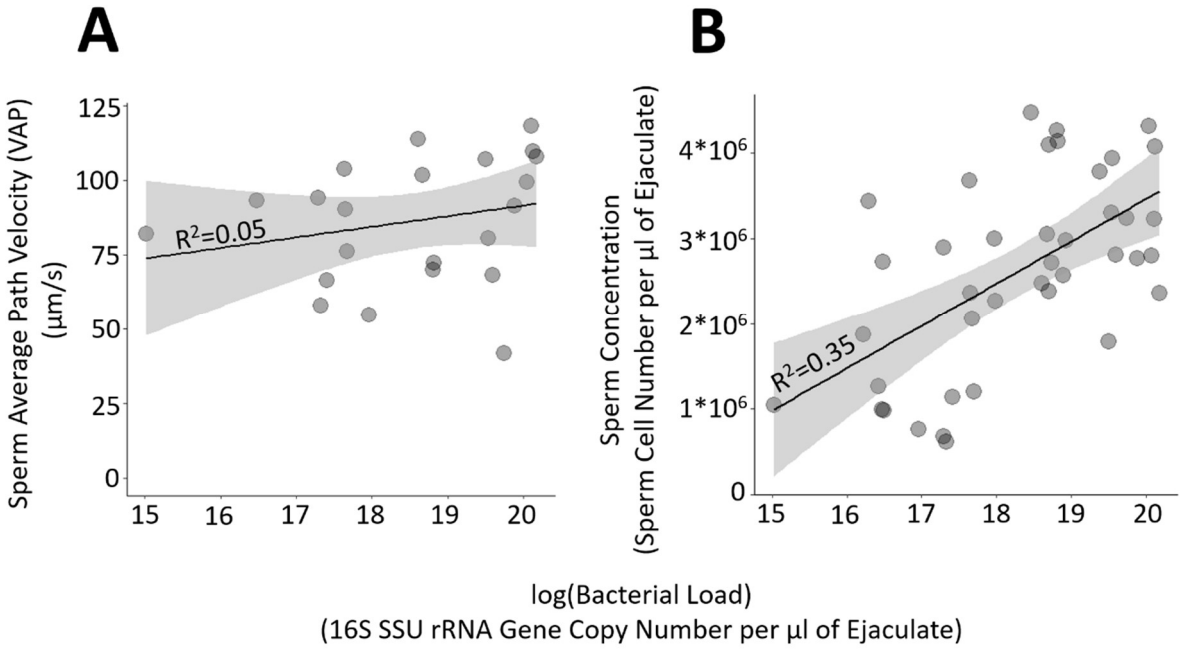


Figure 11. Linear relationships between sperm VAP and log(bacterial load) (n=22; A), and sperm concentration and log(bacterial load) (n=38; B) in red junglefowl ejaculates. Each dot is an ejaculate sample. The shaded area around the best fit regression line represents the 95% confidence interval.

Discussion

Males of polygynandrous species strategically invest sperm and seminal fluid in their ejaculates based on socio-sexual opportunities to increase their reproductive fitness (Pizzari et al., 2003; Alvarez-Fernandez et al., 2019). Sperm number and seminal fluid compounds also vary with male age, during a breeding season, and across successive mating opportunities (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016; Rakha et al., 2017; Wang, 2020c). Ejaculate microbiota is likely to exhibit similar patterns of variation and could affect fertilisation success in polygynandrous species, although empirical evidence is currently lacking.

We investigated this gap in knowledge by exploring the ejaculate microbiota of the polygynandrous red junglefowl. We found that fowl ejaculates harboured a bacterial community dominated by Actinobacteria, which despite sharing bacterial strains with the microbiota of both male and female fowl reproductive tracts, had a distinct bacterial composition. Young and old males harboured a different ejaculate microbiota, with the differences driven by differentially-abundant bacteria. For example, Tenericutes were more prevalent in old fowl. We report no evidence for ejaculate microbiota variation over the course of breeding season nor across mating sequences. Ejaculate total ASV richness did not covary with sperm velocity nor concentration, but ejaculate bacterial load showed moderate covariation with both sperm parameters.

The average ejaculate of the polygynandrous red junglefowl was largely dominated by the phylum Actinobacteria, followed by Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes. Little is known about the ejaculate microbiota of polyandrous avian species, but the cloacae of socially-monogamous male rufous-collared sparrows were found to harbour Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Tenericutes (Escallón, Belden & Moore, 2019), which is congruent with our findings.

Actinobacteria is commonly found on avian cloacae (Kreisinger et al., 2015), and in oral and faecal samples collected from birds (Kropáčková et al., 2017; Michel et al., 2018). Broiler chickens (Choi, Kim & Cha, 2014) and Indian indigenous chickens (Saxena et al., 2016) have been reported to harbour Actinobacteria across their gastrointestinal tract. The phylum includes avian pathogens, such as *Corynebacterium* (van Dongen et al., 2019), and beneficial bacteria, such as *Bifidobacterium* (phylum Actinobacteria; that we also found in fowl ejaculates). While the functional properties of Actinobacteria in fowl ejaculates remain unknown, Actinobacteria have been found in the testes of both normozoospermic (normal sperm) and azoospermic (no sperm) men (Alfano et al., 2018), suggesting it may be a commensal phylum associated with MRT and ejaculates across host taxa.

Two genera from the phylum Actinobacteria, *Janibacter* and *Brachybacterium*, were particularly abundant in fowl ejaculates, although their function in the context of male reproductive health and fitness remains unexplored. Both genera can take the shape of a rod or a coccoid, and have been reported in bacteremia (bacteria in bloodstream) cases (Loubinoux et al., 2005; Tamai et al., 2018). Further research is needed on the role of bacterial cell shape in host-associated microbiota. For example, specific bacterial shapes may provide a benefit in ejaculates. It is worth mentioning that *Janibacter* has also been reported as a potential laboratory contaminant (Salter et al., 2014). Our prevalence-based contaminant removal method did not categorise *Janibacter* as a contaminant strain (Supplementary Figure 1B).

Our results suggest that red junglefowl ejaculates share some bacterial strains with male and female fowl reproductive tract microbiota, while harbouring a considerable amount of exclusive ASVs. Previous studies have reported that seminal microbiota shares bacteria with vaginal microbiota in humans (Mändar et al., 2015) and faecal microbiota in mice (*Mus musculus*; Javurek et al., 2016), but predominantly supports a unique bacterial community.

The most abundant shared bacterial genus was *Lactobacillus*, which is commonly found in the domestic chicken gut and cloaca (Oakley et al., 2014), but has also been shown to negatively affect rooster sperm quality when sperm and bacteria come in direct contact (Haines et al., 2013; Kiess et al., 2016). Similarly, *Lactobacillus* has been associated with increased fertilisation success in human females, but not necessarily in males (Wang et al., 2020a), although it has been found in healthy, fertile human semen (Weng et al., 2014). Further research is needed to characterise the female and male reproductive tract microbiota across taxa, and study the consequences of sexual bacterial transmission for both female and male host.

Comparisons between the microbiota compositions present in fowl ejaculates, and male reproductive tract, cloaca, colon, and faeces revealed significant differences, with ejaculate bacterial communities clustering separately from the rest of the samples. It is worth mentioning that the differences detected may have been due to both biological and methodological reasons. The ejaculates may truly support a unique microbiota, but we also used slightly different laboratory and bioinformatic pipelines for ejaculate, tissue, and faecal samples to maximise bacterial DNA amplification and the quality of sequencing reads.

Ageing from nestling to adulthood has been shown to change the cloacal (van Dongen et al., 2013), faecal (González-Braojos et al., 2012), and gut (Kohl et al., 2019) microbiota of birds, with adults expected to harbour a relatively stable gut microbiota (Kohl, 2012). We were able to extend on these findings by showing temporal changes in the ejaculates of adult red junglefowl of different age class (young=3–4 years, old=5–6 years). While we did not detect variation in ejaculate bacterial richness, diversity, nor load across male age class, we found differentially-abundant bacteria in young and old fowl. Old males showed an increase in Tenericutes that was caused by *Mycoplasma wenyonii*, a haemotrophic (attracted to red blood cells) bacterial strain previously associated with anaemia, inflammation, and infertility in cattle (*Bos taurus*; McAuliffe et al., 2006). *Sphingomonas*, which has been found in the seminal fluids

of mice, where it was associated with metabolic pathway changes (Javurek et al., 2016), was significantly abundant in old fowl. *Lactobacillus*, which has been linked to both beneficial (Weng et al., 2014) and detrimental effects in ejaculates (Haines et al., 2013; Wang et al., 2020a), was significantly absent in old males.

Differential bacterial abundances between young and old red junglefowl may have been due to (i) decreased mating opportunities for old fowl (Dean et al., 2010), which may limit their exposure to bacterial transfers during mating, (ii) differential sperm number and seminal fluid proteome in old males (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016) that may affect ejaculate microbiota composition, and/or (iii) accumulation of mostly pathogenic bacteria across breeding seasons that become more abundant in old birds, as has been reported in fine wool rams (Wiemer & Ruttle, 1987).

We found remarkable microbiota consistency across red junglefowl ejaculates, with no evidence for ejaculate microbiota variation over the course of breeding season nor across mating sequences. Upon ejaculation, seminal fluid is flushed through the reproductive tract and bacteria present in the lumen are incorporated into the ejaculate. Our results show that a consistent bacterial composition and load are incorporated in all red junglefowl ejaculates, with little variation even between individual birds.

Considering the consistent ejaculate bacterial diversity, harbouring an ejaculate microbiota is unlikely to have negative reproductive fitness consequences in the polygynandrous red junglefowl system, although further research is necessary to test for fitness costs and benefits related to ejaculate bacteria. Alternatively, a consistently diverse and abundant ejaculate microbiota may be beneficial, if it increased male fertilisation success. Sexually-transmitted beneficial bacteria could have an advantageous function in the female reproductive tract where they may also be transferred to offspring (Lombardo et al., 1999; Smith & Mueller, 2015). In support of this, the cloacal bacterial diversity of male rufous-

collared sparrows was found to increase before and decrease after the breeding season (Escallón, Belden & Moore, 2019), suggesting minimal costs or even benefits for harbouring a diverse reproductive microbiota in males. It is also worth mentioning that we may have failed to detect any patterns of variation in ejaculate microbiota due to methodological limitations, such as low sample size and/or allowing the experimental males to rest between intense mating days, during which the highly diverse and abundant MRT and ejaculate microbiota of the red junglefowl could have re-established.

Finally, we report that neither bacterial load nor richness covaried significantly with sperm velocity nor concentration in fowl ejaculates. However, ejaculate bacterial load showed moderate covariation with sperm velocity and concentration, with ejaculates that harboured a higher bacterial load also indicating patterns suggestive of higher sperm velocity and concentration. Although we should not overlook the potential beneficial effect of ejaculate microbiota on sperm cells, having a high ejaculate bacterial load is more likely indicative of a trade-off between reproduction and immunity in males. To illustrate, antimicrobial peptides (AMPs), such as lysozyme in birds, have been proposed to clear the male reproductive tract and ejaculates of bacteria (Rowe et al., 2013), while also being cytotoxic to sperm cells (Boman, 2003). In order to maintain their sperm number, males may have to forego producing AMPs and tolerate bacteria in their reproductive tracts. In support of this, male black garden ants (*Lasius niger*) were shown to have minimal AMP-activity in their sperm storage organs, which in turn increased bacterial growth in their accessory testes (Dávila et al., 2018). This strategy could also explain the highly diverse and abundant ejaculate microbiota observed in the red junglefowl, but would need to be confirmed with proteomics. Of additional note, as measuring sperm concentration via spectrophotometry is limited and could also capture bacterial cells present in the ejaculates (bacterial cells are measured at 600 nm), our reported results should be taken with an added caveat.

Takeaways

- Red junglefowl ejaculate microbiota is distinct from male and female fowl reproductive tract bacterial assemblages
- Fowl ejaculates harbour an Actinobacteria-dominated (of note, the genera *Janibacter* and *Brachybacterium*) bacterial composition
- Ejaculate-associated bacterial assemblages vary across fowl age class, but appear consistently diverse over the course of breeding season and across mating sequences
- Combining our results and considering that we found a weak positive association between ejaculate bacterial load, and sperm velocity and concentration, the function and underlying maintenance mechanisms of harbouring a diverse and abundant ejaculate microbiota merit further exploration

References

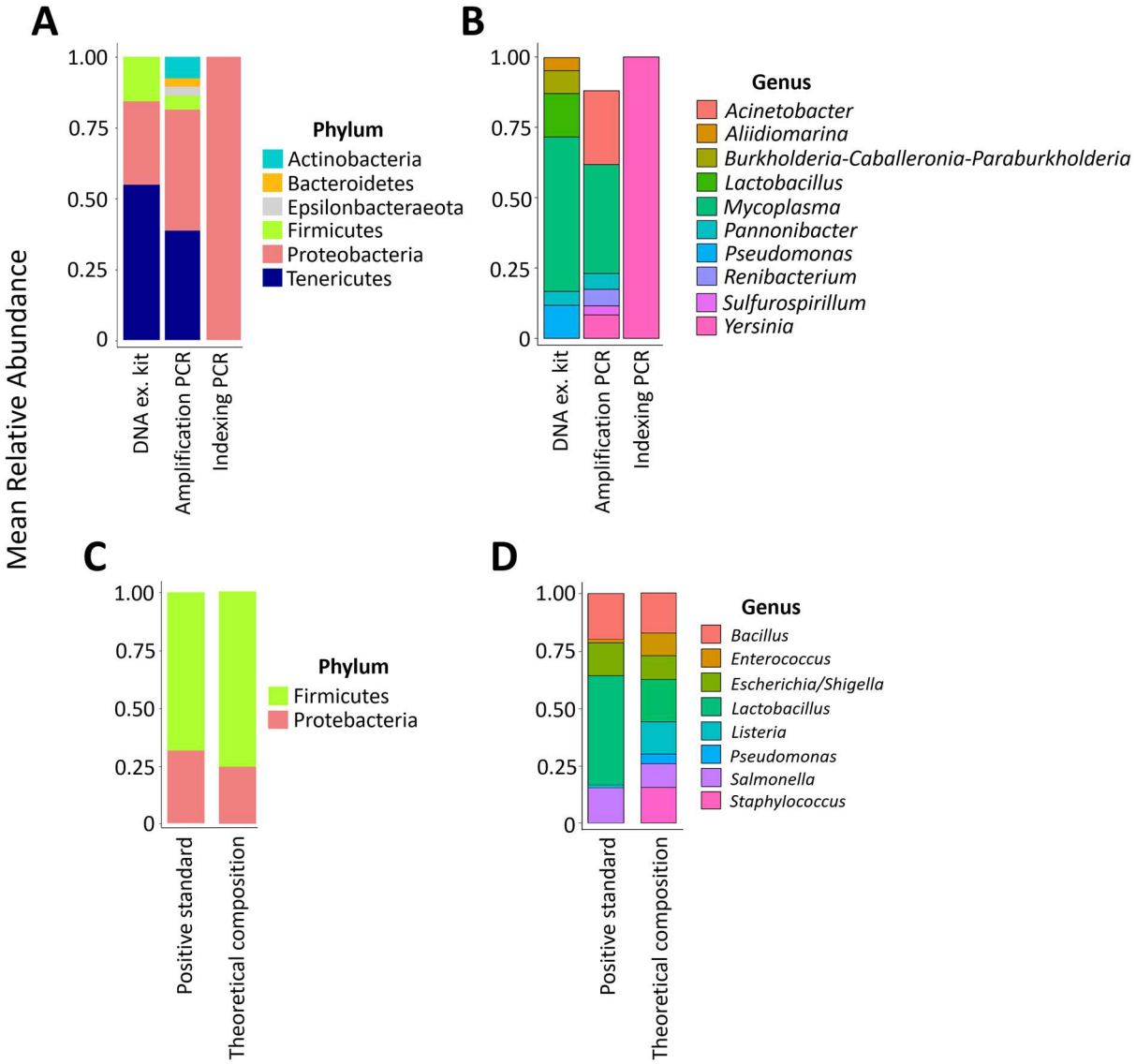
Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

Appendices

Supplementary Table 1. Primer sequences (5'–3' orientation) used in the amplification and indexing PCRs. For clarity, the functional regions of each primer have been separated by a "+". The locus-specific sequence is based on the Earth Microbiome Project (Thompson et al., 2017). The forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) cover the V4 hypervariable region of the bacterial 16S SSU rRNA gene (~254 base pairs). The adapter used for trimming is indicated in bold. The paired-end index combinations were based on Kozich et al. (2013).

Amplification PCR primers	
	Illumina Nextera-compatible overhang + locus-specific sequence
515F	TCGTCGGCAGCGTC**AGATGTGTATAAGAGACAG** + GTGYCAGCMGCCGCGGTAA
806R	GTCTCGTGGGCTCGG**AGATGTGTATAAGAGACAG** + GGACTACNVGGGTWTCTAAT

Indexing PCR primers	
	Flow cell adapter + index + Illumina Nextera-compatible overhang
SA501	AATGATACGGCGACCACCGAGATCTACAC + ATCGTACG + TCGTCGGCAGCGTC
SA502	AATGATACGGCGACCACCGAGATCTACAC + ACTATCTG + TCGTCGGCAGCGTC
SA504	AATGATACGGCGACCACCGAGATCTACAC + CTGCGTGT + TCGTCGGCAGCGTC
SA505	AATGATACGGCGACCACCGAGATCTACAC + TCATCGAG + TCGTCGGCAGCGTC
SA506	AATGATACGGCGACCACCGAGATCTACAC + CGTGAGTG + TCGTCGGCAGCGTC
SA507	AATGATACGGCGACCACCGAGATCTACAC + GGATATCT + TCGTCGGCAGCGTC
SA508	AATGATACGGCGACCACCGAGATCTACAC + GACACCGT + TCGTCGGCAGCGTC
SB501	AATGATACGGCGACCACCGAGATCTACAC + CTACTATA + TCGTCGGCAGCGTC
SB502	AATGATACGGCGACCACCGAGATCTACAC + CGTTACTA + TCGTCGGCAGCGTC
SB503	AATGATACGGCGACCACCGAGATCTACAC + AGAGTCAC + TCGTCGGCAGCGTC
SB504	AATGATACGGCGACCACCGAGATCTACAC + TACGAGAC + TCGTCGGCAGCGTC
SB505	AATGATACGGCGACCACCGAGATCTACAC + ACGTCTCG + TCGTCGGCAGCGTC
SB506	AATGATACGGCGACCACCGAGATCTACAC + TCGACGAG + TCGTCGGCAGCGTC
SB507	AATGATACGGCGACCACCGAGATCTACAC + GATCGTGT + TCGTCGGCAGCGTC
SB508	AATGATACGGCGACCACCGAGATCTACAC + GTCAGATA + TCGTCGGCAGCGTC
SA712	CAAGCAGAAGACGGCATACGAGAT + TCGCTATA + GTCTCGTGGGCTCGG
SB701	CAAGCAGAAGACGGCATACGAGAT + AAGTCGAG + GTCTCGTGGGCTCGG
SB702	CAAGCAGAAGACGGCATACGAGAT + ATACTTCG + GTCTCGTGGGCTCGG
SB703	CAAGCAGAAGACGGCATACGAGAT + AGCTGCTA + GTCTCGTGGGCTCGG
SB704	CAAGCAGAAGACGGCATACGAGAT + CATAGAGA + GTCTCGTGGGCTCGG
SB705	CAAGCAGAAGACGGCATACGAGAT + CGTAGATC + GTCTCGTGGGCTCGG
SB706	CAAGCAGAAGACGGCATACGAGAT + CTCGTTAC + GTCTCGTGGGCTCGG
SB707	CAAGCAGAAGACGGCATACGAGAT + GCGCACGT + GTCTCGTGGGCTCGG
SB708	CAAGCAGAAGACGGCATACGAGAT + GGTACTAT + GTCTCGTGGGCTCGG
SB709	CAAGCAGAAGACGGCATACGAGAT + GTATACGC + GTCTCGTGGGCTCGG
SB710	CAAGCAGAAGACGGCATACGAGAT + TACGAGCA + GTCTCGTGGGCTCGG
SB711	CAAGCAGAAGACGGCATACGAGAT + TCAGCGTT + GTCTCGTGGGCTCGG
SB712	CAAGCAGAAGACGGCATACGAGAT + TCGCTACG + GTCTCGTGGGCTCGG



Supplementary Figure 1. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A & C) and genera (B & D) detected in the negative controls (A & B) and the positive standard (C & D) in the data set consisting of only ejaculate samples. Only taxa that contributed >1% are presented. Mean relative abundances per negative control type (A & B) were calculated using the post-*decontam* untrimmed contaminants-only data set. Positive standard relative abundances (C & D) were calculated using the pre-*decontam* untrimmed data set. Lowering the inclusion threshold to 0.01% in the positive standard was necessary to detect the genus *Listeria* (D). Lowering the inclusion threshold further did not aid in detecting the expected genera *Pseudomonas* and *Staphylococcus* in the positive standard (D). Negative controls (A & B) are a sample of the QIAGEN DNeasy® Blood & Tissue DNA extraction kit (DNA ex. kit; n=1), amplification PCR negatives (n=6), and an indexing PCR negative (n=1). The positive standard (C & D) is a single ZymoBIOMICS® Microbial Community Standard (n=1). For comparison purposes, the theoretical composition of the ZymoBIOMICS® Microbial Community Standard expected by the manufacturer is presented together with the positive standard (C & D). No correction was made to the data set based on the observed and expected positive standard relative abundances. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity.

Supplementary Methods 1. Additional microbiota composition comparisons between ejaculates, and the functionally-distinct sections of the male reproductive tract (MRT), cloaca, colon, and faeces analysed in Chapter III.

Paired-end FASTQ files of the two data sets were processed together with *dada2* (Callahan et al., 2016a; Version 1.12.1) in R (R Core Team, 2019 ; Version 3.6.1). Reads were quality-checked and filtered using standard parameters (maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2) with additional truncLen=c(200, 200), resulting in a ~10% sequencing read loss. The error rates were calculated based on a random subset of samples (randomize=TRUE) and inference was carried out via pseudo-pooling (pool="pseudo"). Non-specific amplicons were removed, retaining only merged, denoised reads with 250–256 base pairs. Chimera check removed ~61% of the merged sequence variants, which accounted for ~16% of the total reads. SILVA reference database (Yilmaz et al., 2014; Version 132) was used for assigning taxonomies (100% identity) to the species level, when possible.

The processed data set including controls contained 152 samples, 6639 amplicon sequence variants or ASVs (241±11 S.E. per sample) and 3,204,158 reads (21,080±995 per sample). *decontam* (Davis et al., 2017) was used for further filtering of potential contaminants, which were detected based on ASV prevalence in negative controls versus biological samples, using a stringency threshold of 0.55 that split ASVs into contaminants (193 ASVs) and non-contaminants (6446 ASVs). There were no biological samples with fewer than 100 reads. ASVs that were present in the taxonomy, but not in the data set due to data filtering (n=111) were excluded. Finally, ASVs that failed to classify at the phylum level (n=93), or that classified as chloroplasts (n=35) or mitochondria (n=20; de la Cuesta-Zuluaga & Escobar, 2016) were removed. The final data set excluding controls contained 142 samples, 6187 ASVs (235±11 per sample), and 2,699,337 reads (19,009±859 per sample). Mean relative bacterial abundance in negative controls and the positive standard were visualised with *ggplot2* (Wickham, 2016), and are presented in the appendices (Supplementary Figure 2).

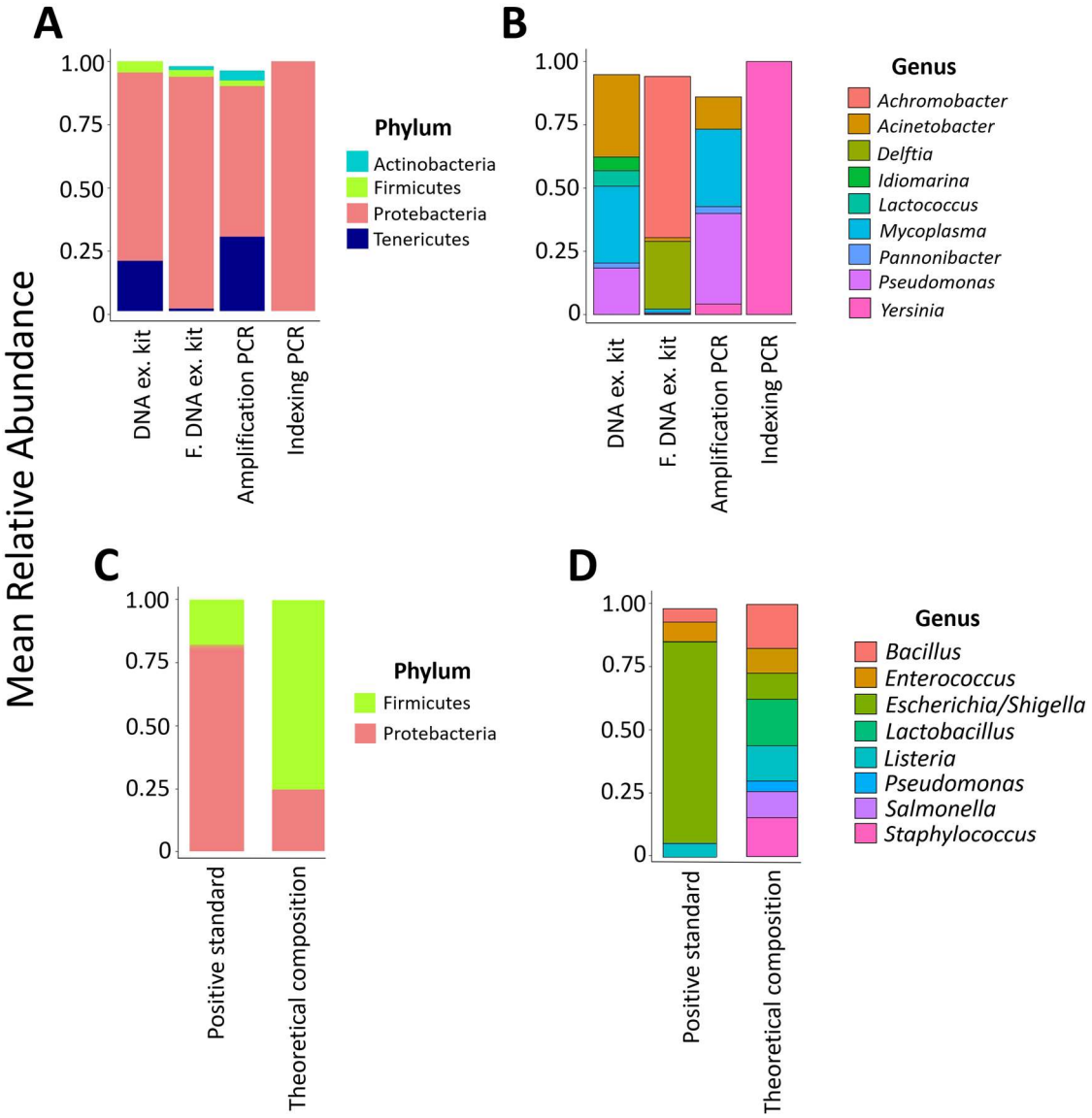
We carried out β-diversity (Whittaker, 1960) modelling to compare microbiota composition variation. *DECIPHER* (Wright, 2016) and *phangorn* (Schliep, 2011; Version 2.5.5) were used to align and create a phylogenetic tree, as proposed by Callahan et al. (2016b). Weighted (wUF) and unweighted UniFrac (uwUF) distances (Lozupone & Knight, 2005) were calculated with *phyloseq* (McMurdie & Holmes, 2013), using the trimmed data set (singletons, i.e. ASVs present only once across all samples, were removed: 16 ASVs, i.e. 0.26% of all the ASVs) that was first agglomerated to either bacterial phylum or genus level, and then normalised via proportions (McMurdie & Holmes, 2014; Callahan et al., 2016b; McKnight et al., 2019). Using the same normalised-by-proportions data set, we also calculated Bray-Curtis dissimilarities and Jaccard (dissimilarity) indices at both the phylum and genus level with *vegan* (Oksanen et al., 2019) for comparative β-diversity analysis.

We did not study bacterial strain-level variation as genus was the finest phylogenetic level that allowed us to assign a taxonomic identity to the majority of our sequencing reads (3953 out of 6187 ASVs, i.e. 63.9%, were assigned a genus identity). This was due to the current bacterial strain databases having limited information about non-human host microbiota, and because of the poor species- and strain-assignment potential of 16S SSU rRNA gene hypervariable region V4 (Gohl et al., 2016; Johnson et al., 2019b). Preliminary statistical tests revealed that strain-level β-diversity analyses with both ANOSIM and PERMANOVA had near-identical results with the genus-level β-diversity analyses, with the strain-level analyses showing slightly lower p-values that did not affect the significance patterns that we were able to observe at the genus-level. This assured us to proceed with our approach, knowing that our results at the phylum and genus level are biologically relevant, and allowing us to pinpoint specific key taxa that contribute to the variation.

Microbiota composition variation comparison between sampling methods ($n_{ejaculate\ sample}$=58, $n_{tissue\ sample}$=72, or $n_{faecal\ sample}$=12) was carried out with a separate ANOSIM analysis (anosim(); permutations=1000) from *vegan*, constraining the permutations within strata=bird ID (n=28).

Microbiota compositions between sample type were statistically compared with a PERMANOVA (adonis(); permutations=1000) from *vegan*, setting sample type ($n_{ejaculate}$=58, n_{testes}=12, $n_{upper\ vasa\ deferentia}$=12, $n_{lower\ vasa\ deferentia}$=12, $n_{papillae}$=12, n_{cloaca}=12, n_{colon}=12, or n_{faeces}=12) as a fixed effect, constraining the permutations within strata=bird ID. β-diversity modelling based on genus-level Bray-Curtis dissimilarities explained the largest amount of variation at the genus level and revealed significant differences between sample type microbiota compositions (Supplementary Table 3B). We therefore decided to follow up the significant genus-level Bray-Curtis dissimilarities PERMANOVA factors with a beta dispersion test (betadisper() followed by permutest(); permutations=1000) from *vegan* to control for significant dispersion differences. Stacked bar plots of mean relative abundance of bacterial phyla and genera per sample type were created with *ggplot2*. Any phylum or genus that contributed <1% across the sample types was considered rare, and was not included in the figure. A non-metric multidimensional scaling (NMDS) ordination indicating the dissimilarities between the sample types was created with *phyloseq* based on genus-level Bray-Curtis dissimilarities.

We were also interested in which bacterial phyla and genera were differentially-abundant in ejaculates in comparison with the MRT and cloaca tissue samples. We excluded colon tissue samples, but decided to include the cloaca as it is a part of the MRT. We explored differentially-abundant bacterial phyla and genera across sampling methods ($n_{ejaculate\ sample}$=58, or $n_{tissue\ sample}$=60) using *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was agglomerated to either phylum or genus level, filtered to include only taxa that contributed >1% to the mean relative bacterial abundance, and analysed with differentialTest(test="Wald", fdr_cutoff=0.05). The effect of sampling method was modelled while controlling for variance across sampling method.



Supplementary Figure 2. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A & C) and genera (B & D) detected in the negative controls (A & B) and the positive standard (C & D) in the combined data set consisting of ejaculate samples, and male reproductive tract, cloacal, and digestive tract samples analysed in Chapter III. Only taxa that contributed >1% are presented. Mean relative abundances per negative control type (A & B) were calculated using the post-*decontam* untrimmed contaminants-only data set. Positive standard relative abundances (C & D) were calculated using the pre-*decontam* untrimmed data set. We could not detect the expected genera *Lactobacillus*, *Pseudomonas*, *Salmonella*, and *Staphylococcus* in the positive standard (D), even when lowering the inclusion threshold to 0.001%. Negative controls (A & B) are a sample of the QIAGEN DNeasy® Blood & Tissue DNA extraction kit (n=1), a sample of the *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (F. DNA ex. kit; n=1), amplification PCR negatives (n=6), and an indexing PCR negative (n=1). The positive standard (C & D) is a single ZymoBIOMICS® Microbial Community Standard (Zymo Research). For comparison purposes, the theoretical composition of the ZymoBIOMICS® Microbial Community Standard expected by the manufacturer is presented together with the positive standard (C & D). No correction was made to the data set based on the observed and expected positive standard relative abundances. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity.

Supplementary Table 2. [ANOSIM] Microbiota composition variation between sampling method ($n_{ejaculate\ sample}$=58, $n_{tissue\ sample}$=72, or $n_{faecal\ sample}$=12) at bacterial phylum and genus level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=28). R=0 indicates high variability both within and between groups, R>0 indicates higher variability between groups than within groups, and R<0 indicates higher variability within groups than between groups. Significant p-values are in bold.

Sampling method	R	p-value
Weighted UniFrac		
Phylum-level	**0.39**	**0.018**
Genus-level	**0.38**	**<0.001**
Unweighted UniFrac		
Phylum-level	**0.33**	**<0.001**
Genus-level	**0.40**	**<0.001**
Bray-Curtis DS		
Phylum-level	0.42	0.105
Genus-level	**0.48**	**<0.001**
Jaccard Index		
Phylum-level	**0.42**	**<0.001**
Genus-level	**0.46**	**<0.001**

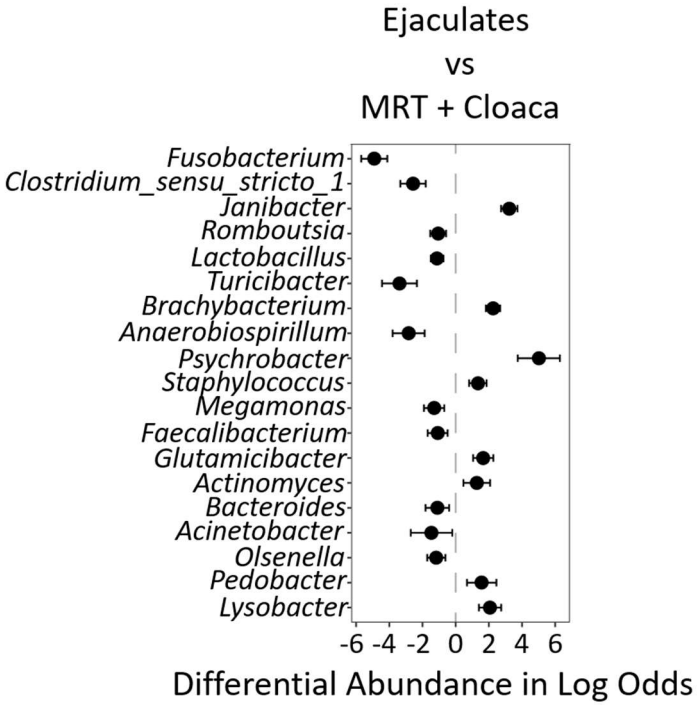
Supplementary Table 3. [PERMANOVA] Microbiota composition comparisons between sample type ($n_{ejaculate}$=58, n_{testes}=12, $n_{upper\ vasa\ deferentia}$=12, $n_{lower\ vasa\ deferentia}$=12, $n_{papillae}$=12, n_{cloaca}=12, n_{colon}=12, or n_{faeces}=12) at bacterial phylum (A) and genus (B) level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=28). Significant p-values are in bold.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**14.72**	**7**	**<0.001**	**35.0%**
Bird ID	**3.21**	**26**	**<0.001**	**28.4%**
Residuals		108		36.6%
Unweighted UniFrac				
Sample type	**7.08**	**7**	**<0.001**	**22.1%**
Bird ID	**2.57**	**26**	**<0.001**	**29.8%**
Residuals		108		48.1%
Bray-Curtis DS				
Sample type	**16.28**	**7**	**<0.001**	**37.3%**
Bird ID	**3.22**	**26**	**<0.001**	**27.4%**
Residuals		108		35.3%
Jaccard Index				
Sample type	**7.25**	**7**	**<0.001**	**22.3%**
Bird ID	**2.65**	**26**	**<0.001**	**30.3%**
Residuals		108		47.4%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sample type	**8.62**	**7**	**<0.001**	**23.8%**
Bird ID	**3.27**	**26**	**<0.001**	**33.5%**
Residuals		108		42.7%
Unweighted UniFrac				
Sample type	**5.06**	**7**	**<0.001**	**17.8%**
Bird ID	**2.12**	**26**	**<0.001**	**27.7%**
Residuals		108		54.5%
Bray-Curtis DS				
Sample type	**8.72**	**7**	**<0.001**	**23.9%**
Bird ID	**3.31**	**26**	**<0.001**	**33.7%**
Residuals		108		42.4%
Jaccard Index				
Sample type	**4.16**	**7**	**<0.001**	**15.6%**
Bird ID	**1.91**	**26**	**<0.001**	**26.6%**
Residuals		108		57.8%



Supplementary Figure 3. Differential abundance (in log odds) of bacterial genera found in male red junglefowl ejaculates compared with male reproductive tract (MRT) microbiota (including the cloaca; Chapter III). The grey dashed line represents the point of comparison, i.e. MRT (+ cloaca) tissue microbiota. Data points with standard error bars depict log odds estimates of the specific bacterial genera. Positive log odds indicate significant enrichment, while negative log odds indicate the significant under-representation of the bacterial genus in ejaculates compared to MRT microbiota.

Supplementary Table 4. [PERMANOVA] Microbiota composition comparisons between experimental group ($n_{start}=n_{middle}=n_{end}=8$, two matings with the same female: $n_{first}=n_{second}=11$, two matings with two different females: $n_{first}=n_{second}=6$) and age class ($n_{young}=34$, $n_{old}=24$) at bacterial phylum (A) and genus (B) level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=16). Significant p-values are in bold. Significant factors were followed up by beta dispersion tests that revealed no significant dispersion variation (p>0.05) across age class regardless of the taxonomic level and β-diversity distance metric.

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	0.68	6	0.886	6.8%
Age class	**3.08**	**1**	**0.014**	**5.1%**
Bird ID	1.19	14	0.216	27.8%
Residuals		36		60.3%
Unweighted UniFrac				
Experimental group	0.85	6	0.710	6.9%
Age class	1.67	1	0.124	2.2%
Bird ID	**2.28**	**14**	**<0.001**	**42.7%**
Residuals		36		48.2%
Bray-Curtis DS				
Experimental group	0.66	6	0.852	6.7%
Age class	**3.70**	**1**	**0.011**	**6.3%**
Bird ID	1.10	14	0.323	26.1%
Residuals		36		60.9%
Jaccard Index				
Experimental group	1.07	6	0.380	8.3%
Age class	1.26	1	0.237	1.6%
Bird ID	**2.41**	**14**	**<0.001**	**43.6%**
Residuals		36		46.5%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Experimental group	0.98	6	0.491	9.2%
Age class	1.84	1	0.072	2.9%
Bird ID	**1.46**	**14**	**0.012**	**31.8%**
Residuals		36		56.1%
Unweighted UniFrac				
Experimental group	0.96	6	0.586	8.7%
Age class	**1.88**	**1**	**0.012**	**2.8%**
Bird ID	**1.62**	**14**	**<0.001**	**34.2%**
Residuals		36		54.3%
Bray-Curtis DS				
Experimental group	1.22	6	0.090	10.5%
Age class	**1.96**	**1**	**0.020**	**2.8%**
Bird ID	**1.74**	**14**	**<0.001**	**35.0%**
Residuals		36		51.7%
Jaccard Index				
Experimental group	1.07	6	0.253	9.7%
Age class	**1.65**	**1**	**0.020**	**2.5%**
Bird ID	**1.54**	**14**	**<0.001**	**33.0%**
Residuals		36		54.8%

Chapter V. Investigating the Relationship Between Cloacal Microbiota and Mating Behaviour in the Polygynandrous Red Junglefowl (*Gallus gallus*)

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Resolving patterns of sexual bacterial transmission is critical in understanding mating behaviour, sexual selection and conflict, and infections in host populations. Little, however, is known about how mating interactions and individual phenotypic variation affect the sexual transmission of bacteria, particularly in species with complex mating systems. Here, we investigated how sexual interactions affect the cloacal microbiota of the polygynandrous red junglefowl, *Gallus gallus*. We assembled replicate experimental groups of different sex ratios, monitored inter-individual mating interactions over the course of six-day mating trials, and collected cloacal microbiota samples from birds immediately before and after a trial. Experimental birds differed significantly in their cloacal microbiota composition in comparison with virgin bird controls, with the non-virgin fowl harbouring more *Corynebacterium* and *Actinomyces*—both pathogens from the phylum Actinobacteria. Recent mating increased the cloacal total ASV richness only in females, suggesting sex-specific patterns in sexual bacterial transmission. Experimental females and males differed in the composition of their cloacal microbiota both before and after the mating trials. There were no pre-existing differences in the cloacal microbiota associated with fowl age class nor personality. The cloacal bacterial diversity of an individual before a trial did not predict the number of partners nor matings that the individual attained in the trial. However, individuals with a pre-existing higher of abundance of *Romboutsia* from the phylum Firmicutes secured more matings in their experimental groups and more partners across the mating population. Some variation in cloacal microbiota after the mating trials was also attributed to the number of partners and matings the birds secured, and fowl age class, and boldness and exploratory behaviour. Analysing the experimental groups as one mating population revealed that the fowl tended to share a homogenous cloacal microbiota, while individuals with fewer partners were significantly different from the rest. Our findings highlight the importance of investigating mating behaviour and individual variation in sexual bacterial transmission, and encourage hypotheses about the potential role of reproductive bacteria in mate choice and host socio-sexual group structure.

Introduction

Previous research on the interdependence between gut bacteria and human behaviour (Gonzalez et al., 2011) has also inspired an interest in the relationship between bacteria and behaviour in non-human hosts (Cryan & O'Mahony, 2011; Davidson et al., 2018; Davidson, Raulo & Knowles, 2020). In the current literature, hypotheses have emerged that suggest the potential for host social behaviour to influence its gut microbiota, while gut bacteria may also affect host social decision making (Archie & Theis, 2011; Ezenwa et al., 2012; Archie & Tung, 2015). To explore this interdependence, bacterial transmission dynamics have been explored in the context of host social behaviours and groups.

A landmark paper by Tung et al. (2015) found that not only do distinct social groups of wild yellow baboons (*Papio cynocephalus*) differ from each other in their gut microbiota, individuals that socialised more within their respective social group harboured a more similar gut bacterial composition than those that socialised less. Analogous findings have been reported in ring-tailed lemurs (*Lemur catta*; Bennett et al., 2016), Verreaux's sifakas (*Propithecus verreauxi*; Perofsky et al., 2017), and red-bellied lemurs (*Eulemur rubriventer*; Raulo et al., 2018) among others, suggesting that host social behaviour may facilitate the transmission of bacteria. The interdependence between host sociality and its microbiota, however, can be detrimental and pathogenic bacterial effects may drive the evolution of non-social host behaviours, such as inter-individual physical avoidance and parasite removal during autogrooming (Hart, 2011; Zylberberg, Klasing & Hahn, 2013; Hart & Hart, 2018).

While social bacterial transmission tends to affect the guts (Münger et al., 2018) and skin (Walke et al., 2011) of hosts, sexual bacterial transfers—that have received far less attention—affect host reproductive organs (Mändar et al., 2015; Bellinvia et al., 2019a; Bellinvia et al., 2019b). Sexual interactions allow direct contact between the reproductive microbiota of mating partners in internally-fertilising species and it is thus likely that a single

mating is enough for successful sexual bacterial transmission. In support of this, a study in bed bugs (*Cimex lectularius*) found that non-mated individuals differed from mated bed bugs in their reproductive organ microbiota, with mating leading to an exchange of novel bacteria between female and male partners (Bellinvia et al., 2020a).

Polygamous mating systems, where at least some individuals mate with multiple partners, offer scope for more complex dynamics of sexual bacterial transmission. Indeed, birds that mate with multiple partners have higher cloacal bacterial diversity and suffer more infections than monogamous individuals (Poiani & Gwozdz, 2002). Mating with multiple partners has also been reported to increase the diversity of vaginal microbiota in deer mice (*Peromyscus maniculatus*; MacManes, 2011) and cloacal microbiota in female viviparous lizards (*Zootoca vivipara*; White et al., 2011) in comparison with monogamous hosts.

Variation across individual phenotype (such as the sex, age, and personality of an individual) and in the social environment (such as social network structure and the sex ratio of a social group) may also directly, or indirectly—via variation in mating behaviour and success—affect host reproductive microbiota. The exact mechanisms through which this may occur, however, are currently poorly understood, but it is highly likely that partners exchange both pathogenic and beneficial bacteria during mating, and that some of these bacterial strains may play a role in mate choice, individual avoidance behaviours, and socio-sexual network structure. To fill this gap in knowledge, controlled experimental studies are necessary to first identify the sexually-transmitted bacterial strains in group-living hosts, and then characterise the patterns of sexual bacterial transmission to understand the way in which host phenotypic variation and social environment are inter-related with the sexual transfer of bacteria.

To shed light on these questions, we investigated the sexual bacterial transmission dynamics in the group-living polygynandrous red junglefowl (*Gallus gallus*)—the main wild ancestor of the domestic chicken (*G. domesticus*; Kanginakudru et al., 2008; Lawal et al., 2020;

Wang et al., 2020b). In nature, junglefowl live in multi-male, multi-female social groups, and both females and males mate frequently with multiple partners during the breeding season (Ligon & Zwartjes, 1995). There is, however, ample variation between individuals in mating behaviour and success, which has been shown to depend on both individual phenotype and the surrounding social environment (McDonald et al., 2019; Pizzari & McDonald, 2019). For example, younger, and more dominant and aggressive males secure more partners and more matings (McDonald et al., 2017; Pizzari & McDonald, 2019). Red junglefowl and domestic chickens also display consistent personality traits, such as boldness and exploratory behaviour (Favati et al., 2016; Favati et al., 2018; Roth, 2019). Roth (2019) found that in female-biased groups, faster-exploring and slower-exploring males secured significantly more partners than intermediate-exploring males. Furthermore, individuals mated disassortatively with respect to boldness behaviour (Roth 2019). Inter-individual variation in mating behaviour is also strongly modulated by social group structure. For example, sex ratio variation significantly affects the mating interactions within a social group of red junglefowl (Løvlie & Pizzari, 2007; Pizzari & McDonald, 2019; Roth, 2019). To study red junglefowl cloacal microbiota variation in association with individual mating behaviour and phenotype, we assembled replicate experimental groups of different sex ratios and monitored the intra-group sexual interactions throughout six-day mating trials. We collected cloacal microbiota samples from individual birds both immediately before and after they completed a mating trial, and also sampled the cloacae of virgin birds independent of the mating trials.

We had four aims. First, we sought to identify changes in cloacal microbiota in relation to mating by comparing virgin birds to non-virgin birds that had been sampled before and after a mating trial. Second, we investigated the association between cloacal bacteria and individual phenotype, such as variation in sex, age class, and boldness and exploratory behaviour, before the first mating trials to explore pre-existing covariance between host phenotype and

microbiota. Third, we tested whether variation in cloacal microbiota diversity and composition before a mating trial predict the mating performance of an individual in terms of the number of partners and matings secured in a trial, while controlling for the effects of individual phenotype and social environment. Fourth and finally, we considered the consequences of a mating trial on cloacal microbiota by investigating whether individual mating performance and phenotype, and the social environment of a group affect cloacal microbiota. We further explored mating trial effects by testing whether cloacal microbiota tend to converge among members of the same group and diverge between groups.

We hypothesised that first, virgin and non-virgin red junglefowl harbour different cloacal microbiota, as has been reported in non-mated and mated bed bugs (Bellinvia et al., 2020a). We also expected the cloacal bacterial diversity of non-virgin females to increase after the mating trial in comparison to their pre-mating state. A study in wild black-legged kittiwakes (*Rissa tridactyla*) reported that sexually-interacting female birds had a higher cloacal bacterial diversity in comparison with their non-mating state when cloacal contact between partners was experimentally blocked (White et al., 2010).

Second, we predicted that hosts of different phenotype harbour distinct cloacal microbiota, as suggested by previous studies showing that the cloacae of female and male birds (Escallón, Belden & Moore, 2019), and chicks and adults (van Dongen et al., 2013) have distinct bacterial communities. Similarly, animal personality, defined as consistent inter-individual behavioural variation (Stamps & Groothuis, 2010; Carter et al. 2013; Sih et al., 2015), may affect host microbiota in various ways. For example, it can affect the position of an individual in social networks (Pike et al., 2008; Croft et al., 2009; Godfrey et al., 2012; Tanner & Jackson, 2012; Aplin et al., 2013) and thus alter its microbiota. To illustrate, being more exploratory helps wild house finches (*Haemorhous mexicanus*) form more and stronger bonds with conspecifics (Moyers et al., 2018). Under the assumption that animal personality is

associated with host microbiota, we expected bolder and more exploratory fowl to have a more diverse cloacal microbiota, as they may acquire more bacteria due to increased socio-sexual interaction rates.

Third, we predicted that birds that secure more matings during a mating trial have relatively few pathogenic bacteria on their cloacae before the trial. For example, *Shigella* (phylum Proteobacteria) is a known chicken pathogen that causes a symptomatic white cloacal discharge (Shi et al., 2014) and such visual cues can be used to avoid infected individuals, as has been previously reported in fowl (Sheldon, 1993) and primates (Paciênca et al., 2019). In support of this, uninfected black garden ants (*Lasius niger*) were found to distance themselves from conspecifics with a fungal-infection, thus restructuring their colony and protecting the rest of the ants from the pathogen (Stroeymeyt et al., 2018).

Fourth and finally, we predicted that an increased number of partners and matings may in turn lead to a more diverse cloacal microbiota (Poiani & Gwozdz, 2002; MacManes, 2011; White et al., 2011). We also expected bolder and more exploratory fowl to be more connected in their socio-sexual networks and exchange more bacteria with their conspecifics (Kulkarni & Heeb, 2007; Moyers et al., 2018). Considering that red junglefowl are polygynandrous, we did not expect cloacal microbiota similarity within breeding pairs, but hypothesised that the mating population may share a similar cloacal microbiota, as has been found in a breeding population of socially monogamous-, yet genetically-polygynous North American tree swallows (*Tachycineta bicolor*; Hernandez et al., 2020). In addition, social environment—such as social network structure and group sex ratio—may affect individual mating behaviour (Sih & Watters, 2005; Sih, Chang & Wey, 2014; Wey et al., 2015; Roth, 2019; but see also Roth et al., 2019) and thus, also alter their sexual bacterial transmission dynamics. Indeed, male mating success is expected to vary more in male-biased populations due to intense male-male competition, while females, often being the choosier sex, have less variable mating success regardless of

group sex ratio (Emlen & Oring, 1977; Jirotkul, 1999; Holveck, Gauthier & Nieberding, 2015). We therefore expected to observe sex-specific differences that emerge due to varying group sex ratios. Finally, we hypothesised that mating among the members of a group increases within-group cloacal microbiota similarity, while increasing between-group dissimilarity—a pattern associated with social bacterial transmission in host networks (Tung et al., 2015; Perofsky et al., 2017; Raulo et al., 2018).

Materials and Methods

Study Population

We studied a captive red junglefowl population at the John Krebs Field Station of the University of Oxford in Wytham. Birds were housed in covered outdoor aviaries and kept in single-sex flocks. Food and water were provided *ad libitum* before, during, and after the experiment. Birds wore unique Darvic rings for individual identification.

All birds used in the study were sexually-mature and had previous mating experience. Birds ranging from 2–9 years of age (mean±S.E.: 4.37±0.25) were grouped into two age classes, "young" (2–4 years) and "old" (5–9 years), based on previous literature. Work on a feral population of domestic chickens showed that female reproductive performance (fecundity and egg size) peaks at the age of 4 and declines in older birds (Cornwallis, Dean & Pizzari, 2014). Some aspects of male reproductive performance (mating behaviour, sperm number) tend to decline more linearly across older cohorts, while others (sperm swimming velocity) peak around the age of 4 (Dean et al., 2010; Cornwallis, Dean & Pizzari 2014). Similar patterns of age-dependent declines in male mating behaviour have been confirmed for the study population (McDonald et al., 2017), and a peak in ejaculate quality at the age of 4 has also been reported in a population of red junglefowl (*G. gallus murghi*) in Pakistan (Rakha et al., 2017).

In total, our study included 19 “young” and nine “old” females, and 16 “young” and 13 “old” males. Each experimental bird had also been assigned an individual boldness and exploratory score based on previous personality assays executed and described in Roth (2019; see Supplementary Methods 1 for a shortened version of methods). All experimental work was conducted under the regulation of a Home Office project license (PIL: P50402706).

Experimental Protocol

During the breeding season of 2017 (experimental period: 9 May–11 July), we studied fowl cloacal bacterial compositions in nine experimental groups of different sex ratios (Table 1). Each experimental group consisted of 12 individuals. Three groups had a female-biased sex ratio with 9 females and 3 males, three had an even sex ratio (6:6), and three were male-biased (3:9). The nine groups (Table 1) were part of a larger study (24 replicate groups in total) described in Roth (2019). Due to limited availability of sexually-mature birds, some of the individuals were tested multiple times across the study design (Table 1).

Each experimental group was observed in the same experimental pen (11.5 m × 7.2 m) during the course of a six-day-mating trial. At 13.00 on the first day of the experiment, males were sampled for cloacal wipes and released into the experimental pen to establish a dominance hierarchy. At 13.00 on the second day of the experiment, females were sampled for cloacal wipes and released into the experimental pen with the males. From the second to the sixth day of each mating trial the behaviour of individual birds was closely monitored for a period of three hours after sunrise and three hours before sunset, which correspond to peaks in sexual activity (Pizzari & Birkhead, 2001; Løvlie & Pizzari, 2007), resulting in six evening and five morning sessions (33 hours per group). All sexual interactions were identified using an established protocol (Løvlie & Pizzari, 2007; Roth, 2019). The ID of the actor and the recipient were recorded for each mating interaction, and the number of partners and the total number of

matings were recorded for each bird. On the seventh and final day of the trial, the birds were captured from the experimental pen, and another cloacal wipe sample was collected from all birds. According to our daily observations, 10 birds did not mate once during the entirety of the experiment, including six old and three young males, and one old female. However, they could have mated outside of the mating trial observation times.

Table 1. List of experimental groups (n_{groups}=9) and birds (n_{female}=28, n_{male}=29) sampled for cloacal wipes (n_{total}=216). Before the nine mating trials started, some of the experimental birds were used in another mating group study (*indicated in italics*). We took these prior interactions into account when determining the occurrence of first mating(s) of the breeding season, and when performing population-level analysis. The nine experimental groups used in this study are presented in their experimentally-chronological order. Female-biased groups are indicated with pink (n=3), even sex ratio groups with green (n=3), and male-biased groups with blue (n=3). In total, we tested 28 female and 29 male birds. 12 females and 10 males were used once in the study. Eight female birds were used twice, six thrice, and two four times across the experimental groups. 14 males were used twice, four thrice, and one four times across the experimental groups. Due to the re-using of birds, some fowl mated with the same partner in more than one experimental group, including 14 mating pairs that mated in two and one mating pair that mated in three experimental groups.

	Group ID	Females ♀	Males ♂
	FB13	*108, E24, J35, J42, M32, M35, M38, M50, L4*	*D3, K40, L38*
Experimental Social Groups	MB14	K20, M35, M40	308, A18, J5, J6, K46, K50, L26, L31, L32
	MB15	J42, M36, M46	J2, J7, J16, K40, K44, L32, L38, L39, L44
	ES16	E24, K18, K20, M31, M37, M38	A18, J1, J6, K32, K50, L26
	FB17	J22, J37, K11, K18, K20, L2, M31, M41, M46	J7, K41, K46
	ES18	E23, E24, J35, J37, M35, M43	308, D3, J2, L31, L38, K39
	FB19	E23, E43, K14, K18, M35, M36, M37, M40, M49	J16, L39, K44
	MB20	K18, M35, M43	D3, G15, J1, J17, K32, K41, K47, K48, L31
	ES21	108, J35, L4, K14, M36, M46	208, 508, J16, K45, L39, L44
	FB22	D48, J35, J37, J42, L4, M39, M44, M43, M50	J2, K32, L31

Cloacal Wipe Sampling in the Field

Cloacal wipe samples were collected by a single experimenter who wore overalls and 70%-ethanol-sterilised nitrile gloves. The cloacae of red junglefowl were gently inverted and a sterile, previously-sealed Petri dish was pressed against it, ensuring that the surrounding feathers did not touch nor contaminate the Petri dish. Immediately upon collection, the Petri

dish was closed and sealed with tape. The cloacal outline was traced with a marker on the outside of the Petri dish, which was then stored at -80˚C. The cloacal wipe collection resulted in two samples per bird per experimental group, 24 samples per group, and 216 cloacal wipe samples across the study.

To obtain a baseline cloacal bacterial composition for female and male fowl, we sampled four virgin individuals of each sex outside of the experimental design. Both female and male virgins were one-year-old and sexually-mature, but without previous mating experience. To account for environmental bacteria that could also come in contact with fowl cloacae (van Veelen, Falcao Salles & Tieleman, 2017), e.g. during dust-bathing, we also sampled the soil (n=1) and water (n=1) in the experimental pen before the first experimental set-up of the breeding season (group ID: FB13; Table 1). The virgin cloacal wipes and environmental source controls were collected, sealed, and stored as the experimental samples.

DNA Extraction and Quantification

Post-fieldwork microbiota processing took place in 2019–2020 at Silwood Park, Imperial College London. Sample handling was carried out in a pre-PCR biological safety cabinet that was sterilised with DNAZap™ PCR DNA Degradation Solutions (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 70% ethanol. DNA extractions from the Petri dishes were carried out with a previously sealed DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany). We followed the manufacturer's protocol with an additional lysozyme lysis step to increase yield from Gram-positive bacteria (Gill et al., 2016). Lysozyme from chicken egg white (final concentration 0.4 mg/ml; Merck, Darmstadt, Germany) was mixed with the ATL Buffer, and 180 µl of the solution was pipetted on each Petri dish, constraining the solution within the marked cloacal outline, pipetting up and down, and scraping the surface of the Petri dish with the pipette tip. The solution was then transferred into a sterile collection

tube on ice. After all the samples had been transferred into tubes, we allowed 30 minutes of incubation at 37°C before proceeding with the Proteinase K step in the manufacturer's protocol. The second incubation at 56°C was carried out for an hour.

Environmental source controls (i.e. soil and water from the experimental pen) were processed with an opened *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, California, USA), according to the manufacturer's protocol. We had to opt for another DNA extraction kit for the environmental samples as preliminary experiments revealed that the DNA concentration and PCR amplification were low and not comparable with the rest of the biological samples when using the QIAGEN DNeasy® Blood & Tissue Kit with the environmental source controls.

Controls from both DNA extraction kits were collected for downstream contamination assessment (DNeasy® Blood & Tissue DNA extraction kit, n=1 (one across four 96-well plates); and *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit, n=1). All samples were quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) with a Qubit® Fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol. DNA extractions were diluted to a standardised concentration of 0.1 ng/µl and all samples were stored at -20°C.

Amplification Polymerase Chain Reactions (PCRs)

The PCR hood was sterilised with DNAZap™ PCR DNA Degradation Solutions and 70% ethanol, followed by a 30-minute UV-sterilisation. A separate 30-minute UV-sterilisation was carried out for the consumables. PCRs were set up on ice with sterile, filtered pipette tips and reagents taken from unopened aliquots.

The V4 region (~254 base pairs) of the bacterial 16S SSU rRNA gene was amplified, following the guidelines of the Earth Microbiome Project (Thompson et al., 2017) and Walters

et al. (2015). Reactions were prepared on 96-well plates in three replicates to minimise amplification bias and ensure the amplification of rare bacterial strains. 0.5 ng or up to 5 µl (if the DNA template concentration was too low) of DNA was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus* (Sigma-Aldrich, St. Louis, Missouri, USA), 5 µl of *Taq* DNA Polymerase PCR Buffer 10X (Sigma-Aldrich), 0.5 µl of Bovine Serum Albumin (Sigma-Aldrich), 0.1 µl of 100 nM dNTP mix (Thermo Fisher Scientific), 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) to make up the rest of the reaction volume.

At least one PCR negative control per 96-well plate was prepared and sealed before template DNA entered the PCR hood. A ZymoBIOMICS® Microbial Community Standard (Zymo Research) was added to each set-up for amplification success assessment.

Parameters for the amplification PCRs were as follows: initial three-minute denaturing at 94°C followed by 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step at 72°C. The same PCR machine was used for all replicate plates. The PCR products were tested on a 1% agarose gel, stained with GelRed™ (Biotium, Hayward, California, USA) according to the manufacturer's protocol, in a 1×TBE buffer at 80 V for 45 minutes.

Quantitative Polymerase Chain Reactions (qPCRs)

A subset of DNA extractions was additionally amplified with qPCRs to quantify and explore bacterial load variation across cloacal wipe samples. We randomly picked the before and after samples of one female and one male bird per experimental group (n=9), and the

cloacal wipe samples of four virgin female and four virgin male fowl, resulting in a total of 44 cloacal wipes.

The PCR hood and consumables were sterilised as for the amplification PCRs. Reactions were prepared with the Thermo Scientific ABsolute qPCR SYBR® Green Mix (Thermo Fisher Scientific) following the manufacturer's protocol, adding 0.2 µM (final concentration) of forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) each (Supplementary Table 1), and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the 25 µl reaction volume. The amount of template DNA was kept congruent with the 16S SSU rRNA gene amplification PCR step for standardised downstream comparisons. Technical replicates were not included.

A three-point serial dilution of a standard (*Escherichia coli*; quantified using the Qubit™ methodology) and a negative control were added to the 96-well format, which was then processed with the LightCycler® 96 Instrument (Roche Holding AG, Basel, Switzerland).

Parameters for the qPCRs were as follows: 15-minute enzyme activation at 95°C followed by 50 cycles of 45 seconds at 95°C, 60 seconds at 50°C, and 90 seconds at 72°C, with a final 10-minute extension step of at 72°C. This was followed by a melt curve: 10 seconds at 95°C, 60 seconds at 65°C, and one second at 97°C. A single set-up was sufficient to process all samples.

Standard curve (primer efficiency=71.9%, R^2=0.87) and concentration analysis (ng/µl) were carried out in the LightCycler® 96 Application (Roche Holding AG). Samples were omitted, if they failed to amplify or amplified less than five cycles before primer dimer formation in the negative control (C_q=30.00). This data filtering resulted in the loss of one virgin male cloacal wipe sample (bird ID: N10) and one male cloacal wipe sample that was collected after exiting a male-biased experimental group (bird ID: D3; group ID: MB20).

DNA concentrations in ng/µl were corrected for DNA elution buffer volume (200 µl) and template DNA volume that was processed with the qPCR (5 µl). The DNA concentrations were then converted into 16S SSU rRNA gene copy number per cloacal wipe sample by setting the average weight of a base pair (bp) to 660 Daltons and using the following equation: $\frac{DNA\ (ng) * Avogadro\ number\ (molecules/mole)}{Template\ full\ lengt\ \ (bp) * 660\ (g/mole) * 1 * 10^9\ (ng/g)}$.

As the sample size of the qPCR-quantified cloacal wipe samples was too low for statistical analysis, we present them in the appendices (Supplementary Figure 1).

Clean-Up, Indexing PCRs, and Normalisation

Replicate biological samples were pooled and cleaned of primer dimers using the ZR-96 DNA Clean-up Kit™ (Zymo Research), following the manufacturer's protocol.

Samples were dual-indexed with unique combinations proposed by Kozich et al. (2013). 20 ng of amplification PCR product was added to a 50 µl reaction volume, consisting of 0.25 µl of *Taq* DNA Polymerase from *Thermus aquaticus*, 5 µl of *Taq* DNA Polymerase PCR Buffer 10X, 0.1 µl of 100 nM dNTP mix, 0.0625 µM (final concentration) of forward and reverse index primer (Supplementary Table 1) each, and UltraPure™ DNase/RNase-Free Distilled Water to make up the rest of the reaction volume.

An indexing PCR negative control (n=1) was processed together with the biological samples. DNA extraction kit controls (DNeasy® Blood & Tissue DNA extraction kit, n=1; and *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit, n=1), amplification PCR negative controls (n=12), and a positive standard (n=1) were indexed as biological samples.

Parameters for the indexing PCRs were as follows: initial two-minute denaturing at 95°C followed by 15 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final 10-minute extension step at 72°C. PCR products were tested on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes.

Dual-indexed samples were quantified using the Qubit™ methodology. 5 ng of each sample was pooled into a DNA LoBind Tube (Eppendorf, Hamburg, Germany) and cleaned with the DNeasy® PowerClean Pro Cleanup Kit (QIAGEN), according to the manufacturer's protocol.

The library was tested for size and quality on a 1% agarose gel, stained with GelRed™, in a 1×TBE buffer at 80 V for 45 minutes. Library purity was assessed from the 260/280 nm ratio, calculated using the Take3™ Micro-Volume Plate (BioTek Instruments, Inc., Winooski, Vermont, USA) in a BioTek microplate reader (BioTek Instruments, Inc.), and the Gen5 programme (BioTek Instruments, Inc.). The quality-checked library was stored at -20°C prior to sequencing. Immediately before sequencing, the library was quantified again using the Qubit™ methodology and diluted to 4 nM.

Next-Generation Sequencing (NGS)

NGS was carried out at the sequencing facility of Silwood Park (Imperial College London), following Illumina's 16S SSU rRNA gene metagenomic sequencing protocol (Illumina, 2013). A 15 pM library with a 5% PhiX control (Illumina, Inc., San Diego, California, USA) was loaded into the V3 chemistry MiSeq cartridge (Illumina, Inc.) to achieve 2*300 bp sequencing reads across 226 biological and 16 control samples.

Bioinformatic Data Processing

Post-sequencing sample demultiplexing was carried out automatically by BaseSpace (Illumina, Inc.). Paired-end FASTQ files were processed with *dada2* (Callahan et al., 2016a; Version 1.12.1) in R (R Core Team, 2019; Version 3.6.1). Reads were quality-checked and filtered using standard parameters (maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2) with additional truncLen=c(200, 200), resulting in a ~10% sequencing read loss. The error rates

were calculated based on a random subset of samples (randomize=TRUE) and inference was carried out via pseudo-pooling (pool="pseudo"). Non-specific amplicons were removed, retaining only merged, denoised reads of 250–256 bp. Chimera check removed ~54% of the merged sequence variants, which accounted for ~18% of the total reads. SILVA reference database (Yilmaz et al., 2014; Version 138) was used for assigning taxonomies (100% identity) to the species level, when possible.

The processed data set with controls contained 242 samples, 11,405 amplicon sequence variants or ASVs (418±13 S.E. per sample) and 6,093,284 reads (25,179±768 per sample). *decontam* (Davis et al., 2017) was used for further filtering of potential contaminants, which were detected based on ASV prevalence in control versus biological samples, using a stringency threshold of 0.55 that split ASVs into contaminants (122 ASVs) and non-contaminants (11,283 ASVs).

There were no biological samples with fewer than 100 sequencing reads. ASVs that were present in the taxonomy, but not in the data set due to data filtering (n=169) were excluded. Finally, ASVs that failed to classify at the phylum level (n=156) or that classified as chloroplasts (n=88) or mitochondria (n=36; de la Cuesta-Zuluaga & Escobar, 2016) were removed.

The final data set without controls contained 226 samples, 10,834 ASVs (432±12 per sample), and 5,511,639 reads (24,388±699 per sample). Mean relative bacterial abundances detected in negative controls and the positive standard were visualised with *ggplot2* (Wickham, 2016) and are presented in the appendices (Supplementary Figure 2).

Statistical Analysis and Data Visualisation

Statistical analysis and data visualisation were carried out in R (Version 3.6.1). Significance threshold was set to $p<0.05$ in all tests. The order of explanatory variables in each

sequential model (such as PERMANOVA) was decided *a priori* based on our expectations of their importance in explaining variation.

(1) *Cloacal microbiota variation between virgin and non-virgin red junglefowl*. For this part of the analysis, we used a subset of data, which consisted of three "mating experience subgroups": (i) virgin individuals (n_{female}=4, n_{male}=4), (ii) individuals with mating experience in previous breeding seasons (n_{female}=19, n_{male}=17), and (iii) individuals that had recently mated in the current breeding season (n_{female}=19, n_{male}=17). Virgin individuals (i) were one-year-old, kept in single-sex flocks, and with no prior mating experience. Birds with mating experience and recently mated birds (i.e. (ii) and (iii)) were the same individuals sampled before (ii) and after (iii) their first mating trial. Matings that occurred during the matings trials were their first matings in the breeding season. Birds with mating experience (ii) had last mated in the previous breeding season (~6–12 months ago). It is important to highlight that the one-year-old virgin birds (i) were substantially younger than the non-virgin birds ((ii) and (iii)), which reflects the fact that virgin birds are not generally kept in single-sex flocks for prolonged periods of time in the study population. Age was thus included as a continuous variable in the analysis performed in aim (1), as we wanted to at least partially control for age-related cloacal microbiota variation.

We also included environmental source controls collected from the bedding soil (n=1) and drinking water (n=1) present in the experimental pen. Environmental samples were included in this part of the analysis to test whether any of the mating experience subgroups shared bacterial strains with the surrounding habitat. The mating experience subgroups and the environmental controls are hereafter referred to as "categories".

Compositional microbiota variation between cloacal wipe and environmental source control samples was analysed by modelling β-diversity, which allowed us to compare local bacterial diversity to regional bacterial diversity (Whittaker, 1960). *DECIPHER* (Wright,

2016) and *phangorn* (Schliep, 2011; Version 2.5.5) were used to align and create a phylogenetic tree, as proposed by Callahan et al. (2016b). Weighted (wUF) and unweighted UniFrac (uwUF) distances (Lozupone & Knight, 2005) were calculated with *phyloseq* (McMurdie & Holmes, 2013), using the trimmed data set (singletons—defined as ASVs present only once across all samples—were removed: 4719 ASVs, i.e. 43.6% of all the ASVs) that was first agglomerated to either bacterial phylum or genus level, and then normalised via proportions (McMurdie & Holmes, 2014; Callahan et al., 2016b; McKnight et al., 2019). Using the same normalised-by-proportions data set, we also calculated Bray-Curtis dissimilarities and Jaccard (dissimilarity) indices at both the phylum and genus level with *vegan* (Oksanen et al., 2019) for comparative β-diversity analysis.

We did not study bacterial strain-level variation as genus was the finest phylogenetic level that allowed us to assign a taxonomic identity to the majority of our sequencing reads (6143 out of 10,834 ASVs, i.e. 56.7%, were assigned a genus identity). This was due to the current bacterial strain databases having limited information about non-human host microbiota, and because of the poor species- and strain-assignment potential of 16S SSU rRNA gene hypervariable region V4 (Gohl et al., 2016; Johnson et al., 2019b). Preliminary statistical tests revealed that strain-level β-diversity analyses with PERMANOVA had near-identical results with the genus-level β-diversity analyses, with the strain-level analyses showing slightly lower p-values that did not affect the significance patterns that we were able to observe at the genus-level. This assured us to proceed with our approach, knowing that our results at the phylum and genus level are biologically relevant, and allowing us to pinpoint specific key taxa that contribute to the variation.

Bacterial compositions were statistically compared with PERMANOVA (adonis(); permutations=1000) from *vegan*, setting category ($n_{virgin\ female}$=4, $n_{experienced\ female}$=19, $n_{mated\ female}$=19, $n_{virgin\ male}$=4, $n_{experienced\ male}$=17, $n_{mated\ male}$=17, n_{soil}=1, or n_{water}=1) as a fixed effect,

constraining the permutations within strata=bird ID (n=44). Significant PERMANOVA results were followed up with beta dispersion tests (betadisper() followed by permutest(); permutations=1000) from *vegan* to control for significant dispersion differences.

To visualise the pairwise differences between categories, a heatmap with average linkage hierarchical clustering was created with *Heatplus* (Ploner, 2019) based on the genus-level Bray-Curtis dissimilarities (the genus level was the finest taxonomic level in our analysis and Bray-Curtis dissimilarities explained the most variation associated with categories; Supplementary Table 2B). Stacked bar plots of mean relative bacterial abundance per category were created with *ggplot2.* Bacterial phyla and genera that were <1% abundant were considered rare and thus excluded from the figure.

Environmental source controls were then excluded from all further statistical analyses due to significant differences from the cloacal wipe samples (Figure 1; Supplementary Table 2). An additional PERMANOVA comparing only the genus-level Bray-Curtis dissimilarities was performed (singletons were removed: 4833 ASVs, i.e. 44.6% of all the ASVs) to investigate microbiota variation between cloacal wipe samples, setting sex (n_{female}=42 or n_{male}=38), mating experience subgroup (n_{virgin}=8, $n_{experienced}$=36, or n_{mated}=36), their interaction, and age as fixed effects, constraining the permutations within strata=bird ID (n=44).

Differential bacterial abundance on female and male cloacae across mating experience subgroups (i)–(iii) was tested using *corncob* (Martin, Witten & Willis, 2020). The untrimmed data set, i.e. including singletons, was split into female or male cloacal wipe samples (females: n_{virgin}=4, $n_{experienced}$=19, or n_{mated}=19; males: n_{virgin}=4, $n_{experienced}$=17, or n_{mated}=17), agglomerated to phylum or genus level, filtered to include only taxa that contributed >1% to the mean relative bacterial abundances, and tested with differentialTest(test="Wald", fdr_cutoff=0.05), while controlling for variance across mating experience subgroup (i)–(iii).

Differential cloacal bacterial abundance associated with the sex of the individual (n_{female}=42 or n_{male}=38) was modelled while first controlling for bacterial abundance across mating experience subgroup (i)–(iii), and variance across mating experience subgroup (i)–(iii) and fowl sex. Results were visualised with *corncob*. Age was not accounted for in the differential abundance analyses as it was not indicated as significant in the genus-level Bray-Curtis dissimilarities PERMANOVA results.

To investigate bacterial richness and diversity variation, we calculated three different α-diversity metrics (Whittaker, 1960), using the untrimmed, i.e. including singletons, data set. Total ASV richness was calculated with *breakaway* (Willis et al., 2018), and Shannon's and inverse Simpson's diversity index estimates with *phyloseq*. Normality of each α-diversity metric was assessed with a Shapiro-Wilk test, and suitable data distributions were determined from histograms, which were then confirmed with *fitdistrplus* (Delignette-Muller & Dutang, 2015), using Cullen and Frey graphs (bootstrap=500).

A linear mixed-effects model (LMM) by restricted maximum likelihood (REML) was fitted to the normally distributed total ASV richness estimates with *lme4* (Bates et al., 2015), setting mating experience subgroup (i)–(iii), sex, their interaction, and age as fixed effects, and bird ID as a random effect. Significant results were followed up by Tukey-adjusted pairwise comparisons from *lsmeans* (Lenth, 2016) to detect differences between mating experience subgroup*sex ($n_{virgin\ female}$=4, $n_{experienced\ female}$=19, $n_{mated\ female}$=19, $n_{virgin\ male}$=4, $n_{experienced\ male}$=17, or $n_{mated\ male}$=17) that were then visualised with *ggplot2*.

Shannon's diversity followed a Gaussian (link="log") distribution and inverse Simpson's diversity a gamma distribution (link="inverse"). A generalised linear mixed-effects model (GLMM) by Laplace approximation was fitted to either α-diversity metric with *lme4*, setting mating experience subgroup (i)–(iii), sex, their interaction, and age as fixed effects, and bird ID as a random effect.

Goodness of model fits was assessed from Q-Q and residual plots. Function Anova() from *car* (Fox & Weisberg, 2019) was used to carry out deviance analyses (type III Wald F-test with Kenward-Roger degrees of freedom for LMM and type III Wald chi-square tests for GLMMs) of the fixed effects, while random effect statistics were obtained from model summaries.

(2) *Individual phenotype association with red junglefowl cloacal microbiota*. We tested whether different phenotypes had inherently distinct cloacal microbiota at the onset of the breeding season. For this part of the analysis, we only considered samples collected before a bird experienced their first mating(s) in the breeding season (n=46).

We investigated the following host characteristics known to affect mating behaviour: sex, age class, and personality traits (boldness and exploratory scores). Considering that the phylum-level variation in cloacal microbiota composition was less pronounced than the genus-level variation (Figure 2A), we decided to focus only on potential genus-level differences in the following analyses.

Singletons were removed (6980 ASVs, i.e. 64.4% of all the ASVs), and PERMANOVA and beta dispersion analyses were performed as in aim (1), setting the matrix of genus-level wUF or uwUF distances, or Bray-Curtis dissimilarities or Jaccard indices as the response variable, and sex (n_{female}=20 or n_{male}=26), age class (n_{young}=29 or n_{old}=17), and boldness and exploratory scores of the individual as fixed effects.

PERMANOVA results indicated the significance of fowl sex (Table 2). We therefore modelled and visualised the differential bacterial genus abundance associated with host sex with *corncob* as performed in aim (1), while controlling for variance across sex. We did not control for age class nor personality traits as they had no significant effect on cloacal microbiota compositions (Table 2).

α-diversity metrics were calculated, modelled, and analysed as in aim (1), unless specified otherwise. A linear model (LM) was fitted to the normally distributed total ASV richness estimates, setting sex, age class, and boldness and exploratory scores of the individual as fixed effects. Fixed effects were tested for significance with Anova() from *car* (type II F-test). Generalised linear models (GLMs) were fitted to Shannon's diversity (Gaussian distribution, link="log") and inverse Simpson's diversity index estimates (gamma distribution, link="log"). Fixed effects were tested for significance with Anova() from *car* (type II Wald chi-square tests).

(3) *Cloacal microbiota association with future mating opportunities*. We investigated whether the composition of cloacal microbiota before a bird joined an experimental group predicted its mating performance (i.e. the number of partners and matings) in the mating trial. For this part of the analysis, we utilised cloacal wipe samples collected from birds before joining any experimental group throughout the breeding season (n=108). To control for variation in individual mating rates across groups, we z-transformed the number of partners and matings an individual bird secured within its experimental group separately for both female and male birds ($z = \frac{(number\ of\ partners\ (or\ matings)\ per\ bird - group\ mean)}{group\ S.D.}$ for each sex).

Standardising allowed us to compare the proportional mating opportunities across experimental groups in association with fowl cloacal microbiota, while removing the effect of group ID. However, we included group sex ratio as a fixed effect, as sex ratio treatment could affect variance in mating opportunities. We also included individual sex, age class, and boldness and exploratory scores in the analysis to account for individual phenotypic variation.

z-transformed number of partners was normally distributed, while the number of matings followed a log-normal distribution. We thus re-scaled the number of matings so that the smallest value =1, and log-normalised the values. We fit LMMs by REML to both variables, setting sex (n_{female}=54 or n_{male}=54), sex ratio ($n_{female\text{-}biased}$=36, $n_{even\ sex\ ratio}$=36, or $n_{male\text{-}biased}$=36),

their interaction, age class (n_{young}=69 or n_{old}=39), boldness and exploratory scores, and α-diversity as fixed effects, and bird ID (n=57) as a random effect. Model outputs indicated that we had successfully removed the effect of sex*sex ratio ($p>0.75$ for both individual and the interaction term) and thus, we fit the final models without the two variables. In support of our decision, the AIC_C scores (Akaike's information criterion corrected for small sample size, k=2; Burnham & Anderson, 1998) that were calculated with *MuMIn* (Barton, 2020) indicated that the models including sex*sex ratio had no statistical support (all $\Delta AIC_C>10$, all weights<0.001).

We investigated the effect of three α-diversity metrics— total ASV richness, Shannon's diversity, and inverse Simpson's diversity—on fowl mating performance. While highly correlated, these metrics capture different aspects of bacterial diversity. Total ASV richness is the sum of observed and unobserved unique bacterial strains (Willis et al., 2018). Shannon's and inverse Simpson's diversity also consider the abundance of these unique bacterial strains, with Shannon's diversity giving more importance to rare bacteria (Spellerberg & Fedor, 2003), while inverse Simpson's diversity to common bacteria (Simpson, 1949). We therefore fitted separate models with each of these three metrics as an explanatory variable and tested them for significance with Anova() from *car* (type II Wald F-tests with Kenward-Roger degrees of freedom). To assess the importance of each of the α-diversity metric, we calculated an AIC_C score per model.

We also investigated whether females and males that secured a higher number of matings showed patterns of differentially-abundant bacterial genera on their cloacae before joining the experimental groups. Based on the z-transformations that were spread around 0, birds were categorised into individuals that mated less ($z<0$; n_{female}=31, n_{male}=29) or more ($z\geq0$; n_{female}=23, n_{male}=25). We carried out sex-specific differential bacterial abundance analyses, as

performed in aim (1), controlling for variance. We did not control for other individual phenotypic variation, as that over-parametrised the differential abundance models.

(4) *Changes in cloacal microbiota after the mating trials*. We analysed the cloacal wipe samples that were collected after birds exited a mating trial (n=108) to test whether mating interactions within the experimental group were associated with changes in cloacal microbiota, also controlling for social environment and host phenotypic variation.

In this part of the analysis, we used the actual number of partners and matings (rather than z-standardised), because each unique partner and additional mating could potentially introduce novel bacteria to the cloacal microbiota.

We also measured each individual's position in the socio-sexual network of its experimental group by calculating two social network metrics: eigenvector centrality, which measures the connectedness of the focal bird, also taking into account the connectedness of the birds connected to the focal bird; and betweenness, which reflects the number of times an individual bird is included in the shortest path connecting two other birds (Sosa, Sueur & Puga-Gonzalez, 2020). The two measures were calculated with *sna* (Butts, 2019) based on mating interaction matrices that indicated the strength of mating interaction between two individual birds as a proportion of all the matings that happened within that experimental group.

We fitted three separate models, one for each α-diversity metric (total ASV richness, Shannon's and inverse Simpson's diversity), which were calculated, modelled, and analysed as in aim (1), unless specified otherwise. Total ASV richness was normally-distributed and fitted with an LMM by REML. Shannon's and inverse Simpson's diversity index estimates followed a Gaussian (link="log") and a gamma distribution (link="log") respectively, and were fitted with GLMMs. To account for pre-existing individual differences, we entered the cloacal α-diversity of a bird before joining the experimental groups as a fixed effect. Thus, the fixed effects were the respective cloacal α-diversity metric before joining the experimental group,

sex (n_{female}=54 or n_{male}=54), group sex ratio ($n_{female\text{-}biased}$=36, $n_{even\ sex\ ratio}$=36, or $n_{male\text{-}biased}$=36), their interaction, number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=69 or n_{old}=39), boldness and exploratory scores. Bird ID (n=57) and group ID (n=9) were set as random effects. α-diversity models including bird ID as a random effect had little statistical support (all ΔAIC_C>2.5, all weights<0.22, except for the model for inverse Simpson's diversity for which even group ID had a negligible effect). As group ID explained all variation associated with random effects, we excluded bird ID from the final α-diversity models. Significant α-diversity modelling factors were visualised with *ggplot2*. The individual boldness and exploratory scores (Supplementary Methods 1) were log-normalised, as performed in aim (3), before visualising the data.

Genus-level cloacal microbiota composition variation after exiting experimental groups was analysed with PERMANOVA, as in aim (1), removing the singletons (3658 ASVs, i.e. 33.8% of the ASVs) and setting group ID (n=9), sex (n_{female}=54 or n_{male}=54), sex ratio ($n_{female\text{-}biased}$=36, $n_{even\ sex\ ratio}$=36, or $n_{male\text{-}biased}$=36), their interaction, number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=69 or n_{old}=39), and boldness and exploratory scores of the individual as fixed effects, constraining the permutations within strata=bird ID (n=57). Due to the large number of variables in these particular PERMANOVA tests, permutations=100,000. A non-metric multidimensional scaling (NMDS) ordination based on Jaccard indices was created with *phyloseq*.

PERMANOVA analyses with Jaccard indices and Bray-Curtis dissimilarities explained more cloacal microbiota variation than PERMANOVA analyses with their phylogenetic equivalents, i.e. unweighted and weighted UniFrac distances respectively (Table 3). Presence/absence β-diversity metrics, such as Jaccard index, may be more indicative of a significant bacterial transmission event than metrics that focus on differential bacterial abundance, such as Bray-Curtis dissimilarity. We therefore decided to focus only on Jaccard

indices in the rest of the analysis in aim (4), while controlling for bacterial abundance variation by performing differential abundance analysis.

Based on Jaccard indices, sex, and the boldness and exploratory scores of the individual had a significant effect on fowl cloacal microbiota composition after exiting the experimental groups, while age class had a moderate effect (Table 3D). We thus performed beta dispersion tests based on Jaccard indices, as in aim (1), for these four variables. To do that, we split the birds into "boldness behaviour groups" and "exploratory behaviour groups" based on the z-transformed boldness (Supplementary Methods 1; $z<0$ is $n_{shyer}=69$, $z\geq0$ is $n_{bolder}=39$) and exploratory scores ($z<0$ is $n_{less\ exploratory}=49$, $z\geq0$ is $n_{more\ exploratory}=59$).

Differential abundance analysis and visualisation was performed as in aim (1), considering all significant phenotypic factors that emerged from the PERMANOVA results (Table 3). Prior to the analysis, the continuous variables (number of partners and matings) were categorised into binary groups based on the means (number of partners: mean±S.E.=2.22±0.18, $n_{fewer\ partners}=71$ or $n_{more\ partners}=37$; number of matings: mean±S.E.=6.41±0.65, $n_{mated\ less}=69$ or $n_{mated\ more}=39$). We tested the differentially abundant bacterial genera across the sex of the individual, number of partners and matings (as categorical variables), age class, and boldness and exploratory behaviour groups, while controlling for bacterial genus abundance and variance across group ID, sex, number of partners and matings, age class, and the boldness and exploratory behaviour groups.

To analyse between experimental group differences, we performed two Mantel tests (method="spearman", permutations=1000) from *vegan* to test for a correlation between the distance matrix of genus-level Jaccard indices and group membership (0=not a member, 1=member of the experimental group) both before and after joining the experimental groups.

To analyse the cloacal microbiota variation within each social group due to mating, the data (n=108) was split into nine experimental group data sets (n=12 per group). A Mantel test

was carried out per group ID, correlating distance matrices of genus-level Jaccard indices and the strength of mating interactions. Network figures were created with *qgraph* (Epskamp et al., 2012) and box plots with *ggplot2*. The newest version of R (R Core Team 2020; Version 4.0.2.) was needed for *qgraph*.

To test whether the cloacal wipe samples collected before and after joining an experimental group differed from each other, and whether the cloacal microbiota of birds became more homogenous after intra-group interactions, the whole cloacal wipe data set (n=216) was split into nine experimental group data sets (n=24 per group). PERMANOVA was performed as in aim (1), removing singletons per group ID data set. Our response variable was a matrix of genus-level Jaccard indices, and we set sex (female-biased: n_{female}=18 or n_{male}=6, even sex ratio: n_{female}=12 or n_{male}=12, and male-biased: n_{female}=6 or n_{male}=18), collection time (n_{before}=12 or n_{after}=12 in each social group), and their interaction as fixed effects, constraining the permutations within strata=bird ID (n=12 in each social group). A beta dispersion test per experimental group was performed as in aim (1). NMDS ordinations was created with *phyloseq*.

Finally, we performed a population-wide analysis, using the cloacal wipe samples that were collected after exiting the experimental groups (n=108), considering the nine groups as one population. This was a justified approach, as the birds were re-used across experimental groups (Table 1), and had had prior mating interactions with each other in the previous breeding seasons. Singletons were removed (3658 ASVs, i.e. 33.8% of the ASVs), the cloacal wipe samples were merged per individual bird (fun=mean; if the fowl had been re-used across experimental groups), and genus-level Jaccard indices were calculated as in aim (1). The final population-wide data set consisted of cloacal microbiota samples from 57 individual birds. We created a population-wide strength of mating interactions matrix. A Mantel test was performed between the distance matrices of genus-level Jaccard indices and the strength of mating

interactions. The data was visualised with a network figure created with *qgraph* and a violin plot created with *ggpubr* (Kassambara, 2020).

Population-wide number of partners and the sum of all matings were recorded per bird, and additional population-wide eigenvector centrality and betweenness estimates were calculated per individual using *sna*. We compared population-wide genus-level Jaccard indices with PERMANOVA, as in section (1), setting sex (n_{female}=28 or n_{male}=29), number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=35 or n_{old}=22), and boldness and exploratory score of the individual as fixed effects. Due to the moderately large number of variables in this particular PERMANOVA, permutations=10,000. Significant factors were tested for significant beta dispersion differences, as performed in aim (1).

PERMANOVA results suggested a significant relationship between cloacal microbiota and the number of partners (Supplementary Table 8). We thus split the birds into two "partner number" groups of individuals that either mated with fewer (n=32) or more partners (n=25) than the population average (mean±S.E.=4.16±0.48) to compare beta dispersion differences (performed as in aim (1)) between less and more sexually-interacting members of the mating population.

To explore what bacteria did the birds that mated with more partners acquire in comparison with the birds that mated with fewer partners, we also carried out a differential abundance analysis, performed as in aim (1), first controlling for bacterial genus abundance across sex (n_{female}=28 or n_{male}=29), and variance across sex and partner number group. To investigate whether individuals that secured more partners at the population level had pre-existing patterns of differentially-abundant cloacal bacteria in comparison with individuals that secured fewer partners, we carried out the same analysis, using the samples collected before the mating trials. However, in this test, we used the z-transformed number of partners as in aim (3)—calculating the means for birds that were re-used—to control for the effect of group sex

ratio and group ID on the number of partners an individual would attain. Based on the z-transformations that were spread around 0, birds were categorised into individuals that went on to secure fewer ($z<0$; n=27) or more ($z\geq0$; n=30) partners across the mating population.

Results

(1) *Cloacal Microbiota Variation Between Virgin and Non-Virgin Red Junglefowl*

Environmental source controls were significantly different from cloacal wipe samples (Figures 1, 2; Supplementary Table 2), and were removed from further statistical analyses.

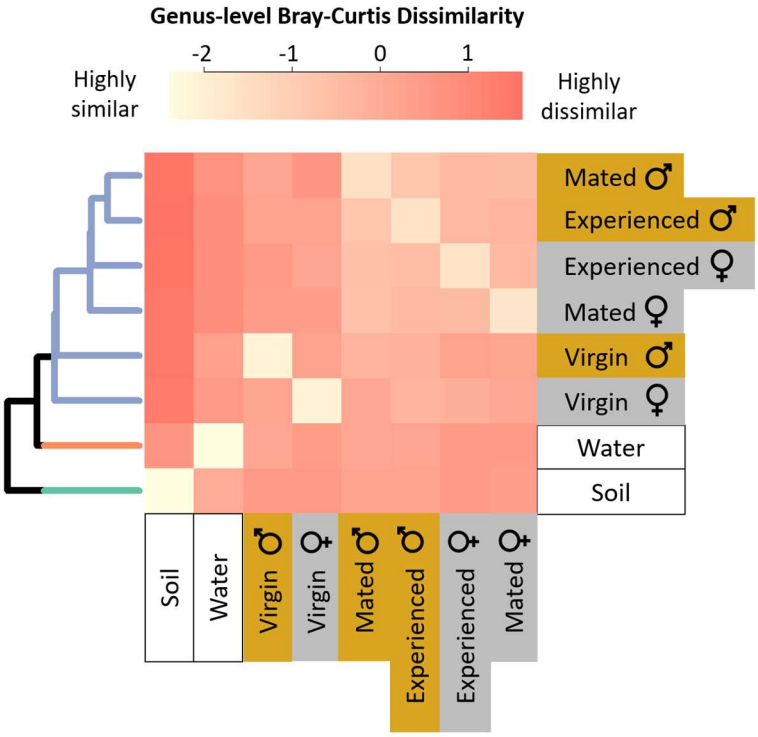


Figure 1. A heatmap with average linkage hierarchical clustering, depicting genus-level Bray-Curtis dissimilarities between mating experience subgroups (virgin=no previous mating experience, experienced=last mating happened in the previous breeding seasons, mated=mating happened in the mating trial in the current breeding season; $n_{virgin\ female}$=4, $n_{experienced\ female}$=19, $n_{mated\ female}$=19, $n_{virgin\ male}$=4, $n_{experienced\ male}$=17, $n_{mated\ male}$=17, n_{soil}=1, or n_{water}=1). Female cloacal samples are indicated by grey and male by beige. Spectrum from similarity to dissimilarity is presented by a range from light yellow to light red. Three separate clusters (dendrogram cutting height=0.55) are highlighted by unique colours.

Cloacal microbiota compositions differed significantly between sexes (PERMANOVA of genus-level Bray-Curtis dissimilarities: pseudo-$F_{1,\ 34}$=3.72, $p<0.001$, R^2=3.5%), mating

experience subgroups (pseudo-$F_{2, 34}$=4.05, p<0.001, R^2=7.6%), and individual birds (pseudo-$F_{40, 34}$=1.45, p<0.001, R^2=54.4%), but not across bird age (pseudo-$F_{1, 34}$=1.93, p=0.193, R^2=1.2%) nor the interaction term between sex and mating experience subgroup (pseudo-$F_{1, 34}$=1.62, p=0.087, R^2=1.5%). These differences were not due to significant beta dispersion differences (p>0.05). In particular, non-virgin individuals harboured significantly different cloacal bacterial compositions than virgin birds (Figures 1, 2).

Regardless of mating experience, cloacal microbiota were dominated by the phylum Actinobacteria, although virgins (females: 35.1±12.7% S.E., males: 39.3±10.2%) harboured a lower and more variable Actinobacteria relative abundance than non-virgin birds (females: 52.4±4.6% and males: 57.8±4.1% with previous mating experience; recently mated females: 49.7±4.0% and males: 59.2±3.2%; Figure 2A).

Females with mating experience had more Fusobacteria (β±S.E.=2.37±0.94, t=2.51, p=0.017) compared with virgin and recently mated females. Fusobacteria was <1% abundant in males. Males with mating experience (β=-2.94±0.69, t=-4.28, p<0.001) and recently mated males (β=-3.01±0.69, t=-4.37, p<0.001) had less Proteobacteria compared with virgin males (Figure 2A). Males had less Firmicutes than females (β=-0.38±0.15, t=-2.61, p=0.011; Figure 2A).

Bacterial genus-level comparison revealed that fowl with previous mating experience and recently mated birds harboured a higher relative abundance of *Corynebacterium* (phylum Actinobacteria) and *Actinomyces* (phylum Actinobacteria) than virgin individuals (*Corynebacterium*: virgin females: 5.1±1.3%, experienced females: 17.4±3.6%, mated females: 17.6±3.3%, virgin males: 5.4±4.4%, experienced males: 15.8±3.8%, mated males: 20.0±2.6%; *Actinomyces*: virgin females: 0.4±0.4%, experienced females: 8.7±2.2%, mated females: 4.8±1.5%, virgin males: 0±0%, experienced males: 5.8±1.4%, and mated males: 7.5±2.4%; Figure 2B).

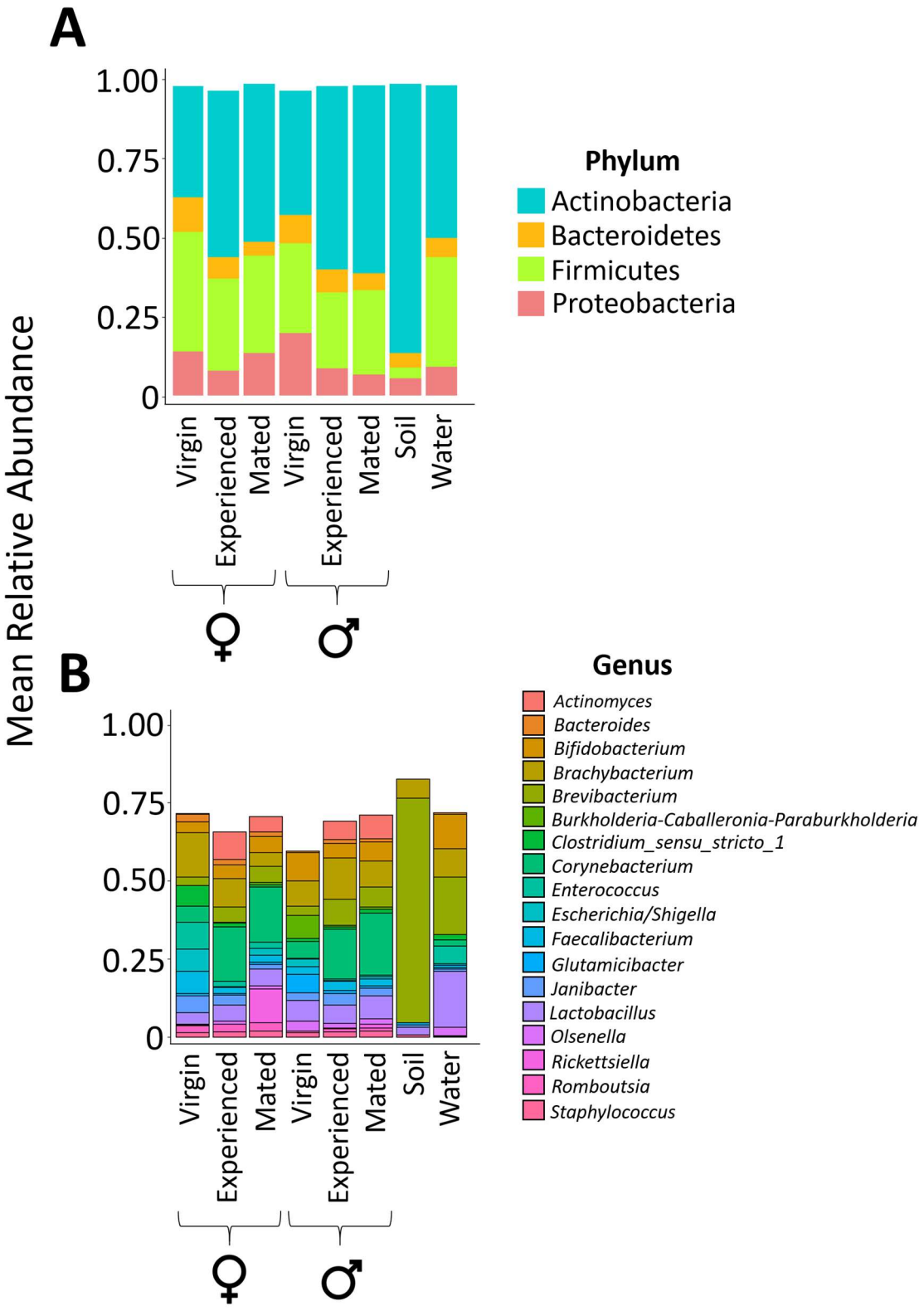


Figure 2. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A) and genera (B) found on female and male red junglefowl cloacae, and in soil and water present in the experimental pen ($n_{virgin\ female}$=4, $n_{experienced\ female}$=19, $n_{mated\ female}$=19, $n_{virgin\ male}$=4, $n_{experienced\ male}$=17, $n_{mated\ male}$=17, n_{soil}=1, or n_{water}=1). Bacterial phyla and genera that were <1% abundant were considered rare and thus excluded from the stacked bar plots. Each taxon is indicated by a unique colour and genera are separated by black lines for clarity (B).

Seven genera in females and four in males were differentially-abundant across mating experience subgroups (Supplementary Figure 3A & B). In particular, *Corynebacterium* was more abundant in non-virgin females (experienced: β=1.28±0.36, t=3.59, p=0.001; recently mated: β=1.51±0.34, t=4.42, p<0.001; Figure 2B; Supplementary Figure 3A). Females with mating experience had more *Fusobacterium* (phylum Fusobacteria; β=2.43±0.87, t=2.78, p=0.009; Supplementary Figure 3A) than virgin and recently mated females.

Eight bacterial genera were differentially-abundant between the two sexes (Supplementary Figure 3C). Of importance, *Escherichia/Shigella* (phylum Proteobacteria; distinguishing between the two genera is not possible based on the V4 hypervariable region) was less abundant in males (β=-0.84±0.36, t=-2.36, p=0.021), while *Lysobacter* (phylum Proteobacteria) was more abundant in male fowl (β=0.80±0.25, t=3.18, p=0.002; Supplementary Figure 3C).

Total ASV richness analysis revealed a significant interaction between mating experience subgroup and sex (Figure 3; Supplementary Table 3A). Recent mating significantly increased female, but not male, cloacal bacterial richness compared to their pre-mating state (Figure 3). Virgin males harboured lower total ASV richness than non-virgin males (Figure 3). Shannon's and inverse Simpson's diversity analyses did not suggest any significant effects (Supplementary Table 3B & C).

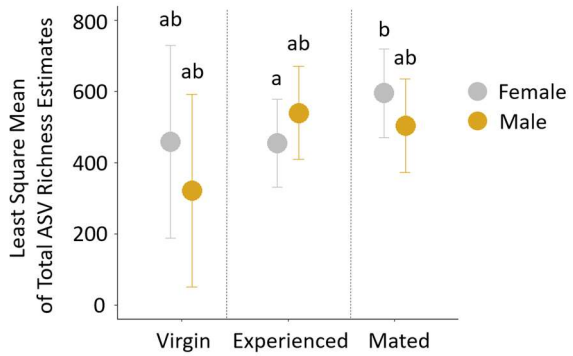


Figure 3. Least square (LS) means of total ASV richness estimates per sex-specific mating experience subgroup ($n_{virgin\ female}$=4, $n_{virgin\ male}$=4, $n_{experienced\ female}$=19, $n_{experienced\ male}$=17, $n_{mated\ female}$=19, and $n_{mated\ male}$=17). Error bars indicate the 95% confidence interval. LS means sharing a letter are not significantly different from each other (Tukey-adjusted comparisons).

(2) *Individual Phenotype Association with Red Junglefowl Cloacal Microbiota*

The sex of the bird had a significant effect on the cloacal microbiota of experienced non-virgin birds, i.e. before entering their first mating trial of the breeding season (Table 2). These differences were likely due to nine differentially-abundant bacterial genera between sexes (Figure 4).

Table 2. [PERMANOVA] Genus-level cloacal microbiota composition comparisons between red junglefowl of different sex (n_{female}=20 or n_{male}=26), age class (n_{young}=29 or n_{old}=17), and boldness and exploratory personality before the first mating trial of the breeding season, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Significant p-values are in bold. Beta dispersion tests (permutations=1000) revealed no significant dispersion variation in any of the β-diversity metrics (p>0.05) between males and females.

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Sex	**2.65**	**1**	**0.014**	**5.6%**
Age class	0.61	1	0.790	1.3%
Boldness score	1.25	1	0.239	2.7%
Exploratory score	1.52	1	0.129	3.2%
Residuals		41		87.2%
Unweighted UniFrac				
Sex	**3.75**	**1**	**<0.001**	**7.8%**
Age class	1.07	1	0.331	2.2%
Boldness score	1.10	1	0.268	2.3%
Exploratory score	0.90	1	0.609	1.9%
Residuals		41		85.8%
Bray-Curtis DS				
Sex	**2.56**	**1**	**0.011**	**5.5%**
Age class	0.66	1	0.772	1.4%
Boldness score	1.31	1	0.203	2.8%
Exploratory score	1.08	1	0.360	2.3%
Residuals		41		88.0%
Jaccard Index				
Sex	**3.24**	**1**	**<0.001**	**6.9%**
Age class	0.87	1	0.683	1.8%
Boldness score	1.20	1	0.154	2.5%
Exploratory score	0.96	1	0.499	2.0%
Residuals		41		86.8%

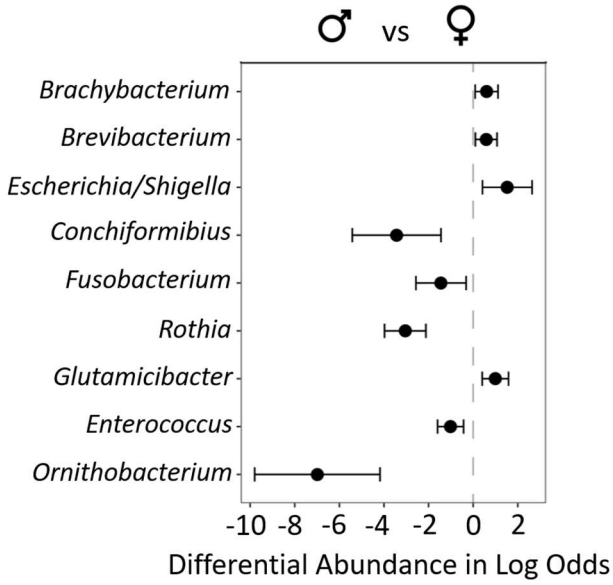


Figure 4. Differential abundance (in log odds) of bacterial genera on the cloacae of male fowl (n=26) in comparison with female birds (n=20) before the first mating trial of the breeding season. The grey dashed line represents the point of comparison, i.e. the cloacal microbiota of female fowl. Data points with standard error bars depict log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds the significant under-representation of the bacterial genus in males compared to females.

We found no sex-specific variation in cloacal total ASV richness, Shannon's, nor inverse Simpson's diversity (Supplementary Table 4). We also report no covariation between cloacal microbiota, and fowl age class, nor boldness nor exploratory score (Table 2; Supplementary Table 4).

(3) *Cloacal Microbiota Association with Future Mating Opportunities*

We found no evidence for a relationship between fowl cloacal bacterial diversity before joining an experimental group, and the number of partners nor matings secured in the mating trial (Supplementary Table 5).

Females that entered the mating trial with a higher abundance of bacteria that are considered pathogenic, and fewer bacterial genera considered commensal/mutualistic attained a larger proportion of matings in the trials (Figure 5A). Both females and males with a higher

abundance of *Romboutsia* (phylum Firmicutes) on their cloacae at the onset of a mating trial obtained more matings in their experimental groups (Figure 5).

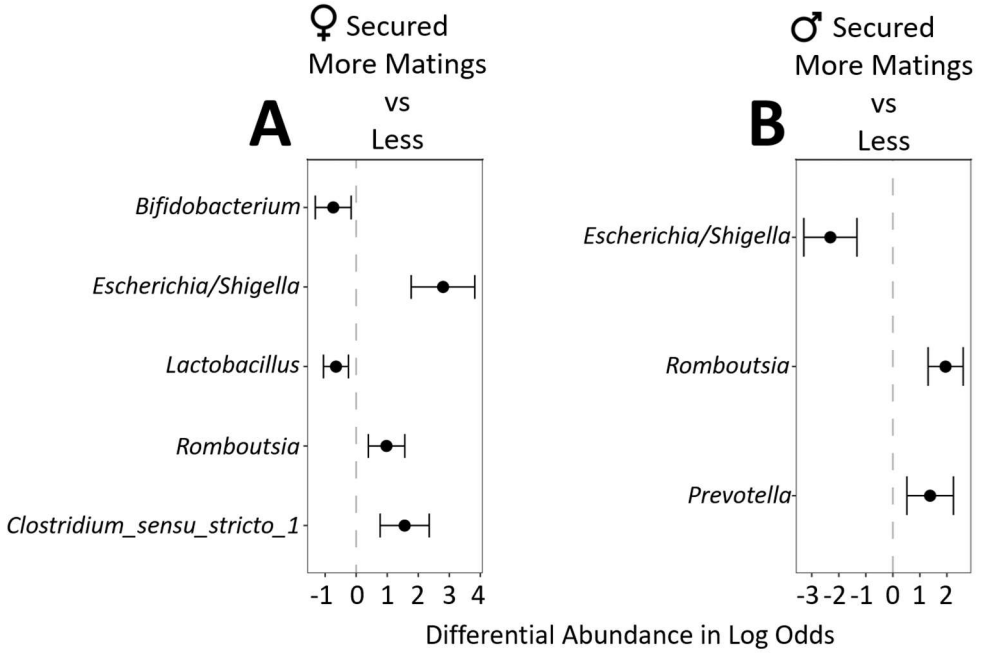


Figure 5. Differential abundance (in log odds) of bacterial genera on the cloacae of female (n=23; A) and male fowl (n=25; B) that went on to secure more matings in their respective experimental groups. The grey dashed line represents the point of comparison, which were the female (n=31; A) and male fowl (n=29; B) that went on to secure fewer matings. Data points with standard error bars depict log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds the significant under-representation of the bacterial genus on the cloacae of birds with higher mating rates in comparison with birds that were able to secure fewer matings in the mating trials.

(4) *Changes in Cloacal Microbiota After the Mating Trials*

After the mating trials, bolder individuals had a decreased cloacal total ASV richness (Figure 6A), while more exploratory birds had a significantly increased total ASV richness on their cloacae (Figure 6B; Supplementary Table 6A). We did not find a significant Pearson's correlation between individual log(boldness) and log(exploratory) scores ($r(55)=0.15$, $p=0.253$).

Males harboured a significantly higher inverse Simpson's diversity on their cloacae than females regardless of group sex ratio (Figure 7; Supplementary Table 6C).

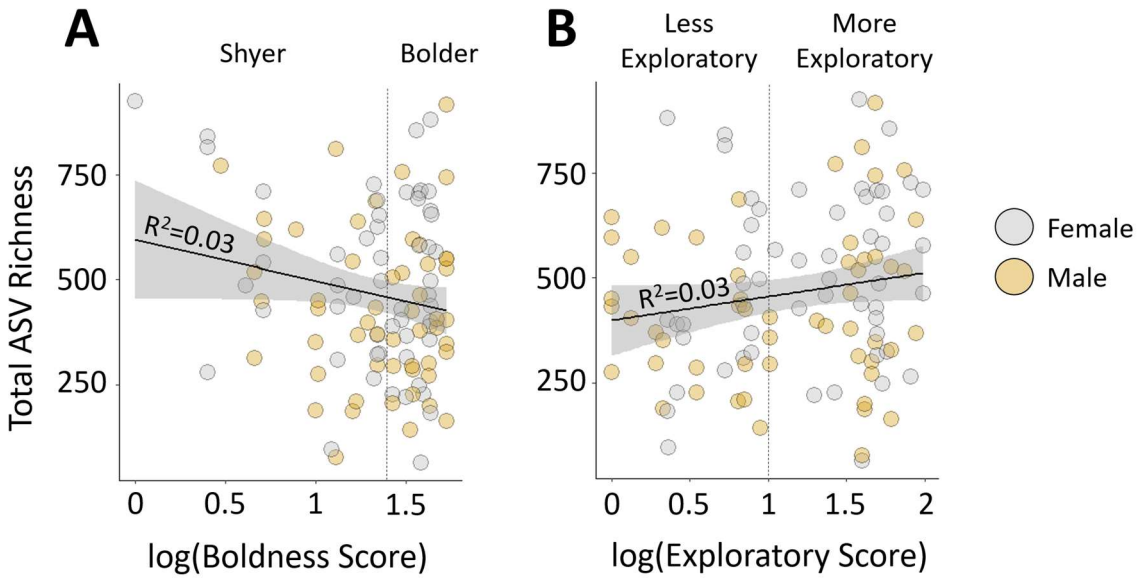


Figure 6. Linear relationships between cloacal total ASV richness estimates and, log(boldness) (A) and log(exploratory) scores (B). Each dot is a cloacal wipe sample (n=108). The shaded area around the best fit regression line represents the 95% confidence interval.

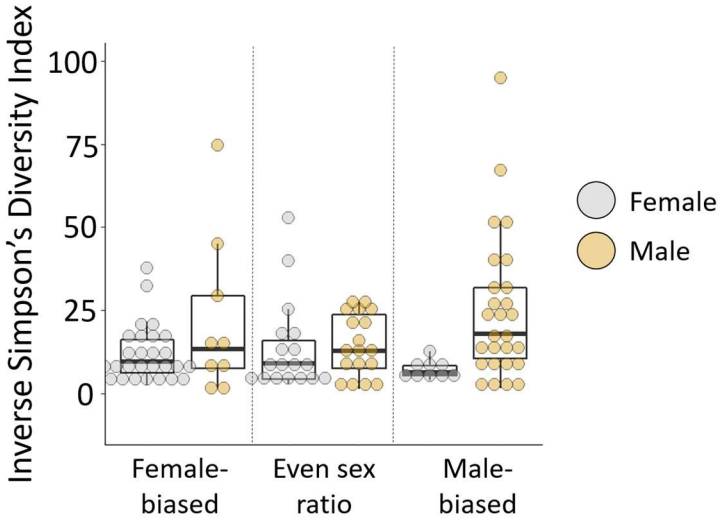


Figure 7. Box plots depicting the inverse Simpson's diversity index estimates for female (n=54) and male fowl (n=54) across the three experimental group sex ratio treatments. Dots represent individual cloacal wipe samples (n=108). Each box plot has an indicated median and interquartile range. The tips depict the minimum and maximum values. Outliers are included.

Indeed, female and male cloacal microbiota compositions differed significantly after the mating trials (Table 3), and this was not due to significant beta dispersion differences between Jaccard indices ($p>0.05$). Seven genera were significantly differentially-abundant on

female and male cloacae (Supplementary Figure 4). Of note, male fowl had acquired more *Fusobacterium mortiferum* after the mating trials (Supplementary Figure 4), while *F. mortiferum* was significantly scarcer in males, but more abundant in females before the trials (Figure 4).

There was no effect of group sex ratio on cloacal microbiota variation (Table 3), but the number of partners and matings affected the cloacal bacterial abundances (Table 3A & C). After controlling for the bacterial genus abundance and variance across group ID, sex, number of partners and matings, age class, and boldness and exploratory behaviour groups, we did not detect any bacterial genera that were significantly differentially abundant between birds that secured fewer or more partners. However, birds that secured more matings had significantly more *Actinomyces* (β=0.84±0.30, t=2.81, p=0.006), but significantly less *Faecalibacterium* (phylum Firmicutes; β=-1.09±0.33, t=-3.30, p=0.001) and *Bacteroides* (phylum Bacteroidetes; β=-0.67±0.22, t=-3.02, p=0.003) on their cloacae after exiting the experimental groups than birds that mated fewer times than the population mean.

While we found no evidence that birds of different age class, boldness nor exploratory behaviour differed in their cloacal microbiota composition before joining the experimental groups (Table 2), both boldness and exploratory behaviour had a significant effect on cloacal microbiota after the mating trials, while the effect of age class on cloacal bacterial variation was more moderate (Table 3), even after controlling for beta dispersion differences between Jaccard indices (p>0.05). After controlling for differential bacterial abundance and variance across group ID, sex, number of partners and matings, age class, and behaviour groups, we were not able to detect any significantly differentially abundant bacterial genera across boldness nor exploratory behaviour groups, while old birds had significantly more *Brachybacterium* (phylum Actinobacteria; β=0.62±0.23, t=2.62, p=0.011), *Escherichia/Shigella* (β=1.21±0.40, t=3.03, p=0.003), *Fusobacterium* (β=1.89±0.54, t=3.47,

p<0.001), and *Rickettsiella* (phylum Proteobacteria; β=1.47±0.42, t=3.47, p=0.001) on their cloacae than young birds. By far the largest amount of cloacal microbiota variation was explained by bird ID (48.5±0.6%; Table 3).

Based on genus-level Jaccard indices, group ID explained 9.8% of cloacal bacterial composition variation after the mating trials (Table 3D). These significant differences were due to near-significant dispersion variation across the nine experimental groups (pseudo-$F_{8, 99}$=1.81, p=0.073; Figure 8). Groups FB22 and ES21 in particular differed in their beta dispersion from the other experimental groups, with group FB22 being relatively homogenous, while group ES21 was relatively heterogeneous (Figure 8).

Mantel tests between the distance matrix of genus-level Jaccard indices and group membership did not reveal significant correlations between cloacal microbiota and group ID before joining (r=-0.03, p=0.998) nor after exiting an experimental group (r=-0.03, p=1.000).

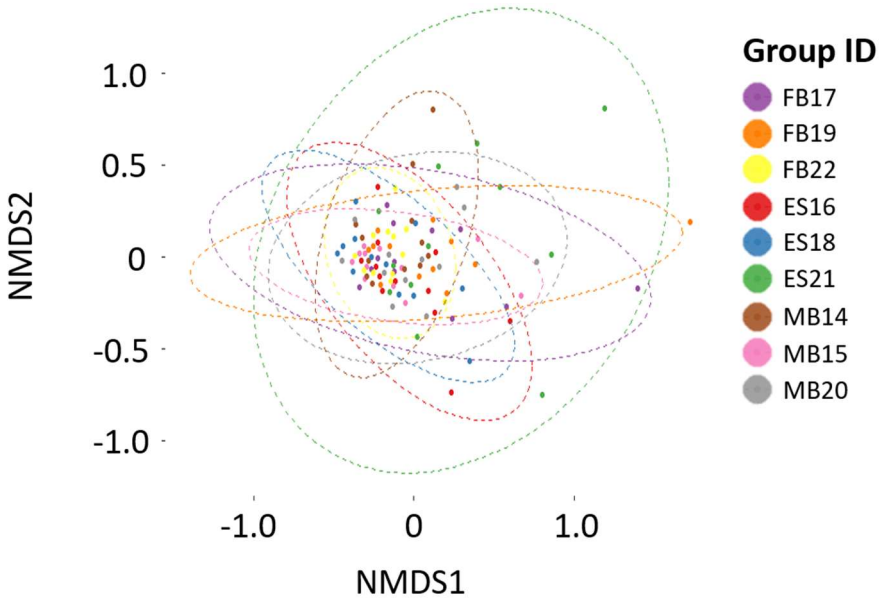


Figure 8. A non-metric multidimensional scaling (NMDS) ordination (stress=0.16) depicting the cloacal wipe samples collected after the 6-day mating trials (FB=female-biased, ES=even sex ratio, or MB=male-biased), with each experimental group indicated by a unique colour. The ordination is based on genus-level Jaccard indices. Each dot is a cloacal wipe sample (n=108). The dashed ellipses indicate the respective multivariate normal distributions of each experimental group.

Table 3. [PERMANOVA] Genus-level cloacal microbiota composition variation across the nine experimental groups after the 6-day mating trials, controlling for sex (n_{female}=54, n_{male}=54), group sex ratio ($n_{female\text{-}biased}$=36, $n_{even\ sex\ ratio}$=36, or $n_{male\text{-}biased}$=36), their interaction, number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=69, n_{old}=39), and boldness and exploratory scores, using weighted (A) and unweighted UniFrac distances (B), and Bray-Curtis dissimilarities (DS; C) and Jaccard indices (D). Permutations (=100,000) were constrained within strata=*bird ID* (n=57). Significant p-values are in bold. Group sex ratio had no effect in any of the models and was thus excluded from the model output.

A

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Group ID	1.21	8	0.172	7.7%
Sex	**3.44**	**1**	**0.005**	**2.7%**
Sex*Sex ratio	0.62	2	0.854	1.0%
# of partners	1.83	1	0.087	1.5%
# of matings	1.97	1	0.069	1.6%
Eigenvector centrality	0.38	1	0.944	0.3%
Betweenness	0.76	1	0.593	0.6%
Age class	1.53	1	0.150	1.2%
Boldness score	2.06	1	0.055	1.6%
Exploratory score	**4.93**	**1**	**<0.001**	**3.9%**
Bird ID	1.18	52	0.133	48.7%
Residuals		37		29.2%

B

Genus-level	pseudo-F	df	p-value	R^2
Unweighted UniFrac				
Group ID	**1.50**	**8**	**<0.001**	**10.0%**
Sex	**3.20**	**1**	**<0.001**	**2.7%**
Sex*Sex ratio	0.83	2	0.806	1.4%
# of partners	1.04	1	0.351	0.9%
# of matings	1.24	1	0.161	1.0%
Eigenvector centrality	1.12	1	0.262	0.9%
Betweenness	1.15	1	0.231	1.0%
Age class	1.11	1	0.264	0.9%
Boldness score	1.49	1	0.059	1.2%
Exploratory score	**2.37**	**1**	**0.002**	**2.0%**
Bird ID	1.10	52	0.095	47.3%
Residuals		37		30.7%

C

Genus-level	pseudo-F	df	p-value	R^2
Bray-Curtis DS				
Group ID	**1.68**	**8**	**<0.001**	**9.3%**
Sex	**5.03**	**1**	**<0.001**	**3.5%**
Sex*Sex ratio	0.89	2	0.614	1.2%
# of partners	**2.71**	**1**	**0.003**	**1.9%**
# of matings	**2.23**	**1**	**0.013**	**1.6%**
Eigenvector centrality	0.79	1	0.653	0.6%
Betweenness	1.32	1	0.193	0.9%
Age class	**2.08**	**1**	**0.020**	**1.4%**
Boldness score	**2.03**	**1**	**0.024**	**1.4%**
Exploratory score	**3.32**	**1**	**<0.001**	**2.3%**
Bird ID	**1.39**	**52**	**<0.001**	**50.1%**
Residuals		37		25.8%

D

Genus-level	pseudo-F	df	p-value	R^2
Jaccard Index				
Group ID	**1.47**	**8**	**<0.001**	**9.8%**
Sex	**3.39**	**1**	**<0.001**	**2.8%**
Sex*Sex ratio	0.86	2	0.839	1.4%
# of partners	1.22	1	0.137	1.0%
# of matings	1.25	1	0.116	1.0%
Eigenvector centrality	1.04	1	0.340	0.9%
Betweenness	1.22	1	0.139	1.0%
Age class	1.32	1	0.079	1.1%
Boldness score	**1.59**	**1**	**0.021**	**1.3%**
Exploratory score	**1.52**	**1**	**0.029**	**1.3%**
Bird ID	**1.11**	**52**	**0.025**	**47.8%**
Residuals		37		30.6%

We found no evidence for a significant positive trend between increased strength of mating interaction and cloacal microbiota similarity in any of the nine experimental groups (Supplementary Figure 5). We report varying significance patterns when comparing the cloacal samples collected before and after the mating trial of each experimental group (Supplementary Table 7A). Fowl cloacal bacterial compositions did not become more homogenous after intra-group mating (Supplementary Table 7B). In fact, some groups (MB20 and ES21) became more dissimilar to each other after the mating trial in comparison with the microbiota present on their cloacae before the mating trial (Supplementary Table 7; Supplementary Figure 6).

Considering the nine experimental groups as one population showed a non-significant, positive trend between increased strength of mating interaction and cloacal microbiota similarity (Figure 9).

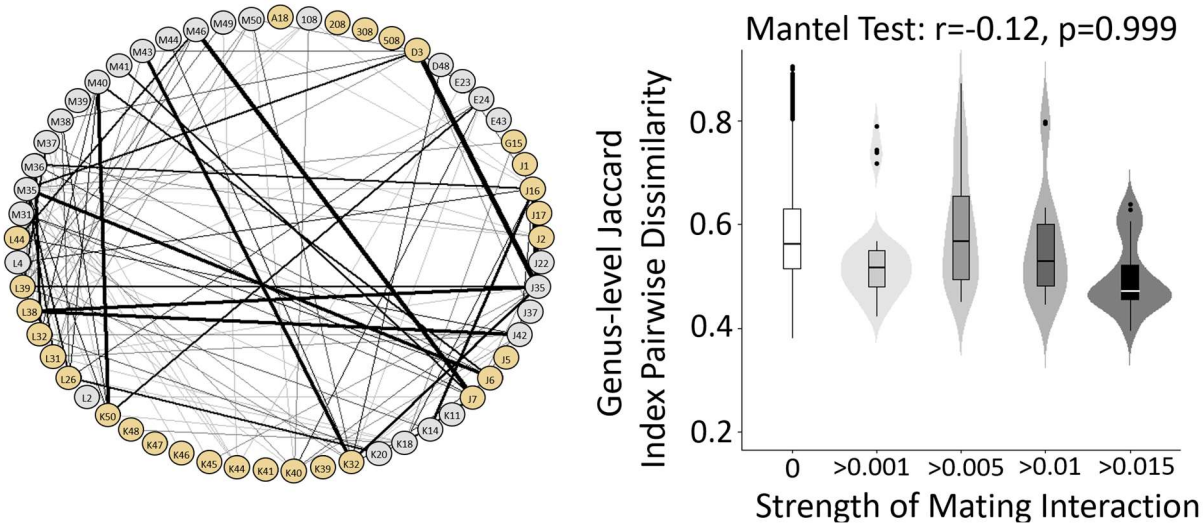


Figure 9. Strength of mating interactions network of the red junglefowl mating population. Nodes represent individual birds (female=grey or male=beige) with IDs. The thicker and darker the edge, the stronger the mating interaction. The violin plots depict the genus-level Jaccard index pairwise dissimilarities across the different strength of mating interaction categories. All box plots within the violin plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values, and the outliers are included in the plot. The shape of the violin plot shows the spread of data, and the colour of the violin plot matches the edge colour in the strength of mating interaction network figure. A Mantel test result between the distance matrices of cloacal microbiota genus-level Jaccard index dissimilarities and the strength of mating interactions is indicated.

Females and males harboured divergent cloacal microbiota compositions (Supplementary Table 8), even after controlling for beta dispersion differences (pseudo-$F_{1, 55}$=0.10, p=0.768). The number of partners secured in the mating population had a significant effect on fowl cloacal microbiota (Supplementary Table 8). Individuals that had fewer partners at the population level diverged significantly from the rest of the mating population that shared a homogenous cloacal microbiota (pseudo-$F_{1, 55}$=6.25, p=0.011).

We found that mating with more partners in the population, as opposed to with fewer partners, led to harbouring significantly less *Escherichia/Shigella* (β=-1.27±0.36, t=-3.48, p=0.001) and *Romboutsia* (β=-1.42±0.0.42, t=-3.40, p=0.001). Fowl that mated with more partners in the mating population had a pre-existing high abundance of *Romboutsia* (β=1.25±0.26, t=4.76, p<0.001) and *Fusobacterium* (β=2.03±0.41, t=5.01, p<0.001) compared with the birds that secured fewer partners.

Discussion

Exploring the patterns of sexual bacterial transmission dynamics could provide important insights into the role of bacteria in host mating behaviour, sexual selection and conflict, and sexually-transmitted infections (Rowe et al., 2020). Surprisingly little, however, is known about the inter-relationship between host mating interactions and phenotypic variation, and the sexual transmission of bacteria. By sampling the cloacal bacteria of polygynandrous red junglefowl before and after experimental mating trials, we were able to explore how mating interactions and individual variation may affect cloacal microbiota.

We found that virgin and non-virgin birds harboured significantly distinct cloacal microbiota, with non-virgin birds showing an increased abundance of two pathogens from the phylum Actinobacteria: *Corynebacterium* and *Actinomyces*. Recent mating significantly increased female, but not male, cloacal total ASV richness compared to before mating. We

report sex-specific cloacal microbiota variation that was independent of mating interactions, but found no evidence that cloacal bacterial diversity before a mating trial predicted the number of partners nor matings that an individual attained in the trial. After six days of intra-group mating interactions, fowl sex, and boldness and exploratory behaviour, but also the number of partners and matings secured, and fowl age class explained a significant proportion of cloacal microbiota variation. More exploratory birds had higher total ASV richness, while bolder individuals had lower total ASV richness on their cloacae. Sex-specific cloacal microbiota differences persisted after the mating trials. We found weak support for intra-group mating interactions leading to between-group differences, and report little evidence for intra-group mating interactions homogenising the cloacal microbiota of more frequently mating individuals. However, analysing the nine experimental groups as one population revealed that the polygynandrous mating population harboured a shared cloacal microbiota, and birds that mated with fewer partners diverged from the rest.

Red junglefowl cloacal microbiota composition was significantly different from the environmental source controls collected from the experimental pen, suggesting that the fowl cloaca supports an independent bacterial ecosystem. Habitat filtering between the host and its environment is not unprecedented, and may be species-specific. Ground-feeding woodlarks (*Lullula arborea*) and skylarks (*Alauda arvensis*) have been reported to horizontally acquire bacteria from soil and nest lining (van Veelen, Salles & Tieleman, 2018), while the plumage of American redstarts (*Setophaga ruticilla*), which typically feed while perched on trees, was different from the surrounding soil (Bisson et al., 2007). Despite frequent dust-bathing and ground foraging, the red junglefowl cloacal microbiota shared few bacterial phyla and genera with its environment. Strong habitat filtering may be beneficial in avoiding pathogenic environmental bacteria, such as feather-degrading bacteria in soil (Grande, Negro & Torres,

2004). It is worth mentioning, however, that we only processed one soil and one water sample, and extracted environmental DNA with a different kit, which could have biased our results.

We found that virgin birds harboured similar cloacal bacterial composition, while significantly differing from non-virgin birds. Regardless of mating experience, however, cloacal bacterial composition was dominated by the phylum Actinobacteria. While Actinobacteria are frequently found on the cloaca of wild (Kreisinger et al., 2015; Escallón, Belden & Moore, 2019; Hernandez et al., 2020) and captive birds (Zhang et al., 2017), their function remains poorly understood in avian species, as the phylum contains both antibiotic-producing pathogens and commensals (Ventura et al., 2007; Barka et al., 2016). In addition to Actinobacteria, the red junglefowl cloacae harboured the phyla Firmicutes, Proteobacteria, and Bacteroidetes, which is congruent with bacterial phyla captured on the cloacae of wild (Kreisinger et al., 2015; Escallón, Belden & Moore, 2019) and captive birds (Zhang et al., 2017).

At the genus level, virgin birds harboured a lower abundance of *Corynebacterium* and *Actinomyces* than birds that had started mating, and it is plausible that the two genera are associated with mating and possibly sexually-transmitted. Indeed, birds that mated more frequently exited the mating trials with a significantly higher abundance of *Actinomyces* on their cloacae than birds that mated less. Both genera can have severe implications in the context of host reproduction: *Corynebacterium*, which was shown to be sexually-transmitted in black-legged kittiwakes, can cause egg hatching failure in birds (van Dongen et al., 2019) and *Actinomyces* induces an inflammatory response in female human reproductive tracts (Kayikcioglu et al., 2005).

Non-virgin females and males shared a relatively similar cloacal microbiota with few evident differences between the cloacal microbiota compositions sampled before and after the mating trials. Convergence of female and male cloacal bacterial communities has also been

reported in breeding pairs of black-legged kittiwakes (White et al., 2010) and in a breeding population of North American tree swallows (Hernandez et al., 2020). However, despite sharing a large abundance of bacterial phyla and genera, sex-specific cloacal microbiota composition variation was evident both before the first mating trial of the breeding season and after the mating trials were over. Interestingly, females harboured significantly more *Fusobacterium mortiferum* before mating, but significantly less after mating, which was the opposite in males. *F. mortiferum* has been found to protect poultry caeca from *Salmonella* (phylum Proteobacteria) infections (Portrait, Cottenceau & Pons, 2000), and may also be a mutualist in the red junglefowl system. Further research is necessary, however, to test whether this bacterial strain is sexually-transmitted, or were the observed patterns caused by mating disturbing the sex-specific cloacal bacterial communities. We also encourage further investigation of the genus *Lysobacter* that was predominantly found on male cloacae (Supplementary Figure 3C). *Lysobacter* is known to produce complex antibiotics (Panthee et al., 2016) and antifungal metabolites (Brucker et al., 2008), and may serve a functional role in fowl.

Our results suggest that the sexual transmission of bacteria in red junglefowl may be asymmetric. Our qualitative comparison of 16S SSU rRNA gene copy number per cloacal wipe revealed that mating females, but not virgin females, harboured higher and more variable bacterial loads on their cloacae than males (Supplementary Figure 1). The asymmetric pattern was also reflected in our finding that males had higher cloacal inverse Simpson's diversity after the mating trials than females. This pattern was not evident before the mating trials had begun. Inverse Simpson's diversity is particularly sensitive to abundant, rather than rare, bacterial strains (Simpson, 1949). Thus, a higher value indicates the presence of more common and uniformly-abundant bacteria. Mating females may have a particularly diverse cloacal microbiota, where multiple bacteria-packed ejaculates from different males may decrease the

abundance of common bacterial strains, thus leading to reduced inverse Simpson's diversity estimates.

The asymmetric nature of sexual bacterial transmission in birds may make females more vulnerable to STIs than males, considering that bacterial infections are often dose-dependent (Rybicki, Kisdi & Anttila, 2018). Severe health implications and reproductive fitness consequences may lead to the evolution of strategies to maintain a healthy reproductive microbiota in female hosts. Unlike males, which showed a consistently high cloacal total ASV richness both before and after mating trials compared to virgin males, the cloacal total ASV richness of females that had mated last in the previous breeding seasons was more similar to the virgin state, and significantly lower than the cloacal richness sampled after the mating trials. It is possible that during the non-breeding season, females attempt to revert their cloacal microbiota back to the virgin state of lower bacterial richness, although further research is necessary to test this hypothesis. In support of our results, however, female black-legged kittiwakes were found to revert to a lower diversity microbiota after cloacal contact was experimentally blocked between mating partners (White et al., 2010). Female striped plateau lizards (*Sceloporus virgatus*; Martin, Gilman & Weiss, 2010) were reported to harbour lower cloacal bacterial diversity than males, suggesting a potential for a bacterial diversity-associated fitness cost and/or a reproductive microbiota maintenance mechanism in female hosts. Indeed, antimicrobial peptides (AMPs) produced by female host have been shown to regulate reproductive bacteria in the humans (Agarwal et al., 2015) and in White Leghorn hens (Silphaduang et al., 2006).

We must, however, emphasise that the reported patterns of cloacal microbiota variation across the mating experience subgroups come with added caveats. First, the sample size for virgin birds was limited (n_{female}=4 and n_{male}=4). Second, the virgin birds were independent of the non-virgin individuals, which were the same birds that were sampled immediately before

and after the mating trials. Third, we were not able to completely disentangle the effects of mating and age. There was a considerable age gap between the one-year-old virgin birds and the non-virgin birds (2–9 years). We could not sample older virgin birds, because fowl are rarely kept virgin for long in the captive population in Wytham. It is thus necessary to repeat this experiment with a larger sample size, following the same bird from virgin state through multiple breeding seasons, also accounting for potential age-related variation. In support of our results, however, the virgin fowl were sexually-mature, interacting with mating birds within same-sex flocks, and eating the same food as the rest of the population. In addition, variation in age was minimally associated with changes in cloacal microbiota composition variation in non-virgin birds. It is therefore unlikely that a significant difference between the cloacal microbiota of virgin and non-virgin birds would stem from anything else but mating experience.

We found no evidence for age- nor personality-related differences in fowl cloacal microbiota prior to mating for the first time in the breeding season. Considering that all the fowl appeared healthy and were sexually-mature adults, it is possible that we failed to detect considerable age-associated cloacal microbiota variation that is present for example between the chicks and adults of black-legged kittiwakes (van Dongen et al., 2013). However, after the mating trials, fowl personality explained a significant proportion of cloacal microbiota variation.

More exploratory fowl, regardless of sex, harboured a significantly higher cloacal total ASV richness after mating. Being more exploratory may aid individuals in forming social connections (Moyers et al., 2018) and securing more matings. The nine experimental groups investigated in this study were a subset of 24 mating groups analysed by Roth (2019) who found that in female-biased groups, faster- and slower-exploring males secured significantly more matings than intermediately-exploring males. However, in our subset—where we did not

control for the effect of group sex ratio on the relationship between mating behaviour and personality—we found no significant effect of exploratory behaviour on mating performance. Roth (2019) found that females solicited the mating attempts by more exploratory males more frequently than the courting of less exploratory males, and reported that more exploratory males spent more time interacting with the females—e.g. they showed higher harassment during courtship than less exploratory males. These differences in female preference and/or male strategy could have somehow influenced their cloacal total ASV richness, although the exact mechanism of how this would happen is unclear. Roth (2019) also detected intersexual allopreening in red junglefowl mating groups, and it is possible that it was the grooming of conspecifics that contributed to the cloacal microbiota variation along the oral-faecal-genital route (Kulkarni & Heeb, 2007).

On the other hand, bolder red junglefowl of both sexes harboured a significantly lower cloacal total ASV richness after mating. Boldness and exploratory behaviour are not correlated in red junglefowl (Favati et al., 2016; Favati et al., 2018; Roth, 2019), and neither we nor Roth (2019) found a correlation between fowl boldness and exploratory behaviour in this study population. As these are distinct personality traits, they may have contributed to the divergent results we found when analysing the covariance between the two personality traits and cloacal total ASV richness. There are few current studies exploring the association between boldness behaviour and host microbiota, however, bolder green frog (*Lithobates clamitans*) tadpoles were found to acquire more bacteria from their conspecifics (Keiser et al., 2019). Considering that our results seem to suggest a different pattern, i.e. bolder individuals lose cloacal bacterial richness despite mating more frequently and securing more partners, we cannot conclude much about the effect of boldness behaviour on fowl cloacal microbiota.

Previous studies in wild baboons (Tung et al., 2015), ring-tailed lemurs (Bennett et al., 2016), Verreaux's sifakas (Perofsky et al., 2017), and red-bellied lemurs (Raulo et al., 2018)

have found that social group membership affects and predicts host faecal bacterial composition. Less is known about how sexual interactions may influence microbiota differences across groups. Our results suggest some association between intra-group mating interactions and increased between-group cloacal microbiota differences. However, these patterns were predominantly driven by considerable beta dispersion variation between the experimental groups and not by group membership.

We did not find significant within-group positive correlations between the strength of mating interaction and cloacal microbiota composition similarity, nor did we detect within-group cloacal microbiota homogenising due to intra-group mating. A considerable amount of cloacal microbiota variation across the study was explained by bird ID, which is a common finding in host-microbiota studies (Laforest-Lapointe, Messier & Kembel, 2016; Antwis et al., 2018), but may have affected our interpretation of results. Additionally, difficult to disentangle within-group patterns may have been due to the short amount of time the fowl spent in the experimental groups, but also because the fowl were re-used across the mating trials and had been mating with each other in previous breeding seasons in the context of the larger captive population. Mating frequently and with multiple partners may lead to a shared cloacal microbiota on the population level, diminishing within-pair and within-experimental group similarities. In support of this, socially- and genetically-monogamous black-legged kittiwakes were found to harbour significantly similar within-pair cloacal bacterial compositions (White et al., 2010), while socially monogamous-, yet genetically-polygynous North American tree swallows shared a population-wide cloacal microbiota (Hernandez et al., 2020).

Our population-wide investigation revealed a non-significant trend, suggesting that the fowl that mated more frequently with each other may also become more similar in their cloacal microbiota. The captive population shared a homogenous cloacal bacterial composition, and individuals that secured fewer partners became more heterogenous from the rest of the birds.

In support of our results, socio-sexual interactions in a wild population of Australian sleepy lizards (*Tiliqua rugosa*) also revealed that increased connectedness between individuals led to an increased number of shared cloacal bacterial strains in comparison with individuals that were less connected in the social network of lizards (Bull, Godfrey & Gordon, 2012).

If population-level shared microbiota provides a benefit (Lombardo et al., 1999; Smith & Mueller, 2015; Rowe et al., 2020a), individuals that are failing to secure matings at the periphery of social groups are likely to suffer from fitness costs. It has even has been hypothesised that sociality and the sharing of bacteria may provide an evolutionary advantage via exposing group-living hosts to small doses of bacteria, thus priming and enhancing their immune responses for future infections (Ezenwa et al., 2016).

On the other hand, if an STI is spreading in the population, individuals that mate with fewer partners may have an advantage (Ashby & Gupta, 2013). In support of this, socially less connected gidgee skinks (*Egernia stokesii*) were found to have fewer ticks and blood parasites (Godfrey et al., 2009). The hosts would thus benefit from being able to distinguish between healthy and pathogen-carrying partners, suggesting that sexually-transmitted bacteria may partake in pre-copulatory sexual selection (Sheldon, 1993; Lombardo, 1998; Nunn, 2003; Beltran-Bech & Richard, 2014).

At first glance, females that secured more matings had a pre-existing high abundance of chicken pathogens, such as *Escherichia/Shigella* (Shi et al., 2014) and *Clostridium_sensu_stricto_1* (phylum Firmicutes; Oakley et al., 2014), and significantly less cloacal bacteria that are traditionally thought of as mutualists in fowl, such as *Lactobacillus* (phylum Firmicutes; Messaoudi et al., 2011; Choi, Kim & Cha, 2014) and *Bifidobacterium* (phylum Actinobacteria; Shang et al., 2018; Figure 5). *Shigella* causes shigellosis in chickens, which manifests in a symptomatic cloacal discharge (Shi et al., 2014) that clearly indicates the health status of the bird to the human eye. Considering that shigellosis can be lethal in chickens

(Shi et al., 2014), birds would benefit from avoiding infected individuals (Sheldon, 1993; Lombardo, 1998). Despite harbouring a seemingly "unhealthy" cloacal microbiota, these females were able to secure more matings, suggesting that birds are unable to distinguish between healthy and symptomatic fowl, or that these specific pathogens may have smaller fitness costs than expected. Similarly, empirical studies in milkweed leaf beetles (*Labidomera clivicollis*; Abbot & Dill, 2001) and decorated crickets (*Gryllodes sigillatus*; Luong & Kaya, 2005) also failed to show host preference for uninfected partners. STIs often follow differential infection pathways and patterns than other infectious disease—for example, STIs tend to have longer infectious periods and lower mortality rates in comparison with other infectious diseases, and predominantly affect the fertility rates of the host (Lockhart, Thrall & Antonovics, 1996)—and thus, may elicit varying host behaviour.

Both females and males that secured more matings in the mating trials had a pre-existing higher abundance of *Romboutsia* on their cloacae in comparison with individuals that secured fewer matings. *Romboutsia* is a newly-established bacterial genus (Gerritsen et al., 2014) that has so far been associated with digestive tracts of healthy hosts, which have an ample amount of carbohydrates, amino acids, and vitamins available as an energy source for bacteria (Gerritsen et al., 2014; Gerritsen, 2015; Ricaboni et al., 2016). *Romboutsia* has probiotic benefits in mouse and human gastrointestinal tracts, and has been found to compete with mucus-degrading pathogens (Gerritsen, 2015). As such, a higher cloacal abundance of *Romboutsia* may be beneficial in the red junglefowl and an indicator of "healthy" cloacal microbiota.

At the population-level, we detected a lower abundance of *Romboutsia* on the cloacae of individuals that mated with more partners, which may suggest that mating compromises the abundance of potentially beneficial bacteria, such as *Romboutsia*. Indeed, based on the samples collected before the mating trials, *Romboutsia*—but also *F. mortiferum*—was more abundant

on the cloacae of birds that went on to mate with more partners in the mating population. Taking into account that red junglefowl perform pre-copulatory sexual selection (Pizzari & McDonald, 2019), we encourage further investigation into the relationship between reproductive bacteria and mate choice (Beltran-Bech & Richard, 2014), but also the sexual bacterial transmission dynamics in host socio-sexual networks.

Takeaways

- Non-virgin birds harboured a higher abundance of pathogenic *Corynebacterium* and *Actinomyces* (phylum Actinobacteria) than virgin birds, but it remains unknown whether these bacterial genera are truly sexually-transmitted in fowl
- Sex-specific differences in cloacal microbiota were evident both before and after the mating trials
- Role of fowl personality in mating behaviour and sexual bacterial transmission is complex: variation in boldness and exploratory personality did not affect cloacal microbiota before the first matings of the breeding season, but had a significant effect after the end of mating trials
- Mating interactions within the fowl population, but not within experimental groups, led to a shared homogenous cloacal bacterial composition, indicating the potential for sexual bacterial transmission in polygynandrous systems
- We encourage further investigation into the role of both mutualistic and pathogenic sexually-transmitted bacteria in host mate choice and host socio-sexual group structure

References

Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

Appendices

Supplementary Methods 1. Assigning boldness and exploratory scores to individual red junglefowl as executed and described by Roth (2019) in their thesis. The methods have been shortened.

Individual fowl were subjected to two behavioural assays, including (1) a novel object test to investigate individual boldness behaviour and (2) a novel arena test to investigate individual exploratory behaviour. Both assays were based on the methods described by Zidar et al. (2017) with some modifications. Before each assay, individual fowl were placed in a 45.5 × 26.5 × 33.5 cm cardboard box for five minutes to standardise individual stress response induced by catching the birds. All assays were video-recorded with a Motorola Nexus 6 64GB smartphone and the videos were replayed to measure individual behaviour. Statistical analyses were carried out in R (R Core Team, 2018; Version 3.3.2). The first axis of a principal component analysis (PCA) performed using princomp() was used to summarise the different behaviours that were recorded in the (1) novel object and (2) novel arena assays. Female and male birds were analysed and assigned behavioural scores (normalised via z-transformation) separately.

(1) Individual boldness scores were recorded in April–September 2016 and March–August 2017. Five consecutive days before the start of each assay, a taupe-coloured reward bowl with mealworms was placed inside the birds' home pens from which all experimental individuals were recorded to eat. Individual fowl were assayed in a familiar 3.08 × 6.66 m pen with gravel grounding, which was divided in half on the long edge using a mesh net that was familiar to the birds. Pens that were adjacent to the experimental pen were visually isolated with forest green or royal blue tarpaulin that the experimental birds were familiar with. The reward bowl was placed 50 cm from one pen corner and angled to make the mealworms visible to the bird upon their exit from the cardboard box. A 0.5 m radius was marked around the bowl, using small twigs on the ground and beige taping on the pen walls. A starting line was marked with tape on the pen walls in the diagonal corner (88 cm from the corner). Each cardboard box was placed on the starting line, opened, and the birds were allowed to exit the box upon which time recording began. Hesitant birds were encouraged to exit by tilting the box. Each assay lasted for 20 minutes and consisted of two parts carried out across two consecutive days: the baseline test without a novel object and a test with the novel object. The first trial aimed to control for inter-individual differences that were unrelated to the novel object. Per bird measurements included (to the nearest second): latency to reach 0.5 m from the reward bowl, latency to reach one body length from the reward bowl, and latency to feed from the reward bowl, both without and with a novel object. The novel object, which was kept constant in all experiments, was a yellow and black rubber duck (10 × 8.5 × 10 cm) that was placed 2 cm to the left of the reward bowl. Individual fowl were allowed 20 minutes in the experimental pen. If they failed to reach the reward bowl and/or feed from it, they were recorded to take 20 minutes to perform the task. All experimental birds were assayed three to four times. All assays were carried out between three to five and a half hours after sunrise to ensure individuals were fed and satiated in a standardised way to avoid hunger motivating their approach towards mealworms. The order of experimental birds in the baseline and novel object assays were randomised in the first trial, reversed in the second, and randomised again in the third and fourth trial. The first two trials were one month apart, the third trial was conducted two to four months after the second trial, and the fourth trial was carried out at the start of the subsequent breeding season, which was approximately a year after the first trial. 0.595 of variance in females and 0.533 variance in males was explained by PC1, and these results were moderately repeatable (interclass correlation) for both females (repeatability=0.355, 95% CI=0.050–0.470) and males (repeatability=0.226, 95% CI=-0.027–0.246). The PC1 values were used to calculate

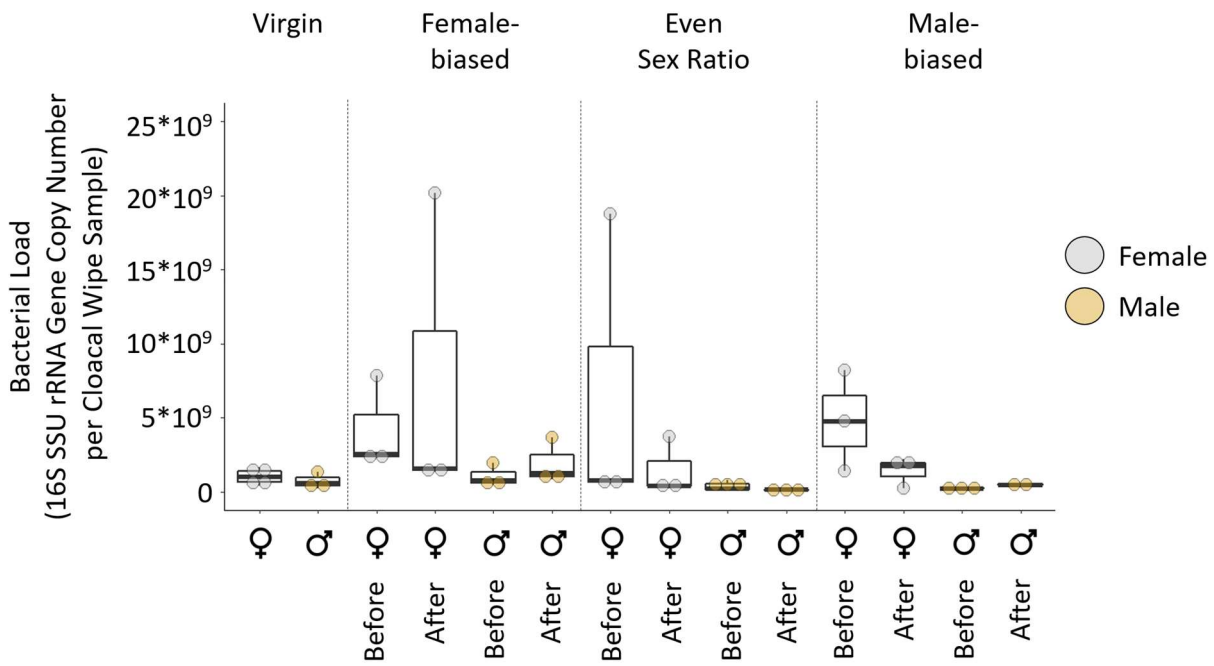
individual boldness scores, with individuals with higher scores having lower latencies in the novel object assay and were thus considered bolder. For binary calculations, bolder individuals had a >0 score and shyer individuals a <0 score.

(2) Individual exploratory scores were recorded in March–September 2016 and March–August 2017. Individual birds were tested in a novel indoor room (4.00 × 3.25 m) that contained five artificial trees (1.54 m) to facilitate exploration. The floor of the novel arena was divided into nine equal quadrats marked with beige tape. In all assays, the focal bird was placed in the middle quadrat, which was right of the central artificial tree, with the lights off. Each assay lasted for 30 minutes, and time recording started once the experimenter had left the room and turned on the lights. Assays were video-recorded from a one-way window. Per bird measurements included (to the nearest second): latency to "unfreeze" (defined as taking three consecutive steps after the room was lit), latency to leave the starting quadrat (defined as at least one foot outside the starting quadrat), latency to visit six quadrats, the total number of unique quadrats visited, and the number of transitions made between quadrats (defined as the number of times the bird moved from one quadrat to the other, placing at least one foot into a neighbouring quadrat). Birds that did not "unfreeze", did not leave the starting quadrat, and/or visited fewer than six unique quadrats were recorded to take 30 minutes to perform the task. All experimental birds were assayed three to four times. All assays were carried out in the morning (starting from half an hour after sunrise to three hours after sunrise) and evening (starting from three hours before sunset until sunset) to capture the red junglefowl daily activity peaks (Løvlie & Pizzari, 2007). Birds that were tested in the morning in the first trial were tested in the evening in the second trial, and *vice versa*. Trial time was randomised across individuals in the third and fourth trial. The first two trials were 10 days apart and were carried out prior to joining the mating group study (Roth, 2019). The third trial was conducted three to five months after the second trial and after the mating group study (Roth, 2019) had begun. The fourth trial was carried out at the beginning of the subsequent breeding season, which was approximately a year after the first trial. 0.822 of variance in females and 0.716 variance in males was explained by PC1, and these results were moderately repeatable for both females (repeatability=0.479, 95% CI=0.260–0.554) and males (repeatability=0.538, 95% CI=0.351–0.591). The PC1 values were used to calculate individual exploratory scores, using the intercept coefficient together with an individual bird parameter taken from a generalised linear model (controlling for bird ID, age, trial number, days since March 1st (exploratory score may change across the breeding season), and whether it was a morning or evening trial). Individuals with higher scores had lower latencies in the novel arena assay, visited more quadrats, and/or made more transitions between quadrats, and were thus considered more exploratory. For binary calculations, more exploratory individuals had a >0 score and less exploratory individuals a <0 score.

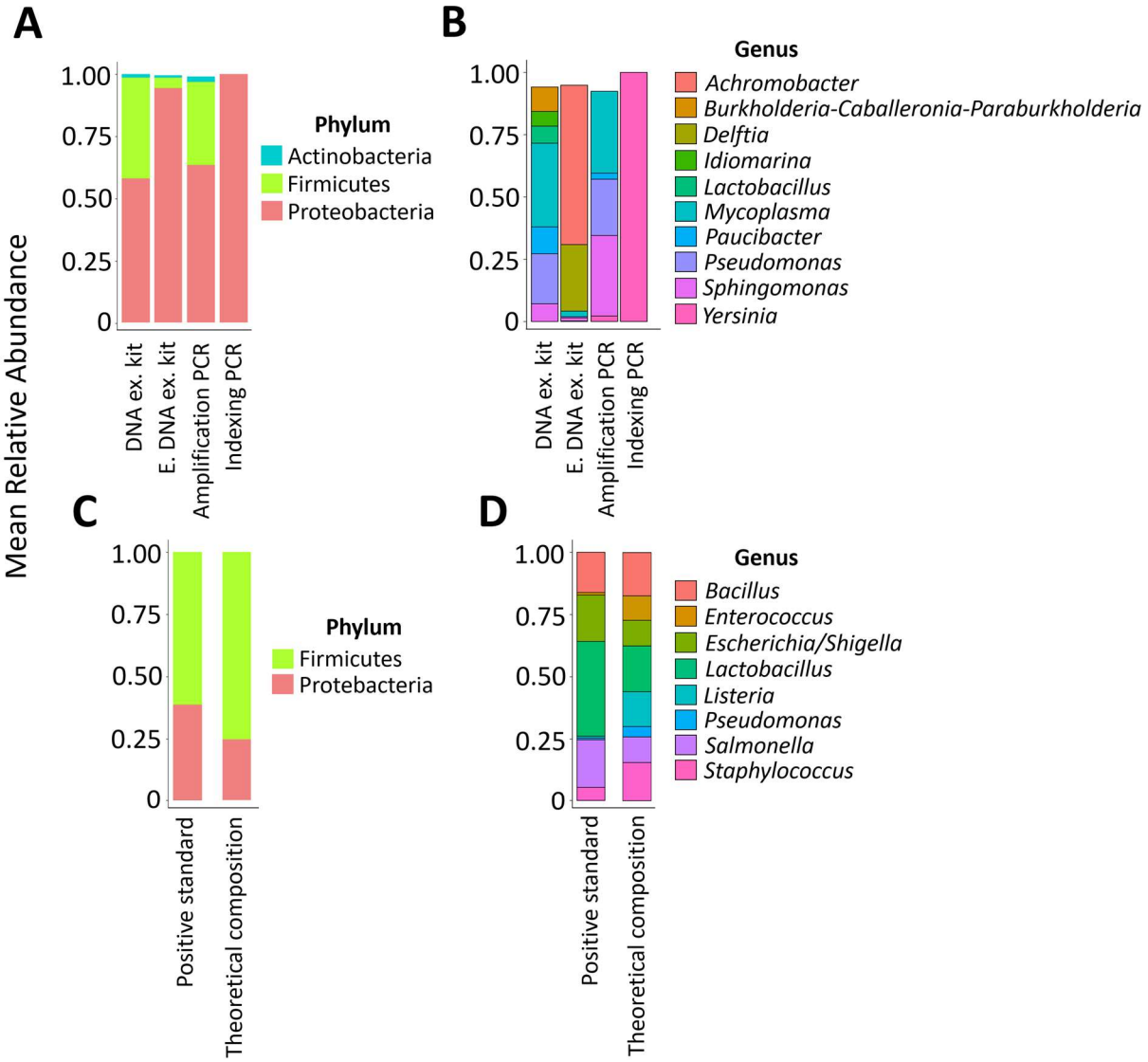
The group composition of fowl exploratory behaviour was experimentally manipulated across the mating group study (Roth, 2019) to allow different compositions of more and less exploratory females and males within each experimental group of different sex ratio. The manipulated compositions were based on the mean values of individual exploratory scores of the first two novel arena trials.

Supplementary Table 1. Primer sequences (5'–3' orientation) used in the amplification and indexing PCRs. For clarity, the functional regions of each primer have been separated by a "+". The locus-specific sequence is based on the Earth Microbiome Project (Thompson et al., 2017). The forward (515F; Parada, Needham & Fuhrman, 2016) and reverse primer (806R; Apprill et al., 2015) cover the V4 hypervariable region (~254 base pairs) of the bacterial 16S SSU rRNA gene. The adapter used for trimming is indicated in bold. The paired-end index combinations were based on Kozich et al. (2013).

Amplification PCR primers	
	Illumina Nextera-compatible overhang + locus-specific sequence
515F	TCGTCGGCAGCGTC**AGATGTGTATAAGAGACAG** + GTGYCAGCMGCCGCGGTAA
806R	GTCTCGTGGGCTCGG**AGATGTGTATAAGAGACAG** + GGACTACNVGGGTWTCTAAT
Indexing PCR primers	
	Flow cell adapter + index + Illumina Nextera-compatible overhang
SA501	AATGATACGGCGACCACCGAGATCTACAC + ATCGTACG + TCGTCGGCAGCGTC
SA502	AATGATACGGCGACCACCGAGATCTACAC + ACTATCTG + TCGTCGGCAGCGTC
SA503	AATGATACGGCGACCACCGAGATCTACAC + TAGCGAGT + TCGTCGGCAGCGTC
SA504	AATGATACGGCGACCACCGAGATCTACAC + CTGCGTGT + TCGTCGGCAGCGTC
SA505	AATGATACGGCGACCACCGAGATCTACAC + TCATCGAG + TCGTCGGCAGCGTC
SA506	AATGATACGGCGACCACCGAGATCTACAC + CGTGAGTG + TCGTCGGCAGCGTC
SA507	AATGATACGGCGACCACCGAGATCTACAC + GGATATCT + TCGTCGGCAGCGTC
SA508	AATGATACGGCGACCACCGAGATCTACAC + GACACCGT + TCGTCGGCAGCGTC
SB501	AATGATACGGCGACCACCGAGATCTACAC + CTACTATA + TCGTCGGCAGCGTC
SB502	AATGATACGGCGACCACCGAGATCTACAC + CGTTACTA + TCGTCGGCAGCGTC
SB503	AATGATACGGCGACCACCGAGATCTACAC + AGAGTCAC + TCGTCGGCAGCGTC
SB504	AATGATACGGCGACCACCGAGATCTACAC + TACGAGAC + TCGTCGGCAGCGTC
SB505	AATGATACGGCGACCACCGAGATCTACAC + ACGTCTCG + TCGTCGGCAGCGTC
SB506	AATGATACGGCGACCACCGAGATCTACAC + TCGACGAG + TCGTCGGCAGCGTC
SB507	AATGATACGGCGACCACCGAGATCTACAC + GATCGTGT + TCGTCGGCAGCGTC
SB508	AATGATACGGCGACCACCGAGATCTACAC + GTCAGATA + TCGTCGGCAGCGTC
SA701	CAAGCAGAAGACGGCATACGAGAT + AACTCTCG + GTCTCGTGGGCTCGG
SA702	CAAGCAGAAGACGGCATACGAGAT + ACTATGTC + GTCTCGTGGGCTCGG
SA703	CAAGCAGAAGACGGCATACGAGAT + AGTAGCGT + GTCTCGTGGGCTCGG
SA704	CAAGCAGAAGACGGCATACGAGAT + CAGTGAGT + GTCTCGTGGGCTCGG
SA705	CAAGCAGAAGACGGCATACGAGAT + CGTACTCA + GTCTCGTGGGCTCGG
SA706	CAAGCAGAAGACGGCATACGAGAT + CTACGCAG + GTCTCGTGGGCTCGG
SA707	CAAGCAGAAGACGGCATACGAGAT + GGAGACTA + GTCTCGTGGGCTCGG
SA708	CAAGCAGAAGACGGCATACGAGAT + GTCGCTCG + GTCTCGTGGGCTCGG
SA709	CAAGCAGAAGACGGCATACGAGAT + GTCGTAGT + GTCTCGTGGGCTCGG
SA710	CAAGCAGAAGACGGCATACGAGAT + TAGCAGAC + GTCTCGTGGGCTCGG
SA711	CAAGCAGAAGACGGCATACGAGAT + TCATAGAC + GTCTCGTGGGCTCGG
SA712	CAAGCAGAAGACGGCATACGAGAT + TCGCTATA + GTCTCGTGGGCTCGG
SB701	CAAGCAGAAGACGGCATACGAGAT + AAGTCGAG + GTCTCGTGGGCTCGG
SB702	CAAGCAGAAGACGGCATACGAGAT + ATACTTCG + GTCTCGTGGGCTCGG
SB703	CAAGCAGAAGACGGCATACGAGAT + AGCTGCTA + GTCTCGTGGGCTCGG
SB704	CAAGCAGAAGACGGCATACGAGAT + CATAGAGA + GTCTCGTGGGCTCGG
SB705	CAAGCAGAAGACGGCATACGAGAT + CGTAGATC + GTCTCGTGGGCTCGG
SB706	CAAGCAGAAGACGGCATACGAGAT + CTCGTTAC + GTCTCGTGGGCTCGG
SB707	CAAGCAGAAGACGGCATACGAGAT + GCGCACGT + GTCTCGTGGGCTCGG
SB708	CAAGCAGAAGACGGCATACGAGAT + GGTACTAT + GTCTCGTGGGCTCGG
SB709	CAAGCAGAAGACGGCATACGAGAT + GTATACGC + GTCTCGTGGGCTCGG
SB710	CAAGCAGAAGACGGCATACGAGAT + TACGAGCA + GTCTCGTGGGCTCGG
SB711	CAAGCAGAAGACGGCATACGAGAT + TCAGCGTT + GTCTCGTGGGCTCGG
SB712	CAAGCAGAAGACGGCATACGAGAT + TCGCTACG + GTCTCGTGGGCTCGG



Supplementary Figure 1. Box plots depicting the 16S SSU rRNA gene copy number per cloacal wipe sample collected from female (n=4) and male virgin fowl (n=3), and female and male fowl immediately before and after joining experimental groups of different sex ratios (n=3 per category; bacterial DNA from one male cloacal wipe sample that was collected after exiting a male-biased experimental group (bird ID: D3; group ID: MB20) failed to amplify). Dots represent individual cloacal wipe samples (n=42). Each box plot has an indicated median and interquartile range. The tips depict the minimum and maximum values.



Supplementary Figure 2. Stacked bar plots depicting the mean relative abundances of bacterial phyla (A & C) and genera (B & D) detected in the negative controls (A & B) and the positive standard (C & D). Only taxa that contributed >1% are presented. Mean relative abundances per negative control type (A & B) were calculated using the post-*decontam* untrimmed contaminants-only data set. Positive standard relative abundances (C & D) were calculated using the pre-*decontam* untrimmed data set. Lowering the inclusion threshold to 0.1% was necessary to detect the genera *Listeria* and *Pseudomonas* in the positive standard (D). Negative controls (A & B) are a sample of the QIAGEN DNeasy® Blood & Tissue DNA extraction kit (DNA ex. kit; n=1), a sample of the *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (E. DNA ex. kit; n=1), amplification PCR negatives (n=12), and an indexing PCR negative (n=1). The positive standard (C & D) is a single ZymoBIOMICS® Microbial Community Standard (n=1). For comparison purposes, the theoretical composition of the ZymoBIOMICS® Microbial Community Standard expected by the manufacturer is presented together with the positive standard (C & D). No correction was made to the data set based on the observed and expected positive standard relative abundances. Each bacterial taxon is indicated by a unique colour and genera are separated by black lines for clarity.

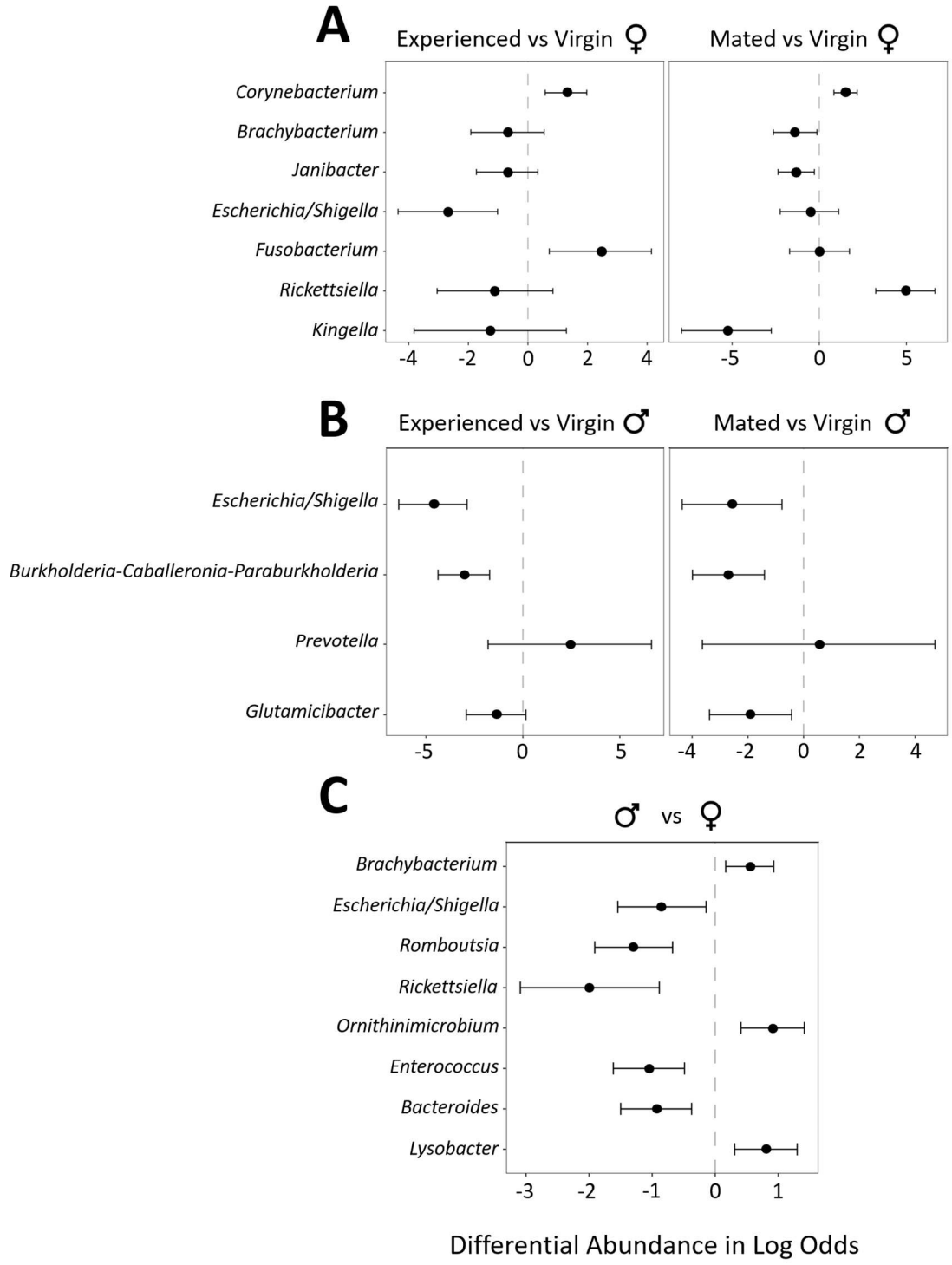
Supplementary Table 2. [PERMANOVA] Cloacal microbiota composition comparisons across category ($n_{virgin\ female}$=4, $n_{experienced\ female}$=19, $n_{mated\ female}$=19, $n_{virgin\ male}$=4, $n_{experienced\ male}$=17, $n_{mated\ male}$=17, n_{soil}=1, or n_{water}=1) at bacterial phylum (A) and genus (B) level, using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarities (DS) and Jaccard indices. Permutations (=1000) were constrained within strata=*bird ID* (n=44). Significant p-values are in bold. Beta dispersion tests (permutations=1000) revealed significant dispersion variation across category for all β-diversity metrics at the genus level ($p<0.05$; with the exception of genus-level wUF distances), but not at the phylum level ($p>0.05$).

A

Phylum-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Category	**3.00**	**7**	**0.011**	**16.7%**
Bird ID	**1.76**	**40**	**0.009**	**56.2%**
Residuals		34		27.1%
Unweighted UniFrac				
Category	**2.64**	**7**	**<0.001**	**16.2%**
Bird ID	**1.54**	**40**	**0.004**	**54.0%**
Residuals		34		29.8%
Bray-Curtis DS				
Category	**3.07**	**7**	**0.009**	**16.7%**
Bird ID	**1.84**	**40**	**0.070**	**57.0%**
Residuals		34		26.3%
Jaccard Index				
Category	**2.37**	**7**	**<0.001**	**15.0%**
Bird ID	**1.48**	**40**	**<0.001**	**54.1%**
Residuals		34		30.9%

B

Genus-level	pseudo-F	df	p-value	R^2
Weighted UniFrac				
Category	**2.46**	**7**	**0.002**	**15.2%**
Bird ID	**1.55**	**40**	**0.004**	**54.7%**
Residuals		34		30.1%
Unweighted UniFrac				
Category	**2.77**	**7**	**<0.001**	**17.8%**
Bird ID	**1.38**	**40**	**<0.001**	**50.9%**
Residuals		34		31.3%
Bray-Curtis DS				
Category	**2.95**	**7**	**<0.001**	**18.5%**
Bird ID	**1.43**	**40**	**<0.001**	**51.1%**
Residuals		34		30.4%
Jaccard Index				
Category	**2.60**	**7**	**<0.001**	**17.6%**
Bird ID	**1.28**	**40**	**<0.001**	**49.5%**
Residuals		34		32.9%



Supplementary Figure 3. Differential abundance (in log odds) of bacterial genera on female (A) and male fowl cloacae (B) compared across the mating experience subgroups (virgin, experienced, or mated); and on male cloacae compared to females (C), controlling for the mating experience subgroup. The grey dashed line represents the point of comparison, which is either the cloacal bacterial abundance in virgin females (A) or virgin males (B), or the female cloacal bacterial abundance (C). Data points with standard error bars depict log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds indicate the significant under-representation of the bacterial genus.

Supplementary Table 3. [ANOVA] Covariation between mating experience subgroup (n_{virgin}=8, $n_{experienced}$=36, or n_{mated}=36), sex (n_{female}=42 or n_{male}=38), their interaction, age, and cloacal total ASV richness estimates (LMM; A), and Shannon's (GLMM; B) and inverse Simpson's diversity index estimates (GLMM; C). Type III Wald F-test with Kenward-Roger degrees of freedom (A) and type III Wald chi-square tests (B & C) were used for the deviance analyses of the fixed effects. Significant p-values are in bold. It is worth mentioning that bird ID had a considerable effect on total ASV richness estimates (S.D.=141.90), but a small effect on Shannon's (S.D.=0.18) and inverse Simpson's diversity index estimates (S.D.=0.03).

A

Total ASV Richness	F	df	p
Mating experience	2.68	2, 50.0	0.078
Sex	0.29	1, 47.8	0.595
Mating experience*Sex	**3.98**	**2, 51.8**	**0.025**
Age	0.47	1, 36.4	0.498

B

Shannon's Diversity	χ^2	df	p
Mating experience	0.36	2	0.834
Sex	0.02	1	0.877
Mating experience*Sex	1.20	2	0.548
Age	0.05	1	0.825

C

Inverse Simpson's Diversity	χ^2	df	p
Mating experience	1.37	2	0.504
Sex	0.85	1	0.358
Mating experience*Sex	0.83	2	0.660
Age	0.01	1	0.928

Supplementary Table 4. [ANOVA] Covariation between fowl sex (n_{female}=20 or n_{male}=26), age class (n_{young}=29 or n_{old}=17), boldness and exploratory scores, and cloacal total ASV richness estimates (LM; A), and Shannon's (GLM; B) and inverse Simpson's diversity index estimates (GLM; C) before mating for the first time in the breeding season. Type II F-test (A) and type II Wald chi-square tests (B & C) were used for the deviance analyses of the fixed effects.

A

Total ASV Richness	F	df	p
Sex	1.63	1, 41	0.208
Age class	1.48	1, 41	0.230
Boldness score	0.29	1, 41	0.590
Exploratory score	2.22	1, 41	0.144

B

Shannon's Diversity	χ^2	df	p
Sex	0.48	1	0.487
Age class	0.83	1	0.362
Boldness score	1.68	1	0.195
Exploratory score	0.31	1	0.581

C

Inverse Simpson's Diversity	χ^2	df	p
Sex	0.63	1	0.426
Age class	1.01	1	0.316
Boldness score	2.54	1	0.111
Exploratory score	0.41	1	0.523

Supplementary Table 5. [ANOVA] Covariation between fowl age class (n_{young}=69 or n_{old}=39), boldness and exploratory scores, α-diversity, and the number of partners (LMM; A–C) and matings (LMM; D–F) the fowl would go on to secure in the mating trials. Total ASV richness (A, D), Shannon's (B, E), and inverse Simpson's diversity (C, F) were modelled separately due to strong correlations between the three α-diversity metrics. Type II Wald F-tests with Kenward-Roger degrees of freedom were used for the deviance analyses of the fixed effects. Significant p-values are in bold. The AIC_C score and the random effect *bird ID* (n=57) *standard deviation* are indicated per model.

A

Number of Partners	F	df	p
Age class	0.72	1, 49.5	0.399
Boldness score	**4.03**	**1, 52.9**	**0.050**
Exploratory score	0.03	1, 45.5	0.859
Total ASV richness	0.03	1, 97.1	0.854
AIC_C=314.92		*bird $ID_{S.D.}$=0.56*	

B

Number of Partners	F	df	p
Age class	0.75	1, 49.3	0.391
Boldness score	**4.10**	**1, 52.6**	**0.048**
Exploratory score	0.04	1, 45.1	0.884
Shannon's diversity	<0.01	1, 91.8	0.948
AIC_C=304.48		*bird $ID_{S.D.}$=0.55*	

C

Number of Partners	F	df	p
Age class	0.73	1, 49.2	0.398
Boldness score	3.92	1, 52.9	0.053
Exploratory score	0.02	1, 45.2	0.877
Inverse Simpson's diversity	0.58	1, 90.1	0.448
AIC_C=309.37		*bird $ID_{S.D.}$=0.55*	

D

Number of Matings	F	df	p
Age class	0.19	1, 49.3	0.663
Boldness score	**5.75**	**1, 52.8**	**0.020**
Exploratory score	0.33	1, 45.3	0.568
Total ASV richness	0.02	1, 97.9	0.869
AIC_C=100.65		*bird $ID_{S.D.}$=0.19*	

E

Number of Matings	F	df	p
Age class	0.20	1, 49.3	0.674
Boldness score	**5.72**	**1, 52.5**	**0.020**
Exploratory score	0.36	1, 45.0	0.553
Shannon's diversity	0.03	1, 92.3	0.859
AIC_C=90.17		*bird $ID_{S.D.}$=0.19*	

F

Number of Matings	F	df	p
Age class	0.18	1, 49.2	0.673
Boldness score	**5.66**	**1, 52.8**	**0.021**
Exploratory score	0.36	1, 45.1	0.551
Inverse Simpson's diversity	0.02	1, 90.5	0.888
AIC_C=95.67		*bird $ID_{S.D.}$=0.19*	

Supplementary Table 6. [ANOVA] Covariation between prior cloacal α-diversity, sex (n_{female}=54 or n_{male}=54), sex ratio ($n_{female\text{-}biased}$=36, $n_{even\ sex\ ratio}$=36, or $n_{male\text{-}biased}$=36), their interaction, number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=69 or n_{old}=39), boldness and exploratory scores, and cloacal total ASV richness (LMM; A), Shannon's (GLMM; B), and inverse Simpson's diversity (GLMM; C) after the mating trials. Type III Wald F-test with Kenward-Roger degrees of freedom (A) and type III Wald chi-square tests (B & C) were used for the deviance analyses of the fixed effects. Significant p-values are in bold. The random effect *group ID* (n=9) had a considerable effect on total ASV richness estimates (S.D.=72.53), but a negligible effect on Shannon's (S.D.<0.01) and inverse Simpson's diversity index estimates (S.D.<0.01).

A

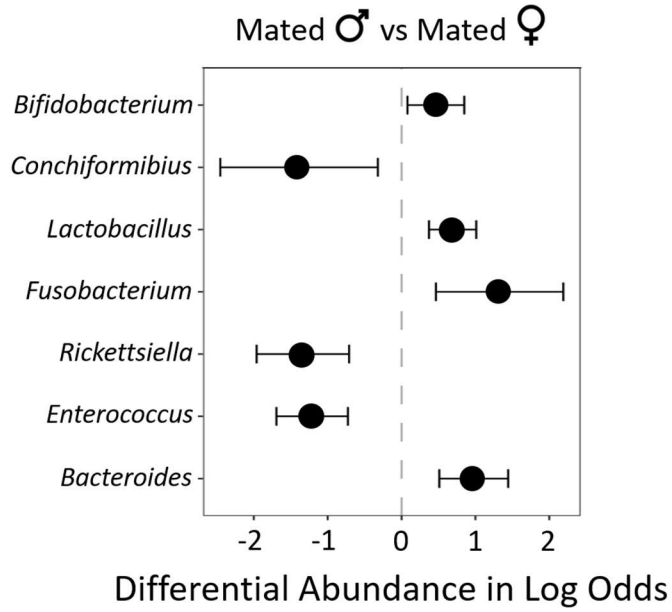
Total ASV Richness	F	df	p
Total ASV richness before	0.16	1, 93.1	0.693
Sex	0.93	1, 89.2	0.338
Sex ratio	0.13	2, 6.8	0.884
Sex*Sex ratio	1.40	2, 89.5	0.253
# of partners	0.50	1, 92.1	0.483
# of matings	2.85	1, 83.9	0.095
Eigenvector centrality	1.48	1, 93.0	0.227
Betweenness	0.10	1, 88.9	0.750
Age class	1.75	1, 90.8	0.190
Boldness score	**4.58**	**1, 91.4**	**0.035**
Exploratory score	**5.47**	**1, 90.3**	**0.022**

B

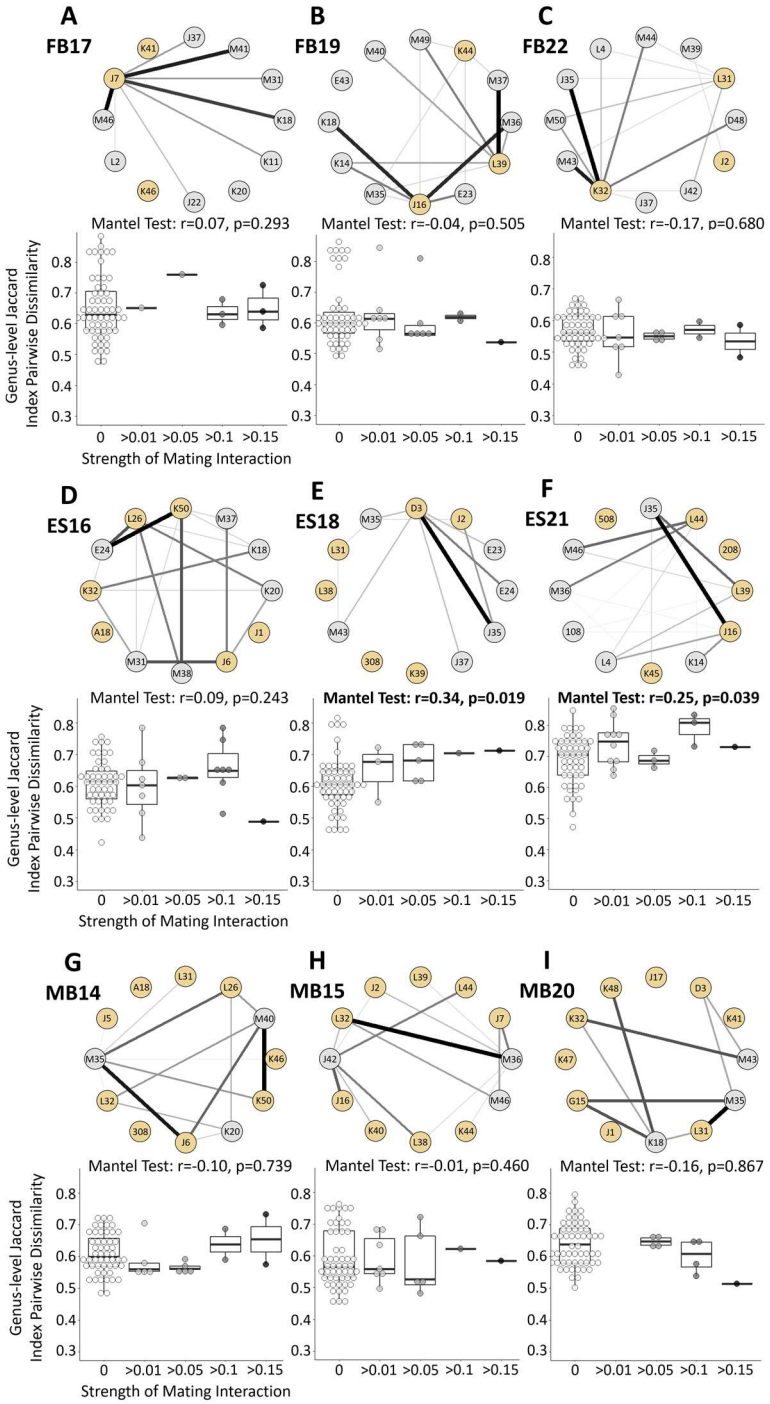
Shannon's Diversity	χ^2	df	p
Shannon's diversity before	0.14	1	0.708
Sex	1.00	1	0.318
Sex ratio	0.28	2	0.871
Sex*Sex ratio	2.30	2	0.317
# of partners	0.10	1	0.751
# of matings	0.98	1	0.321
Eigenvector centrality	0.25	1	0.619
Betweenness	<0.01	1	0.956
Age class	0.30	1	0.586
Boldness score	0.42	1	0.515
Exploratory score	2.62	1	0.105

C

Inverse Simpson's Diversity	χ^2	df	p
Inverse Simpson's diversity before	1.46	1	0.226
Sex	**10.77**	**1**	**0.001**
Sex ratio	0.91	2	0.636
Sex*Sex ratio	4.85	2	0.088
# of partners	0.30	1	0.581
# of matings	1.15	1	0.283
Eigenvector centrality	0.01	1	0.916
Betweenness	0.01	1	0.913
Age class	0.80	1	0.372
Boldness score	0.53	1	0.467
Exploratory score	3.70	1	0.054



Supplementary Figure 4. Differential abundance (in log odds) of bacterial genera on male cloacae (n=54) after the mating trials compared to female fowl cloacae (n=54), while controlling for the differential bacterial abundance and variance across group ID (n=9), number of partners ($n_{fewer\ partners}$=71 or $n_{more\ partners}$=37) and matings ($n_{mated\ less}$=69 or $n_{mated\ more}$=39), age class (n_{young}=69 or n_{old}=39), and fowl boldness (n_{shyer}=69 or n_{bolder}=39) and exploratory behaviour group ($n_{less\ exploratory}$=49 or $n_{more\ exploratory}$=59). The grey dashed line represents the point of comparison, i.e. the female red junglefowl cloacal microbiota after the mating trials. Data points with standard error bars depict log odds estimates of the specific genera. Positive log odds indicate significant enrichment, while negative log odds the significant under-representation of the bacterial genus.



Supplementary Figure 5. Strength of mating interactions networks of nine red junglefowl experimental groups of different sex ratios, including female-biased (A-C), even sex ratio (D-F), and male-biased (G-I) groups. Nodes represent individual birds (female=grey or male=beige) with IDs. The thicker and darker the edge, the stronger the mating interaction. The box plots depict the genus-level Jaccard index pairwise dissimilarities across the different strength of mating interaction categories. All box plots have an indicated median and interquartile range, with the tips depicting the minimum and maximum values. Outliers are included. Each dot is a pairwise cloacal microbiota composition comparison between two birds after exiting an experimental group (n=132 per group). The colour of the dot matches the colour of the edge in the network figure. Mantel test results between the distance matrices of cloacal microbiota genus-level Jaccard index dissimilarities and strength of mating interactions are indicated per experimental group. Significant p-values are in bold.

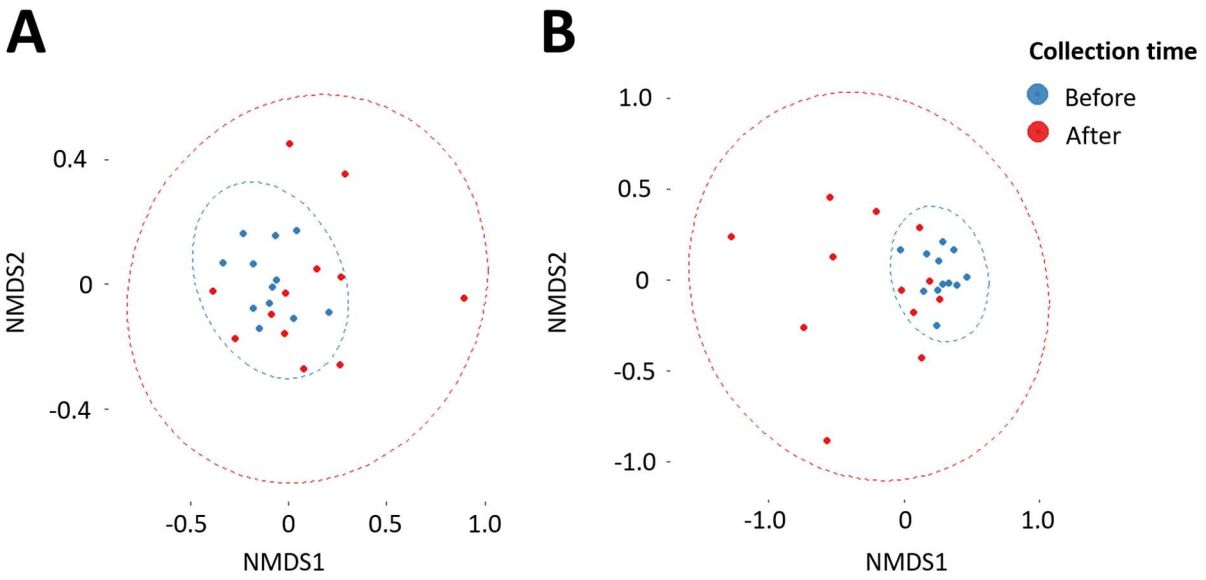
Supplementary Table 7. [PERMANOVA & beta dispersion] Cloacal microbiota composition variation in each experimental group, controlling for sex (female-biased: n_{female}=18 or n_{male}=6; even sex ratio: n_{female}=12 or n_{male}=12; male-biased: n_{female}=6 or n_{male}=18), collection time (n_{before}=12 or n_{after}=12 in each experimental group), and their interaction (A); and the effect of collection time on beta dispersion (B). Both analyses were based on genus-level Jaccard indices**.** Permutations (=1000) were constrained within strata=*bird ID* (n=12 in each experimental group). Significant p-values are in bold.

A

PERMANOVA	pseudo-F	df	p-value	R^2
Female-biased FB17				
Sex	**1.64**	**1**	**0.026**	**5.4%**
Collection time	**1.71**	**1**	**0.017**	**5.7%**
Sex*Collection time	**1.99**	**1**	**0.006**	**6.6%**
Bird ID	**1.48**	**10**	**<0.001**	**49.2%**
Residuals		10		33.1%
Female-biased FB19				
Sex	**1.51**	**1**	**0.032**	**6.0%**
Collection time	**1.80**	**1**	**<0.001**	**7.1%**
Sex*Collection time	1.08	1	0.312	4.3%
Bird ID	1.08	10	0.142	43.0%
Residuals		10		39.6%
Female-biased FB22				
Sex	1.17	1	0.192	4.9%
Collection time	**2.35**	**1**	**<0.001**	**9.8%**
Sex*Collection time	1.28	1	0.122	5.3%
Bird ID	0.92	10	0.777	38.4%
Residuals		10		41.6%
Even Sex Ratio ES16				
Sex	1.23	1	0.140	5.1%
Collection time	**2.15**	**1**	**0.004**	**9.0%**
Sex*Collection time	1.19	1	0.183	5.0%
Bird ID	0.93	10	0.784	39.0%
Residuals		10		41.9%
Even Sex Ratio ES18				
Sex	**2.21**	**1**	**0.002**	**8.0%**
Collection time	1.39	1	0.081	5.0%
Sex*Collection time	**1.19**	**1**	**0.004**	**7.2%**
Bird ID	1.99	10	0.057	43.3%
Residuals		10		36.5%
Even Sex Ratio ES21				
Sex	**2.03**	**1**	**0.006**	**7.4%**
Collection time	**3.04**	**1**	**<0.001**	**11.1%**
Sex*Collection time	1.09	1	0.272	4.0%
Bird ID	1.13	10	0.113	41.2%
Residuals		10		36.3%
Male-biased MB14				
Sex	**1.46**	**1**	**0.041**	**5.9%**
Collection time	**1.89**	**1**	**0.003**	**7.6%**
Sex*Collection time	1.82	1	0.183	4.8%
Bird ID	1.03	10	0.342	41.6%
Residuals		10		40.1%
Male-biased MB15				
Sex	1.38	1	0.060	5.3%
Collection time	**2.20**	**1**	**<0.001**	**8.4%**
Sex*Collection time	1.16	1	0.352	4.0%
Bird ID	1.05	1	0.069	44.2%
Residuals		10		38.1%
Male-biased MB20				
Sex	**1.36**	**1**	**0.036**	**5.7%**
Collection time	**1.53**	**1**	**0.007**	**6.4%**
Sex*Collection time	0.86	1	0.359	3.6%
Bird ID	1.02	10	0.789	42.6%
Residuals		10		41.7%

B

Beta Dispersion	pseudo-F	df	p-value
Female-biased FB17			
Collection time	0.68	1, 22	0.440
Female-biased FB19			
Collection time	1.47	1, 22	0.314
Female-biased FB22			
Collection time	0.16	1, 22	0.700
Even Sex Ratio ES16			
Collection time	0.06	1, 22	0.786
Even Sex Ratio ES18			
Collection time	0.10	1, 22	0.754
Even Sex Ratio ES21			
Collection time	**19.43**	**1, 22**	**0.002**
Male-biased MB14			
Collection time	1.13	1, 22	0.295
Male-biased MB15			
Collection time	0.48	1, 22	0.505
Male-biased MB20			
Collection time	**4.73**	**1, 22**	**0.036**



Supplementary Figure 6. Non-metric multidimensional scaling (NMDS) ordinations depicting the cloacal wipe samples collected before joining (n=12) and after exiting (n=12) the male-biased experimental group MB20 (A; stress=0.16) and the even sex ratio experimental group ES21 (B; stress=0.11). The ordinations are based on genus-level Jaccard indices. Each dot is a cloacal wipe sample (n=24 per experimental group). The dashed ellipses indicate the respective multivariate normal distributions.

Supplementary Table 8. [PERMANOVA] Red junglefowl population-wide cloacal microbiota composition variation after the mating trials, controlling for sex (n_{female}=28 or n_{male}=29), population-wide number of partners and matings, eigenvector centrality, betweenness, age class (n_{young}=35 or n_{old}=22), and boldness and exploratory scores of the individual, using genus-level Jaccard indices. Permutations=10,000. Significant p-values are in bold.

Genus-level	pseudo-F	df	p-value	R^2
Unweighted UniFrac				
Sex	**1.99**	**1**	**0.006**	**3.4%**
# of partners	**2.45**	**1**	**0.002**	**4.2%**
# of matings	0.81	1	0.791	1.4%
Eigenvector centrality	0.91	1	0.535	1.6%
Betweenness	0.80	1	0.817	1.4%
Age class	0.85	1	0.743	1.5%
Boldness score	1.07	1	0.267	1.8%
Exploratory score	1.10	1	0.248	1.9%
Residuals		48		82.8%

Chapter VI. General Discussion: Key Findings and Implications of the Thesis, Limitations, and Future Areas of Research

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Female and male human reproductive tracts have been found to harbour bacterial communities that not only differ from each other, but also from the microbiota of other body sites (Lozupone et al., 2013; Mändar, 2013; Chen et al., 2017; Altmäe, Franasiak & Mändar, 2019). These bacteria can be exchanged between sexual partners (Mändar et al., 2015), and transmitted along the mother-offspring axis (Mändar & Mikelsaar, 1996; Dominguez-Bello et al., 2010). Inspired by human medical studies (Hou et al., 2013; Reid, 2016; Champer et al., 2018), hypotheses have emerged about the role of reproductive microbiota in the evolutionary ecology of animal hosts (Rowe et al., 2020a). In my thesis, I investigated the ecology of reproductive tract microbiota of the polygynandrous red junglefowl, *Gallus gallus*—the main wild ancestor of the domestic chicken (*Gallus domesticus*; Kanginakudru et al., 2008; Lawal et al., 2020; Wang et al., 2020b)—and its patterns of intra- and inter-individual variation. My work was inspired by previous research on red junglefowl reproductive microbiota that had established the presence of bacteria in fowl ejaculates and on female cloacae (Eccleston, 2017). My thesis started by investigating whether bacteria also inhabit the internal reproductive tracts of female (**Chapter II**) and male birds (**Chapter III**). To do this, I developed a standardised laboratory pipeline for 16S SSU rRNA gene next-generation sequencing of low biomass samples collected from fowl reproductive tracts, which was used in all subsequent data chapters. The thesis work was then extended to study the temporal variation patterns in ejaculate microbiota across male age class, over the course of breeding season, and across a sequence of matings (**Chapter IV**). Finally, I explored the effect of mating interactions and phenotypic variation on cloacal microbiota composition in replicate experimental groups of birds (**Chapter V**). By using a well-studied, captive red junglefowl population, I had a wealth of information about the inter-individual variation between the birds at my disposal, such as variation in health status, reproductive status, age, and personality (Roth, 2019), which were accounted for in my analyses. Combined, my thesis provides a comprehensive investigation of microbiota that inhabit the reproductive tracts of polygynandrous red junglefowl, and encourages further research on the role of reproductive bacteria in host mating behaviour, and sexual selection and conflict (Rowe, et al., 2020).

Key Findings and Implications of the Thesis (Also Summarised in Figure 2)

We found that bacteria are common in both female and male red junglefowl (*Gallus gallus*) reproductive tracts, where they inhabit various niches. Across the four integrated data chapters—which characterised the female (**Chapter II**) and male red junglefowl reproductive tract microbiota (**Chapter III**), fowl ejaculate bacterial assemblages (**Chapter IV**), and female and male bird cloacal microbiota (**Chapter V**)—we detected 19,461 ASVs (Figure 1) that we were able to assign to 50 phyla and 1123 genera.

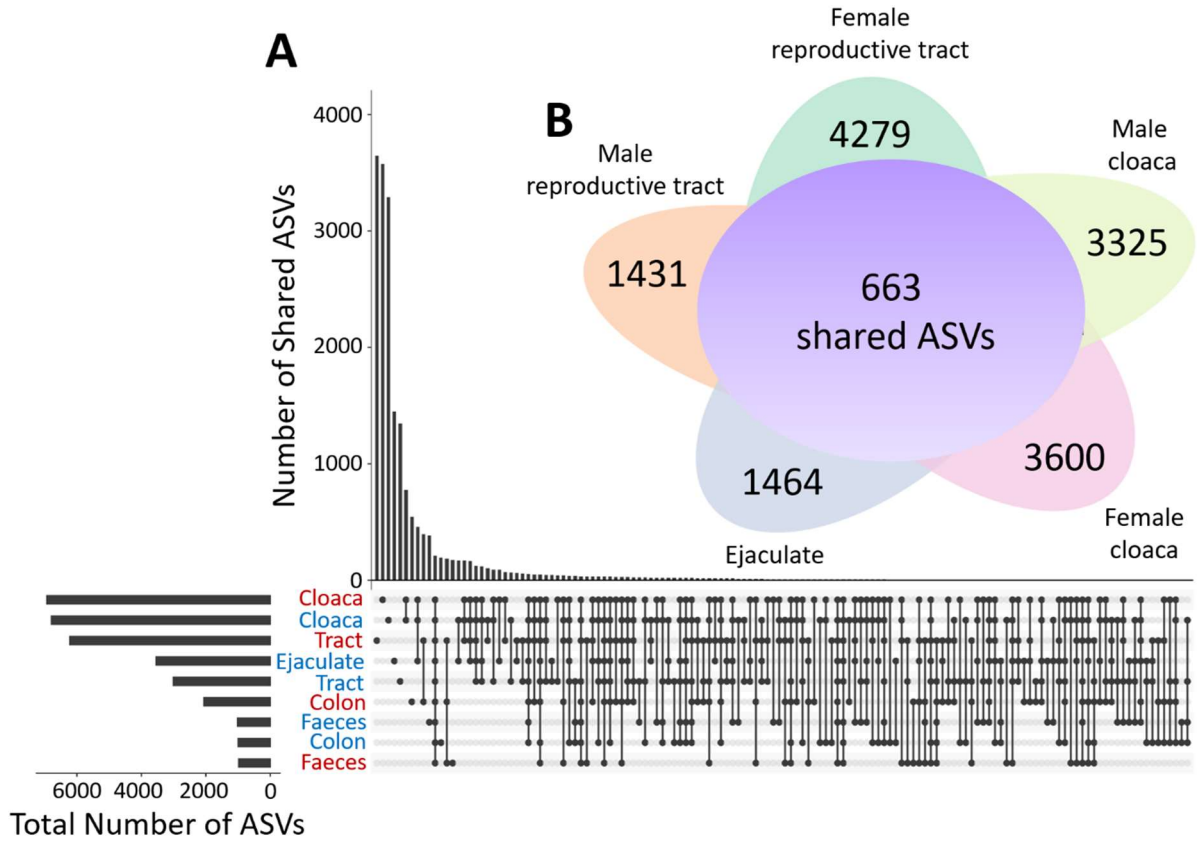

Figure 1. The number of shared ASVs across the samples processed in Chapters II–V (A), and the number of unique and universally-shared ASVs across red junglefowl reproductive microbiota (B). Female-associated samples are indicated by red and male-associated samples by blue (A). The Venn diagram (B) excludes female and male colon and faeces, but includes ASVs that were associated with colon and faecal samples, if they were also found in any of the reproductive microbiota categories. Samples associated with reproductive microbiota shared more ASVs than samples associated with gut microbiota (A). The large number of ASVs found in the female reproductive tract (A & B) may be attributed to collecting both lavage and tissue samples that allowed for a more in-depth microbiota characterisation. We used *UpSetR* (Gehlenborg, 2019; A) and *VennDiagram* (Chen, 2018; B) to create the figures in R (R Core Team, 2019; Version 3.6.1).

Whilst some of these ASVs were shared, a considerable number of bacterial strains were unique to a single host ecosystem (Figure 1), highlighting the importance of an in-depth investigation of host bacterial communities. Oftentimes, microbiota/microbiome researchers have to opt for an incomplete set of samples. To illustrate, faecal samples are typically used as a proxy to describe gut microbiota, but the bacterial communities may in fact be vastly different, as was reported in juvenile ostriches (*Struthio camelus*; Videvall et al., 2018). Our findings suggest that an incomplete sampling will likely result in a compromised understanding of host microbiota.

In birds, the reproductive, digestive, and urinary tracts converge at the cloaca, the shared outlet. Despite the anatomical overlap, the female fowl reproductive tract microbiota (**Chapter II**) was distinct from the female digestive tract microbiota, with the shared outlet cloaca harbouring an intermediate bacterial assemblage between the two tracts. Male fowl reproductive tract microbiota (**Chapter III**) was different from faecal microbiota, but shared a considerable number of bacteria with the cloaca and colon, which may be suggestive of the potential for bacteria to translocate between the tracts (Balzan et al., 2007). This could serve a functional role, if beneficial bacteria are shared across host body sites, but may also have a detrimental effect, if the bacteria are pathogenic. For example, *Escherichia coli* (phylum Proteobacteria) is an established gut commensal, but may cause female and male infertility in the human reproductive tract (Cools, 2017). While the results from **Chapters II-III** form an important step forward, further research is required to understand the mechanism of bacterial crossovers between host body sites and their potential role in microbiota community assembly.

Comparing the female and male reproductive tract microbiota (**Chapters II-III**) revealed that the sexes support divergent reproductive bacterial communities, as is the case with human reproductive tracts (Mändar, 2013; Chen et al., 2017). The most dominant phyla in female and male fowl were shared, including Actinobacteria, Bacteroidetes, Firmicutes,

Fusobacteria, and Proteobacteria, which are all commonly associated with wild and captive bird cloacal microbiota (Kreisinger et al., 2015; Zhang et al., 2017; Escallón, Belden & Moore, 2019). However, these phyla were present in different abundances: Proteobacteria were dominant in females, while Firmicutes dominated in male reproductive tracts. At the genus level, female reproductive tracts harboured a high abundance of *Escherichia/Shigella* (phylum Proteobacteria; distinguishing between the two genera is not possible based on the V4 hypervariable region), while *Fusobacterium* (phylum Fusobacteria) was the most dominant genus in males. The sex-specific phylum- and genus-level variation could be attributed to different reproductive tract anatomy and function, but may have also arisen from the fact that some of the females sacrificed for the reproductive tract dissections were symptomatic with a white cloacal discharge, although we did not in fact detect numerous bacterial genera that were differentially-abundant in sick females (e.g. *Fusobacterium* was more abundant in symptomatic birds). Interestingly, *Fusobacterium mortiferum*—which was the strain that we were able to associate with both female and male fowl across the thesis—has been found to inhibit the pathogenic *Salmonella enteritidis* (phylum Proteobacteria) in poultry caeca (Portrait, Cottenceau & Pons, 2000). We encourage further investigation of its function in female and male red junglefowl reproductive microbiota. Consistent with these sex-specific differences, we found that the cloacal microbiota, additionally investigated via the collection of cloacal wipe samples, differed between sexes (**Chapter V**), and these experimental birds appeared healthy. Although mating interactions altered the cloacal bacterial assemblages in both females and males in comparison with their pre-mating composition, sex-specific cloacal microbiota differences persisted.

The sex-specific differences were also evident when comparing bacterial richness and diversity across the reproductive tracts. We detected a decrease in bacterial diversity in the upper female reproductive tract tissues compared with the lower tract (**Chapter II**), while

bacterial diversity remained uniformly high across male reproductive tract tissues (**Chapter III**). The sex-specific variation in reproductive microbiota composition and spatial structure may suggest a potential for bacteria-mediated sexual conflict between female and male fowl, with the fitness interests of each sex showing divergent optima (Parker, 2006). To illustrate, pathogens can cause severe oviduct inflammation in hens (Keller et al., 1995; Neubauer et al., 2009; Shi et al., 2014), be incorporated within and on their eggshells (De Buck et al., 2004; Olsen, Christense & Bisgaard, 2012; Ahmed et al., 2017), and potentially affect female reproductive success in a negative manner. For example, a strain of *Corynebacterium* (phylum Actinobacteria) detected on the cloacae of mating black-legged kittiwakes (*Rissa tridactyla*) was associated with higher incidences of egg hatching failure (van Dongen et al., 2019). Reproductive tract microbiota maintenance may therefore be of high importance in females. The maintenance may be mediated via behavioural changes (e.g. avoiding infected partners; Sheldon, 1993; Lombardo, 1998), but also via physiological changes (e.g. producing antimicrobial peptides, AMPs; Silphaduang et al., 2006).

On the other hand, we found a consistently-diverse microbiota in fowl ejaculates (**Chapter IV**) and across the male reproductive tract (**Chapter III**), which may suggest minimal microbiota maintenance in males due to potentially low bacteria-induced fitness costs. However, it is important to emphasise here that the production of AMPs, such as lysozyme in birds (Rowe et al., 2013), is likely to be energetically costly and may also be cytotoxic to sperm cells (Boman, 2003), suggesting a potential trade-off between immunity and reproduction in males. Male fowl may thus forego producing AMPs and tolerate bacteria in their reproductive tracts to maintain a high number of healthy sperm. In support of this theory, male black garden ants (*Lasius niger*) showed minimal AMP-activity in their sperm storage organs, which in turn increased bacterial growth in their accessory testes (Dávila et al., 2018). The authors suggested that limiting AMP-activity may serve to protect male ants' sperm cells. On the other hand, the

sperm storage organ in female ants—the spermatheca—was reported to have high AMP-activity (Dávila et al., 2018), suggesting that each sex may have different fitness interests and costs when it comes to reproductive microbiota. Similar patterns seem to emerge in the red junglefowl, with females harbouring lower bacterial diversity in the upper sections of their reproductive tracts, while males do not.

One possible extension of the results in **Chapter IV** would be to investigate how males may manage bacterial growth in their ejaculates, and whether there is truly a trade-off between immunity and reproduction. Interestingly, we detected non-significant, positive associations between fowl sperm velocity and concentration, and ejaculate bacterial load (**Chapter IV**). Whilst this does not indicate causality, ejaculate bacteria may have a negligible or even beneficial effect on sperm performance (Lombardo, Thorpe & Power, 1999; Lombardo & Thorpe, 2000; Smith & Mueller, 2015; Rowe et al., 2020a). Under the assumption that ejaculate-associated bacteria are not costly for males, male hosts are expected to invest less in maintaining their reproductive tract and ejaculate microbiota.

This theory is somewhat supported by our findings in **Chapter V**, where we compared the cloacal microbiota of virgin fowl with the cloacal bacterial assemblages of non-virgin birds before and after a mating trial. The cloacal total ASV richness sampled from non-virgin females before the mating trial was more similar to the virgin state, and significantly lower than the cloacal richness sampled from non-virgin females after the mating trial. Non-virgin male red junglefowl harboured a consistently high cloacal total ASV richness regardless of their participation in a mating trial. Our results suggest that female cloacal microbiota may revert back to the virgin state of lower total ASV richness during the non-breeding season. Similar patterns were reported in female black-legged kittiwakes where females receive bacteria from males during mating (White et al., 2010). However, when cloacal contact between mating partners was experimentally blocked, female cloacal microbiota returned to its pre-copulatory

state of lower diversity (White et al., 2010). It would be worthwhile to investigate the exact mechanisms of reproductive microbiota maintenance by the host, and we propose that the first focus should be on the host-produced AMPs that have been found to limit bacterial growth in both females and males across animal species (Com et al., 2003; Silphaduang et al., 2006; Otti et al., 2009; Rowe et al., 2013; Rowe et al., 2020b).

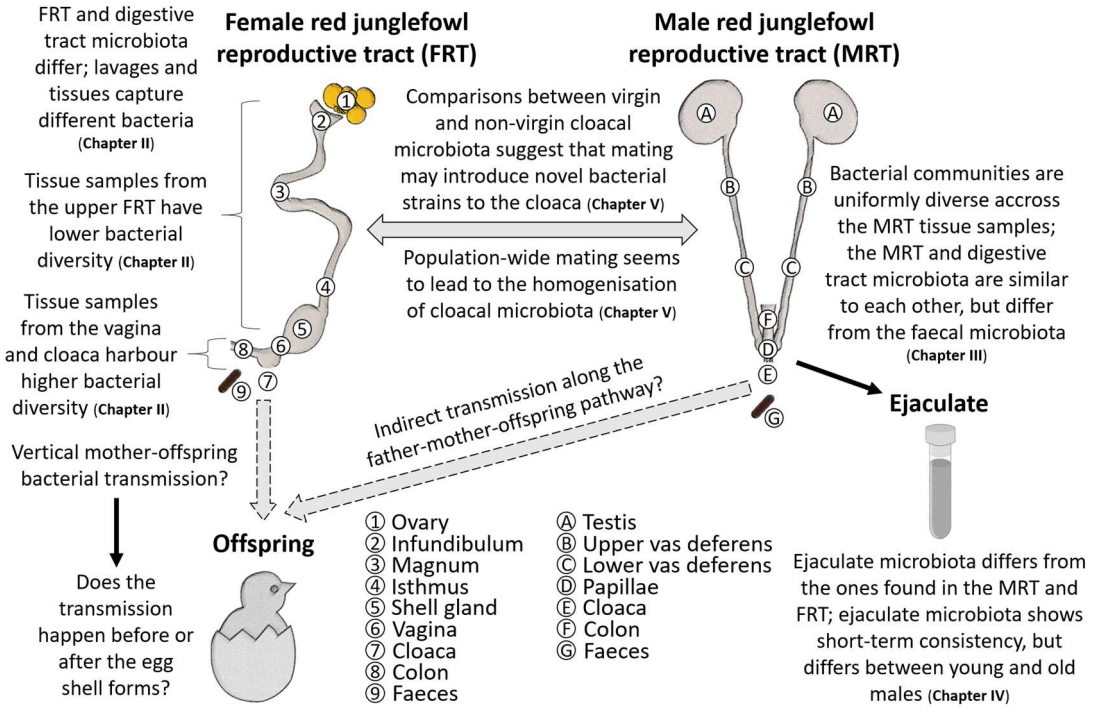

Figure 2. Findings of the thesis (Chapters II–V**) summarised as an illustration.** By using the red junglefowl (*Gallus gallus*) as a model system, we were able to address several previously unanswered questions surrounding the ecology of reproductive tract microbiota (Chapter I, Figure 1): We report that the female (FRT) and male reproductive tract (MRT) differ from one another when processed with standardised methodology. While faeces and ejaculates are oftentimes used as proxy samples for the digestive tract and the MRT respectively, we found that they harbour a divergent microbiota and should be used as proxies with caution. As the shared outlet cloaca harbours bacteria from both the reproductive and digestive tract, one must take extra care when using it as a proxy in microbiota studies. We also highlight the importance of characterising reproductive tract microbiota across host taxa, as the bacteria found in the FRT and MRT of red junglefowl are different from the previously explored human reproductive tract bacteria. For example, the genus *Lactobacillus* (phylum Firmicutes) dominates the lower FRT in humans, while the main emerging pattern in the red junglefowl, particularly when compared with the digestive tract, is that all reproductive tract samples harbour a higher abundance of the phylum Actinobacteria. However, questions remain about the generational transmission of bacteria in oviparous hosts. The FRT was drawn based on a photo by John Anderson**,** MRT based on field dissections, and the offspring (original author: FreeVector) and ejaculate sample in the tube (original author: iStock) based on the vector art of the original authors.

We found a large abundance of Actinobacteria associated with the red junglefowl reproductive tracts in comparison with fowl digestive tract microbiota (**Chapters II–V**). Actinobacteria were especially abundant in fowl ejaculates (**Chapter IV**), and on female and male cloacae (**Chapter V**). Considering that ejaculates and cloacae come in contact during mating, and that ejaculate bacteria have been found to disturb female red junglefowl cloacal microbiota (Eccleston, 2017), it is likely that the two host-provided ecosystems share bacterial strains (Figure 1). Indeed, to name a few, both ejaculates and the cloacae of females and males harboured *Actinomyces*, *Bifidobacterium*, and *Janibacter* from the phylum Actinobacteria. The cloacal microbiota of barn swallows (*Hirundo rustica*) is also dominated by Actinobacteria (Kreisinger et al., 2015), but the function of this bacterial phyla—which contains both antibiotic-producing pathogens and commensals (Ventura et al., 2007; Barka et al., 2016)—remains poorly understood in avian hosts. It has been hypothesised, however, that beneficial bacteria may protect birds from pathogenic bacteria (Soler et al., 2010), and therefore the role of Actinobacteria should be explored further.

As reported in humans (Mändar, 2013; Chen et al., 2017; Altmäe, Franasiak & Mändar, 2019), fowl ejaculate microbiota (**Chapter IV**) was distinct from bacteria inhabiting the male and female reproductive tracts (**Chapters II-III**), although more work needs to be done to understand the way in which bacteria are incorporated into ejaculates. Male red junglefowl have been shown to alter their sperm (Pizzari et al., 2003; Alvarez-Fernandez et al., 2019), seminal fluid (Cornwallis & O'Connor, 2009), and seminal fluid proteome (Alvarez-Fernandez et al., 2019) investment in their ejaculates over a sequence of matings. Substantial variation in ejaculate characteristics also occurs over the course of breeding season (Rakha et al., 2017; Wang, 2020c), and across age classes, with older male fowl typically producing fewer sperm of lower motility, and a distinct seminal fluid proteome (Dean et al., 2010; Noguera et al., 2012; Cornwallis, Dean & Pizzari, 2014; Borziak et al., 2016). We hypothesised that the variation in

ejaculate characteristics may also affect ejaculate microbiota. For example, sperm cells and seminal fluid compounds have been hypothesised to serve as an energy source for bacteria (Dávila et al., 2018), and their availability in ejaculates may affect semen-associated bacteria. Contrary to our hypotheses, we found no evidence for temporal variation in ejaculate microbiota over the course of breeding season nor across a mating sequence (**Chapter IV**). Overall, fowl ejaculates harboured a remarkably consistent bacterial richness, diversity, composition, and load. Investigating the importance of ejaculate microbiota consistency would be a valuable next step. Alternatively, similar patterns may emerge from males simply not exerting ejaculate microbiota maintenance, as we already hypothesised in the context of male reproductive tract microbiota in **Chapter III**.

Across **Chapters II-V**, we found considerable intra- and inter-individual variation in the reproductive microbiota of red junglefowl, highlighting the importance of accounting for phenotypic variation in microbiota studies. Of note, old female (**Chapter II**) and male fowl (**Chapter III**) harboured lower total ASV richness in their reproductive tracts, which may be a result of decreased mating rates (Dean et al., 2010) and the consequent decreased sexual bacterial transmission, but also physiological changes in older fowl (Cornwallis, Dean & Pizzari, 2014). Whether the reduced reproductive bacterial diversity in older individuals is beneficial or detrimental for the host remains currently understudied. Menopausal women, however, were reported to harbour a lower vaginal *Lactobacillus*-diversity (phylum Firmicutes) than pre-menopausal women, which is expected to negatively affect their vaginal homeostasis (Muhleisen & Herbst-Kralovetz, 2016). Age-related variation has also been reported in the cloacal microbiota of adult and chick black-legged kittiwakes (van Dongen et al., 2013), and in the faecal microbiota of parent and offspring barn swallows (Kreisinger et al., 2017). While there is an increased interest in linking animal behaviour and personality to microbiota variation (Davidson, Raulo & Knowles, 2020), we found complex associations

between fowl boldness and exploratory personality, and cloacal microbiota, and were therefore not able to report anything conclusive (**Chapter V**). It is possible that personality-related microbiota variation and bacterial transmission dynamics are more evident in the context of host social behaviour (Keiser et al., 2019), rather than sexual behaviour. Indeed, the cloacal microbiota variation we detected between fowl of different personality may have been due to social contact between the hosts, e.g. allopreening, rather than sexual interactions. We therefore suggest that future host-microbiota network studies should account for multiple host socio-sexual interactions.

Intra-group social interactions increase within-group gut microbiota similarity—while decreasing between-group similarity—and individuals with stronger social bonds share a more homogenous gut microbiota (Tung et al., 2015; Perofsky et al., 2017; Raulo et al., 2018). Whilst we were not able to convincingly show similar patterns between intra-group mating interactions and the homogenising of group-level cloacal microbiota, we found some evidence that increased mating frequency between two hosts may have the potential to homogenise their cloacal microbiota composition (**Chapter V**).

Individuals that mated with fewer partners diverged from the core mating population, which included birds that secured more partners and shared a more homogeneous cloacal microbiota (**Chapter V**). We encourage further investigation of intra-group socio-sexual interactions and their effect on bacterial transmission dynamics within host groups of varying mating behaviour. Indeed, different mating systems have been hypothesised to have distinct effects on sexual bacterial transmission (Ashby & Gupta, 2013; Rowe et al., 2020a). To illustrate, socially- and genetically-monogamous black-legged kittiwakes harbour similar within-pair cloacal microbiota (White et al., 2010), while socially-monogamous, yet genetically-polygynous North American tree swallows (*Tachycineta bicolor*) share a population-wide cloacal microbiota (Hernandez et al., 2020). To this end, we found that the

polygynandrous red junglefowl harbour a more similar cloacal microbiota at the population level (**Chapter V**).

Resolving patterns of sexual bacterial transmission is critical in understanding host mating behaviour, sexual selection and conflict, and sexually-transmitted infections in host populations. We found a potentially interesting result in birds that secured more partners in the mating population (**Chapter V**). Before the mating trials, *Romboutsia* (phylum Firmicutes) and *F. mortiferum* were more abundant on the cloacae of fowl that secured more partners in the population in comparison with individuals that mated with fewer partners. *Romboutsia* is a recently-established genus that has so far been associated with probiotic properties in host guts (Gerritsen et al., 2014; Gerritsen, 2015; Ricaboni et al., 2016). As such, the presence of *Romboutsia* and *F. mortiferum* may be an indicator of "healthy" cloacal microbiota. Taking into account that red junglefowl perform intense pre-copulatory sexual selection (Pizzari & McDonald, 2019), the role of reproductive bacteria in mate choice and socio-sexual group structure merits further exploration.

Limitations of the Thesis

Apart from the evident issue of low sample size, the limitations of my thesis are three-fold. The first limitation is that I was not able to test for causality, but rather, investigated the patterns of reproductive microbiota variation in association with intra- and inter-individual variation. As such, I could not imply much about the potential interdependence between the host and its reproductive microbiota. The reason behind carrying out a more exploratory investigation was the fact that non-human hosts are vastly understudied, and before conducting manipulated experiments, we must first understand the general trends.

The second limitation is that I was not able to assign bacterial "species" nor strains to my sequencing reads, and therefore focused solely on bacterial phyla and genera. This was

partially due to the current databases of bacterial strains lacking information about the wealth of bacteria associated with non-human hosts (e.g. SILVA; Yilmaz et al., 2014), but also due to methodological reasons. I used the amplification primers suggested by the Earth Microbiome Project (Thompson et al., 2017), and while that gave me the benefit of comparing my data across the majority of microbiota literature, the sequenced 16S SSU rRNA gene hypervariable region V4 is known for its poor species- and strain-assignment potential (Gohl et al., 2016; Johnson et al., 2019b). In fact, even genus-level assignments were limited, and we failed to assign genera for 39.7% of the ASVs detected across **Chapters II-V**. Moreover, distinguishing between some bacterial genera was complicated. To illustrate, it is impossible to distinguish between *Escherichia/Shigella* based on the sequenced V4 hypervariable region. Although we were able to explore the higher-level patterns of microbiota variation in a previously understudied host, questions remain about what bacterial "species" and strains inhabit the reproductive tracts of red junglefowl.

The third limitation is that I focused solely on the host-associated bacterial assemblages, but not their function. Microbiota function can be explored by incorporating other -omics tools. However, that was not within the scope of my thesis and as such, I had to rely on the previously established literature that is predominantly focused on human reproductive bacteria and their function, which limited the interpretation of my thesis results. Both the second and third limitation highlight how little is currently known about host-associated bacteria, and the need for better solutions and databases for research that focuses on host microbiota.

Of additional note, there has been criticism in the past about whether low biomass host ecosystems—such as the reproductive tract and in particular the more internal organs of the tract—harbour bacteria (Salter et al., 2014; Perez-Muñoz et al., 2017; Goffau et al., 2019). While we should remain wary in our interpretation of results, we incorporated a rigorous negative control system and removed contaminants based on statistical prevalence (Davis et

al., 2018), adding confidence to our findings. Moreover, our results were congruent with the domestic chicken gut and cloacal microbiota studies (Yeoman et al., 2012; Oakley et al., 2014; Shaufi et al., 2015).

Considering the pace at which new technologies are being developed in the field of microbiota/microbiome research, it is likely that some of the outlined limitations will be overcome in the near future. To illustrate the fast turnover of recommended methods during the course of this DPhil degree alone, we witnessed the transition from the bioinformatics pipeline QIIME™ to QIIME™ 2 (Bolyen et al., 2019) and DADA2 (Callahan et al., 2016a), and the SILVA rRNA database (Yilmaz et al., 2014; Glöckner et al., 2017) was updated multiple times (Versions 128, 132, 138). There has been a considerable effort by statisticians (McMurdie & Holmes, 2013, 2014; Callahan, et al., 2016b; Davis et al., 2018; Willis, 2019), but also by biotechnology companies to create solutions specifically tailored for microbiota/microbiome research. For example, manufactured reagents are being cleaned of pre-existing bacterial contaminants (Kim et al., 2017; Salter et al., 2014), and have a higher standard and specificity than ever. Indeed, I also encountered issues with contamination at the start of the thesis (**Chapter II**), but not in the later stages (**Chapter III-V**). With the wealth of possibilities, staying on top of the state-of-the-art developments poses another challenge in this rapidly-advancing field.

Future Areas of Research

In addition to the aforementioned ideas, we would like to highlight two more potential areas of interest. First, there is increasing evidence that reproductive tracts harbour other microorganisms in addition to bacteria, such as fungi (Chen et al., 2018) and viruses (Gautam et al., 2015; Klase et al., 2015), but also potentially archaea and protists. Little, however, is currently known outside the realm of reproductive bacteria, and whether there is crosstalk

between reproductive microorganisms. Considering that reproductive bacteria can affect human host health and fertility (Weng et al., 2014; Franasiak & Scott, 2015; Amato et al., 2020; Łaniewski, Ilhan & Herbst-Kralovetz, 2020), a better understanding of the reproductive microbiome as a whole is needed to support and potentially engineer "healthy" microbiomes for the benefit of human health, but also for animal welfare and husbandry, and possibly species conservation (Lawson et al., 2019; Trevelline et al., 2019; West et al., 2019).

Second, we encourage increased interest in the mother-to-offspring bacterial transmission (Mändar & Mikelsaar, 1996; Dominguez-Bello et al., 2010), but also investigating the indirect effect of male reproductive tract and seminal fluid bacteria that may be involved in the vertical transmission of bacteria. This could be studied by a carefully-conducted experiment that incorporates ejaculate microbiota composition manipulations, artificial inseminations, and fertilisation success assays. We also want to emphasise the importance of studying the female-male-offspring bacterial transmission across animal taxa and ovoviviparity (egg-laying vs live-bearing reproduction), as the mechanisms and potential functions are likely to differ. Resolving the role of reproductive bacteria in the female-male-offspring context could aid in understanding the interdependence between host evolutionary ecology and reproductive microbiota.

Conclusion

Exploring the ecology of the polygynandrous red junglefowl reproductive microbiota in this thesis revealed remarkable bacterial diversity across the internal reproductive tracts of female and male fowl, but also in the ejaculates and on the cloacae of these birds. Our findings demonstrate that investigating both the female and male reproductive tract in parallel, incorporating different sampling methods—such as lavage, tissue, ejaculate, and cloacal wipe samples—and accounting for intra- and inter-individual variation in the analysis is important

and yields a more integrated understanding of reproductive microbiota. Whilst we were able to provide a thorough survey of reproductive microbiota variation in red junglefowl and indicate preliminary avenues of interest in the realm of sexual bacterial transmission dynamics, future work must investigate the true causality associated with the interdependence between the host and its reproductive microbiota. Collectively, my thesis has interdisciplinary implications for both bacterial ecology and host evolutionary ecology research, and encourages further exploration and validation of hypotheses about the role of reproductive bacteria in host mating behaviour, and sexual selection and conflict.

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Imperial College London – Harvard referencing style is used throughout the thesis. To avoid repetition, a full list of references is provided at the end of the thesis.

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