A microbial take on bird life

Illuminating environmental context of
bird microbiota dynamics and immune function

Pieter van Veelen

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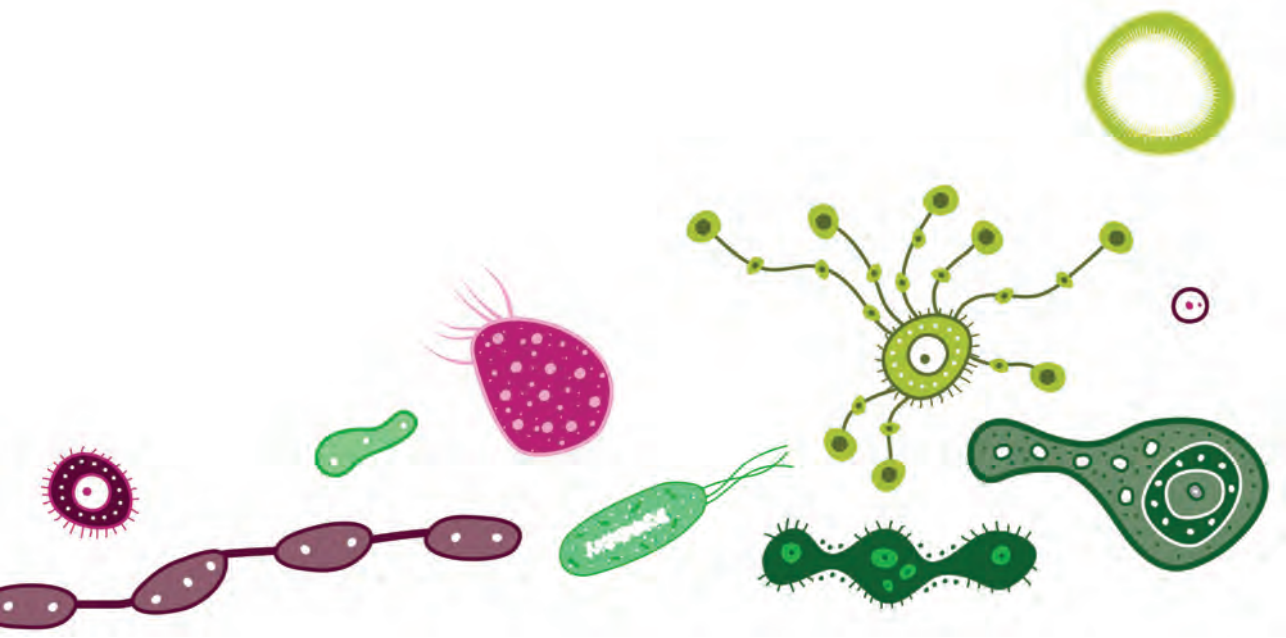

Animals as microbial ecosystems

"Plants, fungi and animals emerged from the microcosm. Beneath our superficial differences we are all of us walking communities of bacteria. The world shimmers, a pointillist landscape made of tiny little beings" – "Microcosmos: Four Billion Years of Evolution from Our Microbial Ancestors" (1986), Lynn Margulis & Dorion Sagan

An animal's soma and a plethora of microorganisms are increasingly considered as an ecosystem in a dynamic equilibrium during homeostasis. The animal's immune system functions as a gatekeeper between the animal's internal and external environment. Traditionally, immune function has been studied as a mechanism of defence against parasites and pathogenic viruses, fungi and bacteria. This emphasis on eliminating pathogens as the primary purpose of immune function disregards its importance for maintaining and regulating complex animal-associated microbiotas (i.e. microbial assemblages associated with animals) that provide beneficial functions (Evans *et al*., 2017; Horrocks *et al*., 2011a; Tieleman, 2018). It is becoming increasingly clear that animal-microbial interactions are of critical importance to development, performance and fitness of animals (McFall-Ngai *et al*., 2013). Time has come to develop our understanding of the ecological interactions between immune systems and microorganisms, without making the somewhat ambiguous distinction between pathogens and non-pathogens. Ecological interactions between microorganisms and larger macroorganisms have gained attention since a few decades (Methé *et al*., 2012; Turnbaugh *et al*., 2007) causing an upsurge to the frontline of scientific discovery in recent years. The limited historical focus on these interactions was likely due to our inability to observe microorganisms in natural ecosystems. Yet, ongoing advancement in sequencing and imaging technologies create unprecedented opportunities to take a detailed look at microbial assemblages in and on animals and their surroundings.

A systematic integration of animal-microbial interactions in ecological immunology may reshape our understanding of eco-evolutionary dynamics of immune systems. Because immune systems are critical components of vertebrate life history evolution, the causes and consequences of variation in immune function have been a focal topic in evolutionary biology for a while. Over the last four decades this devotion has given rise to the field of ecological immunology (Sheldon & Verhulst, 1996). Because we now can assess the diversity of microorganisms in animal microbiota in detail, associations between animal

immune function and animal microbiota could be investigated beyond the pathogens that received most attention in studying how immune systems evolved. So far, comparative studies that assessed covariation between host phylogenies and similarities among host-associated microbiota to understand if coevolution could explain interspecific variation of microbiota composition (e.g., Hird *et al*., 2015; Moeller *et al*., 2016), resulted in inconsistent patterns. At the same time, numerous ecological factors can correlate with microbiota variation among and within species and individuals, supporting strong influence of ecological processes shaping animal microbiotas (Groussin *et al*., 2020; Mazel *et al*., 2018). With several processes potentially at play simultaneously, how ecological community assembly processes shape animal microbiotas remains unresolved. Hence, it is imperative to gain more insights in the factors and processes that drive animal microbiota assembly. Subsequently, more studies are needed to determine how animal microbiotas are maintained, and thus which traits - of host and microbiota - confer resistance to perturbations from (microbial communities in) the external environment, and which traits assist in resilience of animal microbiotas after perturbations.

With this thesis, my aim was to investigate how bird microbiota, bird immune function and the microbial communities in the bird's environment interact at ecological time scales. By studying how microbial diversity modulates immune investment, my ultimate goal is to contribute to a more comprehensive synthesis of the evolutionary ecology of immune function that includes microbial ecology.

In this thesis, I made use of birds as a model system for vertebrate animals that enabled me to (i) simultaneously investigate intraspecific and interspecific variation of host-associated microbiotas and interpret this variation in the context of environmental variation (i.e. horizontal acquisition); (ii) explore if eggshell microbiome assembly (i.e. vertical acquisition) and immunological priming of eggs are shaped by the microbial environment and function as non-genetic maternal effects onto the next generation. To disentangle intrinsic and external influences, (iii) my colleagues and I also sought to investigate if environmental microbial communities affect the microbiota and immune function of adult birds in a phenotypically flexible manner, and to explore if animals show consistent individual differences irrespective of their response to the environment. In order to do so, we focused on bacterial communities as a proxy for the broader group of microorganisms that make up the microbial communities that interact with immune

systems. In general, my colleagues and I believe that avian model systems provide unique opportunities to integrate host-microbiome interactions with ecological immunology. This is partly based on a rich history in empirical avian research in ecological immunology, which helped develop foundational underpinnings of theoretical models in animal ecology and evolutionary biology. Moreover, birds appear popular study subjects for host-microbiota studies. This thesis is another demonstration of the broad utility of birds for studying ecological processes and routes of microbiota acquisition, factors driving host-associated microbiota variation and immunological variation. Although outside the of scope of this thesis, I am convinced that birds will provide opportunities to further study fitness consequences of (co)variation in microbiota (functioning) and immune function.

The current paradigm in ecological immunology

The immune system of vertebrates has evolved as a complex physiological mechanism that contributes to self-maintenance and survival through protection against infection and disease. The immune system could be defined as an integrated assemblage of specialized organs, tissues, cells and molecules that both passively and actively function to maintain an organism's health. Logically, adequate immune defences should be beneficial to an organism and positively contribute to its fitness.

The organization of immune function is far from fixed and varies greatly among species, populations and individuals, across space and over time (e.g., Ardia, 2007; Lee, 2006; Martin *et al*., 2008; reviewed in Demas & Nelson, 2012; Eikenaar & Hegemann, 2016; Hegemann *et al*., 2012; Horrocks *et al*., 2012). Ecological immunology research has elucidated a suite of behavioural, physiological and environmental factors that contribute to variation in immune function in wild animals. While these efforts made vast contributions to our understanding of the significance of immunity in wild populations, most work was embedded in a framework built around two major concepts: the concept of pace-of-life (Promislow & Harvey, 1990; Ricklefs, 2000) and life history theory (Roff, 1992; Stearns, 1992). Common to both concepts lies the central premise that trade-offs are imposed by the costs of immunity, which forms the basis of an underlying framework to investigate and interpret covariation of immune function with ecological and evolutionary axes (Bonneaud *et al*., 2003; Klasing & Leshchinsky, 1999; Lochmiller & Deerenberg,

2000; Martin *et al*., 2006; Norris & Evans, 2000; Ricklefs, 2000; Sheldon & Verhulst, 1996; Tieleman *et al*., 2005; Zuk & Stoehr, 2002). Specifically, the fundamental prediction was that investment in immune defences is balanced among multiple demanding aspects of physiology and life history when energy or nutritional resources are limited (Lochmiller & Deerenberg, 2000; Norris & Evans, 2000; Sheldon & Verhulst, 1996). Optimal resource allocation between demanding physiological processes and life-history traits is, however, not fixed during an organism's life and depends both on the availability of resources and an individual's life-history stage (Drent & Daan, 1980; Roff, 1992). To optimize fitness, life history theory predicts that individuals should invest more in survival early in life as compared to later in life, when prospects for future reproduction typically diminish (Martin *et al*., 2006) (Fig. 1.1). In order to maximize fitness, individuals should optimally distribute available resources among demanding life-history traits for both survival (i.e., self-maintenance for which immune function is vital) and reproduction (Drent & Daan, 1980; Norris & Evans, 2000). However, the observed natural variation in immune function as well as experimental data provide inconsistent support for these predictions (see Tieleman, 2018 for a synthesis).

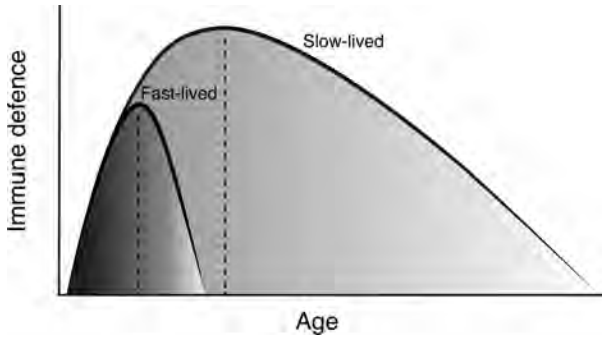

Figure 1.1: Predicted development and age-dependent decline of immune function in fast and slow living species. The concept of pace-of-life predicts differences in immune investment between fast and slow living species. Slow living species are predicted to invest more in development of immune defences as a means of self-maintenance than fast living species. Slow living species also are predicted to start to reproduce (dashed lines) later in life than fast living species, and to rely throughout their lives more on acquired immunity (light shade) than on non-specific innate defences (dark shade) compared with fast living species. This figure is a simplified recreation of a figure presented by Martin *et al*., 2006.

Microbial pressure as potential driver of variation in immune function

The protective benefits of immune systems warrant more emphasis. The evolution of immune function is a process driven by various concurrent selection pressures. These includes promoting defences for preventing infection and disease but also for maintaining and optimizing the benefits of host-microbe interactions that enable animals to flourish in dynamic microbial environments. While the research field of ecological immunology centred on identifying the causes and consequences of natural variation in immunity, the actual agents that trigger immune systems, such as bacteria and other microorganisms, have not been thoroughly investigated in this context. Microorganisms may prove of critical importance to unravelling the factors and underlying processes that cause variation in immune function.

Disease ecologists investigated the effects of specific (macro-)parasites or specific pathogens on immune function (Siikamäki *et al*., 1997; Tschirren & Richner, 2006; Whiteman *et al*., 2006). Spatiotemporal variation in the distribution of parasites and pathogens (Bensch & Åkesson, 2003; Spurgin *et al*., 2012) and its influence of on immune investment (Lazzaro & Little, 2009; Lindström *et al*., 2004; Sandland & Minchella, 2003) already emphasised the importance of this subset of immune-stimulatory agents on variation in immune function in wild animals. Apart from specific parasites and pathogens, simultaneous measurement of immune function and diverse microbial assemblages has remained limited. Nonetheless, several studies indicated immunological variation among different environments (Buehler *et al*., 2008; Horrocks *et al*., 2012, 2015; Martin *et al*., 2004; Matson *et al*., 2006), suggesting that – yet undefined – environmental characteristics triggered changes of immunity. Research that included environmental bacteria as a generally non-pathogenic class of agents to affect immune function detected a correlation between bacterial load and immune function across different environments (Horrocks *et al*., 2012) and among seasons (Horrocks *et al*., 2012). Several other studies also implied that variation in immune function observed in nature does not necessarily result from either balancing costs associated with life history trade-offs or differences in pace-of-life (Hegemann *et al*., 2012; Tieleman, 2018).

By contrast, immune investment in wild animals could be mediated by disease risk. Horrocks *et al*. (2011a) expanded on this idea and suggested that general 'antigenic

pressure' (i.e. the combined pressure from all antigenic entities that can interact with an immune system) from the environment may be an important factor. The 'antigen-exposure' hypothesis predicts that variation in immune function arises from differential antigenic pressure exerted by the collective assemblage of antigenic agents (e.g., bacteria, fungi, viruses and parasites, including but not limited to pathogens) interacting with an animal and its immune system (Horrocks *et al*., 2011a; Schulenburg *et al*., 2009, 2015). Specifically, differential patterns in the diversity and composition of communities of antigenic agents are thus predicted to cause variation in immune defences, both throughout annual cycles and across environments. Such flexible immune defences are expected to provide protection against potential threats (Horrocks *et al*., 2011a) and concurrently enable immunological surveillance of other (non-pathogenic) potentially beneficial organisms from the experienced environment.

Examination of eco-evolutionary dynamics of the interactions between a more comprehensive assemblage of microorganisms and immune functioning of host organisms thus requires a multidisciplinary approach. Contingent on the level of analysis (i.e. evolved responses, individual responses and phenotypic plastic responses), it is imperative to simultaneously assess variation in immune function and microbial pressure among species and populations, among individuals and during a lifetime (Horrocks *et al*., 2012, 2015; Tieleman, 2018; Tschirren & Richner, 2006); a major challenge that demands integration of animal ecology, ecological immunology and microbial ecology. Only such interdisciplinary endeavours may illuminate spatiotemporal covariation between host immunity and a diverse microbial world, and could provide insights into the phenotypic plasticity and adaptive potential of microbial pressure-dependent immune function.

Genetic and non-genetic inheritance of immune function in light of microbial pressure

At the level of species, the antigen-exposure hypothesis predicts that evolution of immune function may be driven by the biogeography of microbial pressure (Guernier *et al*., 2004). Current evidence based on parasite distributions indicates that geographical differences in parasite pressure correlate with parasite prevalence and reproductive performance (e.g. marine vs. freshwater, Mendes *et al*., 2005; and latitudinal clines, Bordes *et al*., 2011; Clark *et al*., 2016). Moreover, comparative analysis of parasite

pressure across a wide range of animal taxa showed that exotic species harboured half as many parasite species than native species (Torchin *et al*., 2003), suggesting clear biogeographical structure of parasite distributions and their evolved host-specificity. In general, 'pathogenic' microbial pressure is considered inversely related with latitude (e.g. Bordes *et al*., 2011; Clark *et al*., 2016; Guernier *et al*., 2004). Whether these patterns apply to the non-pathogenic microorganisms, however, remains unexplored (discussed below). Assuming biogeographical structure of microbial pressure, diversification and biogeographical structuring of animal species may have concurred with local adaptation to microbial pressure on evolutionary time scales. Simultaneous diversification in the genetic architecture of immune systems could then be expected. A comparison of allelic diversity in the immune gene encoding the major histocompatibility complex class I (MHC-I) receptors in African and Palearctic congeneric bird species has been made (O'Connor *et al*., 2018). This work indeed supported genetic adaptation of immune function that correlated with the expected reduction of microbial pressure at higher latitudes. Similar associations have been suggested between microbial pressure and diversity of Toll-like receptor (TLR) genes that encode pattern-recognition receptors on mucosal epithelial cells involved in innate immunity (Ishengoma & Agaba, 2017), which are known to influence the composition of gut microbiota (Wang *et al*., 2010).

On shorter time scales, such as among generations or during individual life times, microbial pressures may vary greatly along spatiotemporal axes (e.g. Carey *et al*., 2013). For example, seasonal variation in microbial pressure may be due to climatic shifts in abiotic selective landscapes. Spatial variation can range from differential microhabitat features across short distances to the different zoogeographical realms that migrating animals face while traveling between continents on an annual basis. In addition, a growing number of studies implicates that changes in diet composition can induce ingestion of different microbial communities (e.g., Amato *et al*., 2013; Ezenwa *et al*., 2012; Youngblut *et al*., 2019); communities which are often typical for particular types of diet (Delsuc *et al*., 2014; Muegge *et al*., 2011). These and other environmental factors that generate variation in the microbial pressure may have direct or indirect effects on an animal's development and health, with potential fitness consequences (Round & Mazmanian, 2009). An animal's ability to optimize the benefits from environmental or its own microbial communities may depend on its immune function. If immune function differs among individuals, and given such differences provide a selective advantage at the population level, then

adaptation through natural selection may follow if these differences are transferable across generations (Räsänen & Kruuk, 2007).

Transgenerational immunological priming can enhance offspring development and survival by transferring antimicrobial peptides and antibodies to offspring as non-genetic maternal effects (Bernardo, 1996; Grindstaff *et al*., 2003). Such non-genetic maternal effects represent a female's ability to transmit her phenotypically plastic response to the environment to her offspring. Transgenerational immunological priming has typically been studied on the basis of immune challenges with single antigens (Grindstaff *et al*., 2006; Saino *et al*., 2002; reviewed in Hasselquist & Nilsson, 2009). In this thesis, my colleagues and investigate if that idea could be extended to microbial pressure as a broad sense stressor, which may have implications for adaptive value of maternal effects at ecological (Boulinier & Staszewski, 2007) and evolutionary time scales (Groussin *et al*., 2020). Accordingly, following the antigen-exposure hypothesis, if microbial pressure can shape immune function in adults as a phenotypically plastic response, then transgenerational immunological priming of offspring may indirectly depend on microbial pressure as well. In this thesis, my colleagues and I made use of birds to investigate if microbial pressure from the environment determines maternal deposition of immune components to eggs (**Chapter 6**).

Innate and acquired immunity: implications of variation in microbial pressure

As animals cope with a countless array of antigens at any given moment and throughout their life most are likely non-pathogenic. Therefore, by acknowledging the omnipresence and great diversity of microbial taxa in natural environments (Martiny *et al*., 2006; Roesch *et al*., 2007), characterisation of microbial communities may contribute to future conceptual advancements and novel approaches to understand effects of the microbial pressure on immune function. Characterising the microbial pressure as the collection of organisms that may interact with an immune system thus constitutes an essential but complex step to answering the question of how animals shape their immune defences.

Lee (2006) proposed a theoretical framework of immune investment across a life history axis to explain immune variation across species and populations. Based on this framework slow-living species are predicted to invest more in immune function for self-maintenance

and survival than fast-living species. Slow-living species were thus predicted to invest more in adaptive immunity (e.g., B- and T-cell mediated immunity), which are nutritionally rather costly, and which take longer to develop than constitutive innate defences (Klasing, 2004; Lochmiller & Deerenberg, 2000; McDade *et al*., 2016). Fast-living species, with relatively short development times are conversely predicted to rely more on energetically costly innate defences (Klasing, 2004). Microorganisms, viruses and parasites interact with both slow- and fast-living species alike, and one might expect that the costs and benefits of (innate and acquired) immune function for both slow and fast living species change depend on interactions with microbes (Klasing, 2004; Zuk & Stoehr, 2002). Therefore, if microbial biogeography is structured similarly to the biogeographical distribution of animals, constitutive levels of innate immunity may have evolved to fit average levels of antigenic pressure at the large spatial scale. From an ecological perspective, energetic and nutritional budgets always depend on resource availability and the life history stage of an individual. Therefore, an individual's immune defences are likely shaped as a function of life history stage, resource availability, and microbial pressure.

Yet, the innate and acquired arms of the vertebrate immune system differ in their predicted response to variation in microbial pressure, a distinction that could depend on which characteristics of microbial communities vary. Non-specific immune responses of the innate immune system act independent of antigen recognition. Conversely, the acquired immune system relies on a broad, but randomly formed array of antigen receptors situated extracellularly on T and B lymphocytes for antigen recognition. A brief summary of vertebrate innate and acquired immunity as well as quantitative methods used in ecological immunology is provided in **Box 1.1**. As the development, maturation and activation of acquired immunity depends on binding of antigens to receptors, the acquired immune system may adjust to local microbial pressure (McDade *et al*., 2016). However, the antigen-exposure hypothesis predicts that microbial pressure serves as key determinant for how animals should optimally invest along the innate-acquired immune axis: priority toward innate immunity is predicted under poor nutritional condition and low microbial pressure, whereas investment priority should shift toward acquired immune function under sufficient resource conditions and increased microbial pressure (Horrocks *et al*., 2011a; McDade *et al*., 2016). In this thesis, my colleagues and I empirically evaluated the proposal by Horrocks *et al*. (2011a) through explicitly considering bacterial communities in the environment, and those associated with animals, as a more inclusive proxy of

microbial pressure than studied thus far. We tested relationships between immune function and the bacterial communities in the environment that animals encounter, and we explored how bacterial communities associated with animals are affected by the microbial environment. (**Chapter 2, 3 and 5**)

Box 1.1: Useful markers of immunological variation in birds

The complexity of immune systems makes meaningful measurement and interpretation of variation of immune function a major challenge (Klasing, 2004; Matson *et al*., 2006), especially for wild animals under field settings (Hegemann *et al*., 2013). Ecological immunologists have developed several measures to meaningfully interpret cellular and humoral immune function, components of which can be expressed constitutively or induced by particular triggers (Gan & Marquardt, 1999; Matson *et al*., 2012, 2005; Millet *et al*., 2007; Tieleman *et al*., 2005; Yamanishi *et al*., 2002). Using these methods, variation of immune function has been related to animal physiology and ecology, but how animals invest in immune defences based on the microbial antigens and other antigens they encounter during their lifetime remains elusive (Horrocks *et al*., 2011a; McDade *et al*., 2016).

Innate immunity

The genetically encoded but nonspecific innate immune system responds relatively fast to microbial invasion as the first line of defence. Innate immunity comprises the physical barriers of the host body (e.g. epithelia), humoral broad-spectrum defences (e.g. complement, acute phase proteins and lysozyme), and cellular responses by an array of leucocytes that mediate inflammation and execute phagocytosis (Janeway *et al*., 2001; Klasing, 2004; Lee, 2006). When mounting an immune response, the innate immune system could induce both local and systemic inflammatory responses. The systemic response consists of energetically costly acute phase protein production and induction of fever (Klasing & Leshchinsky, 1999). The pleiotropic complement system directly eliminates invading microbes through enzymatic breakdown of cell membranes, it promotes phagocytosis by marking infected cells for antibody recognition, and it activates the second line of defence, the acquired immune response (Janeway *et al*., 2001). In addition, nonspecific natural antibodies (immunoglobulin M in birds) also circulate constitutively in the bloodstream and confer immediate protection after infection. Haptoglobin signals inflammation and is an acute phase protein that binds iron (haem), which protects the body against harmful end products of the immune response (Dobryszycka, 1997). Complement comprises aspects of the humoral innate immune response, and is essential in non-specific clearance of invaded bacteria through opsonisation for phagocytosis and enzymatic breakdown of bacterial cell walls (Janeway *et al*., 2001). Lysozyme functions as an antimicrobial peptide that disintegrates gram-positive bacterial cells by degradation of the cell wall peptidoglycan layer (Callewaert & Michiels, 2010). Ovotransferrin is an acute phase protein present both in the blood and in egg albumen, synthesized in the liver and oviduct respectively. Ovotransferrin binds iron molecules, consequently limiting the availability of iron for bacterial growth (Horrocks *et al*., 2011b), reducing the risk of infection of a broad range of bacterial taxa (both Gram-negative and Gram-positive bacteria). Most of these innate components are constitutively present in the bloodstream, and their energetic burden is relatively low when not mounting an immune response (Klasing & Leshchinsky, 1999; Lee, 2006).

The acquired immune response

Acquired immune responses are typically slower than innate responses, but are antigen-specific and allow for fast secondary responses to subsequent infections of a previously encountered invader. The acquired immune response can be categorized into two components: the humoral and the cell-mediated response. Key players in the humoral response, the antibody production focused on in this thesis, are B lymphocytes and T-helper (type Th2) lymphocytes. B lymphocytes recognize invaders and subsequently

produce specific antibodies that have multiple functions. Antigen-specific antibodies (immunoglobulin Y (IgY) in birds) neutralize (products of) pathogens and guide immune cells to the infection site by marking the invader cell surface with antibodies (Janeway *et al*., 2001). Th2-lymphocytes coordinate antibody production by stimulating B lymphocytes to differentiate and proliferate. Th2-lymphocytes also aid in immunological memory for a fast response to secondary infections, and regulate chemotaxis and the activation of leukocytes.

Immune function of eggs
Microbial infection represents one of the most significant threats to eggs (e.g., Cook *et al*., 2003). To minimise the risk of infection, eggs are protected by physical barriers and biochemical protection from antimicrobial compounds. Maternal deposition of antimicrobial compounds in albumen and antibodies in yolk reflect the maternal phenotype and signals maternal immunological experience (Grindstaff *et al*., 2003; Saino *et al*., 2002). This maternal transmission benefits offspring through direct protection, and priming immunological development (Grindstaff *et al*., 2006). Eggs additionally represent a relatively simple model to study immune function compared with the highly complex immune system of a bird.

Quantification of immune function in blood and eggs
In the field, the amount of blood plasma that can be collected from a bird at a given time limits the amount of immunological information to generate a complete picture of immune function. Although a single sample constitutes merely a snapshot of an animal's immunological state, different immune measures enable biologically meaningful comparison among individuals, or within an individual over time (e.g., Hegemann *et al*., 2012). In ecological immunology, widely used methods to quantify immune function in blood plasma samples include a commercial haptoglobin assay (Matson *et al*., 2012). A haemagglutination-haemolysis assays has been developed to assess both natural antibody concentrations (IgM) and complement activity in blood plasma samples (Grindstaff *et al*., 2006; Matson *et al*., 2005). Several methods have been developed to measure concentrations of lysozyme in egg albumen (D'Alba *et al*., 2010; Horrocks *et al*., 2014) and to quantify ovotransferrin in blood plasma and albumen (Horrocks *et al*., 2011b; Yamanishi *et al*., 2002).

Several methods have been developed to quantify acquired immune function. A method based on antigen challenging includes injection with keyhole limpet hemocyanine (KLH) as an antigen and is used to measure specific T-cell mediated responses. Injection with phytohaemagglutinine (PHA) is used to measure unspecific T-cell mediated response (Hasselquist, 2007; Smits *et al*., 1999). Sheep red blood cells (SRBC) or diphtheria-tetanus vaccines are often used as antigens to trigger an induced humoral response (i.e. antigen-specific antibody production of B-cells) (Ardia, 2007; Grindstaff *et al*., 2006). An alternative and more inclusive method that does not rely on single-antigen challenges, the total antigen-specific antibody concentrations (IgG and IgY) can be quantified using enzyme-linked immunosorbent assays (ELISA) in blood and egg yolk samples (Demas & Nelson, 1996; Grindstaff *et al*., 2005).

Compartments of the antigenic universe: environmental and host-associated microbiotas

The rapid technological advances in sequencing technology over the last decades vastly improved the detectability of microorganisms and paved the road for analysis of microbial communities at high resolutions. As a result, the ecological processes shaping microbial communities and the functional services that they provide to ecosystems have become key

topics in microbiology and microbial ecology (Christian *et al*., 2015; Dini-Andreote *et al*., 2015; Gibbons & Gilbert, 2015; Mallon *et al*., 2018; Martiny *et al*., 2006). This study of microbial communities has also started to penetrate the research fields studying ecology and evolution of animals (McFall-Ngai *et al*., 2013). The pioneering work in these fields made it progressively evident that microorganisms constitute vital components to animal development, performance and fitness (e.g., Human Microbiome Project Consortium *et al*., 2012; Round & Mazmanian, 2009; Turnbaugh *et al*., 2007). These early insights inspired a continuation of efforts to enhance our understanding of ecological interactions between vertebrates and microorganisms; an emerging paradigm in ecology and evolutionary biology that is still in its infancy (Hird, 2017; McFall-Ngai *et al*., 2013; Sharpton, 2018; Tieleman, 2018).

Viruses, Archaea, bacteria, fungi and protists are ubiquitously present in the aquatic and terrestrial biomes on Earth (Auguet *et al*., 2010; Nemergut *et al*., 2011), and given their sheer diversity, it is generally assumed that only a fraction of taxa has been identified thus far. Assumptions about the distributional patterns of microorganisms are integral to testing the antigen-exposure hypothesis. However, the understanding of microbial biogeographical distributions is limited and varies with the spatial scale that is considered. A brief account on the spatial distribution of free-living bacteria is provided in **Box 1.2**. Interpreting vertebrate-microbiota covariation patterns from comparative analyses is hampered by the lack of consensus on microbial biogeographical distributions (and microbial pressure in general). Empirical study of the relationships between environmental and host-associated microbial communities at the population or individual level is possible by simultaneous analysis of both of these components (**Chapter 2**).

Box 1.2: Spatial variation of free-living bacterial communities

The diversity of bacterial communities on Earth is tremendous (Fenchel *et al*., 1997; Horner-Devine *et al*., 2004; Martiny *et al*., 2006) and spatial heterogeneity of diversity is caused by variation in local conditions as well as geographical structure (De Deyn & Van der Putten, 2005; Fierer & Jackson, 2006; Lozupone *et al*., 2007; Nemergut *et al*., 2011; Ramette & Tiedje, 2007). Several studies assessed the global distribution of free-living microbes, such as in soil, aiming to uncover potential driving factors (e.g., Delgado-Baquerizo *et al*., 2018; Fierer & Jackson, 2006).

With a growing body of literature on microbial biodiversity patterns spanning various spatial and temporal scales, researchers sought to explain these patterns using classical ecological theory grounded on plant and animal biogeographical distributions. However, as opposed to our understanding of global

biogeography of vertebrates (Lamoreux *et al*., 2006), consensus remains elusive among microbial ecologists on whether global microbial diversity (far) exceeds local diversity, or that most microbial taxa are omnipresent in suitable habitats around the globe (Foissner, 2006; van der Gast, 2015; Meyer *et al*., 2018; Whitaker *et al*., 2003). While some evidence for a latitudinal diversity gradient of microorganisms exists (Andam *et al*., 2016; Fuhrman *et al*., 2008), abiotic conditions, such as soil pH (Chu *et al*., 2010; Fierer & Jackson, 2006; Lauber *et al*., 2009) and aridity (Maestre *et al*., 2015), shape soil bacterial community composition at local and global scales. The general desire to explain spatial patterns of microbial biodiversity has led to an explosion of studies linking spatial and temporal patterns of microbial diversity to a suite of climate variables, such as precipitation levels, temperature, and physicochemical properties of the environment (e.g., Fierer & Jackson, 2006; Horrocks *et al*., 2012; Lauber *et al*., 2009; Martiny *et al*., 2006; Serna-Chavez *et al*., 2013). Overall bacterial diversity seems to differ among macro-scale habitats such as saline vs. non-saline ecosystems (Dini-Andreote *et al*., 2014), and among sediment types (Lozupone *et al*., 2007; Pasternak *et al*., 2013; Wang *et al*., 2013), which indicates niche-specificity of bacterial community diversity and composition. In addition to this macro-scale community structuring, it seems that more abundant bacterial taxa are also (geographically) more widely distributed than rarer taxa (Humbert *et al*., 2009; Nemergut *et al*., 2011), suggesting that some taxa do have a cosmopolitan distribution, while others do not. Because most taxa are far from abundant (Sogin *et al*., 2006), such taxa might be more dependent on local environmental conditions, which as a consequence may restrict their dispersal opportunities. The different factors that successfully explain variation in microbial abundance, diversity and community composition, across biomes and locally, and at various taxonomic scales, highlight the complexity of predicting microbial biogeographical patterns.

Large numbers of microorganisms adopted a host-associated lifestyle (Braendle *et al*., 2003; Colston & Jackson, 2016; Grond *et al*., 2017; Neish, 2009; Nicholson *et al*., 2012; Waite & Taylor, 2015; Yatsunenko *et al*., 2012). However, it is currently poorly understood where host-associated microbial communities originate from; a topic of ongoing debate. Some authors propose that host-associated microbes coevolve with their hosts (Bordenstein & Theis, 2015; Brooks *et al*., 2016; Zilber-Rosenberg & Rosenberg, 2008; but see Groussin *et al*., 2020), whereas others propose that environmental factors are the key drivers of host-associated microbial community assembly (Douglas & Werren, 2016; Moran & Sloan, 2015; Rothschild *et al*., 2018). To test if and how the microbial pressure drives variation in immune function it is of crucial importance to study the characteristics of microbial communities in the environment, how they affect host-associated microbiota, and how each of these components influences immune function, separately, or in concert.

Mirroring the description of macroorganismal natural history over the last centuries, (microbial) ecologists have begun to describe the communities of microorganisms that associate with animals and plants. Both the environmental and host-associated microbiotas can be conceptually viewed as two components of the antigenic universe

that may not be independent. Insights in the (relative) role of environmental and host-associated microbial communities shaping host defences can only be obtained when light is shed on their mutual relationships. Using sequencing technology and statistical tools, we can study the dynamic nature of host-associated microbial communities and evaluate the ecological factors and processes that govern their assembly. In this thesis, my colleagues and I sought to link bird-associated microbial communities to the communities in the bird's surroundings as an essential first step towards decoupling their effects on avian immune function (**Chapter 2 and 4**).

Study system – birds and their eggs

The research presented in this thesis was conducted on nine species of wild passerine birds of the family Alaudidae (Larks) and on captive zebra finches *Taeniopygia guttata*. Larks provided a particularly interesting perspective on the interactions between animals and their environment: in addition to striking behavioural and ecological similarities among different lark species as typically terrestrial birds, larks inhabit a wide range of environments across a large geographical range including deserts, temperate and tropical grasslands (del Hoyo *et al*., 2004). The species distributions across these biomes are, however, not phylogenetically structured (Alström *et al*., 2013; del Hoyo *et al*., 2004), and analysis of interspecific host-associated microbiome variation thus allowed interpretation of the impact of the environmental differences. A rich body of work similarly utilized this group of species to investigate other aspects of their physiology in an eco-evolutionary context (e.g., Hegemann *et al*., 2013, 2012; Horrocks *et al*., 2012, 2015; Tieleman *et al*., 2003). Sympatric breeding populations of woodlark *Lullula arborea* and skylark *Alauda arvensis* at Aekingerzand, The Netherlands (N 52°55'; E 6°18'), provided opportunities to examine if different host species harbour distinct microbial communities under the same environmental conditions. Their sympatry allowed assessment of the direct relationships between their host-associated microbiotas and the microbial communities in their environment. Finding nests and sampling females and eggs in these populations allowed us to investigate the relationships between maternal and egg microbiotas to explore the potential of eggs as transgenerational carriers of maternal microbes.

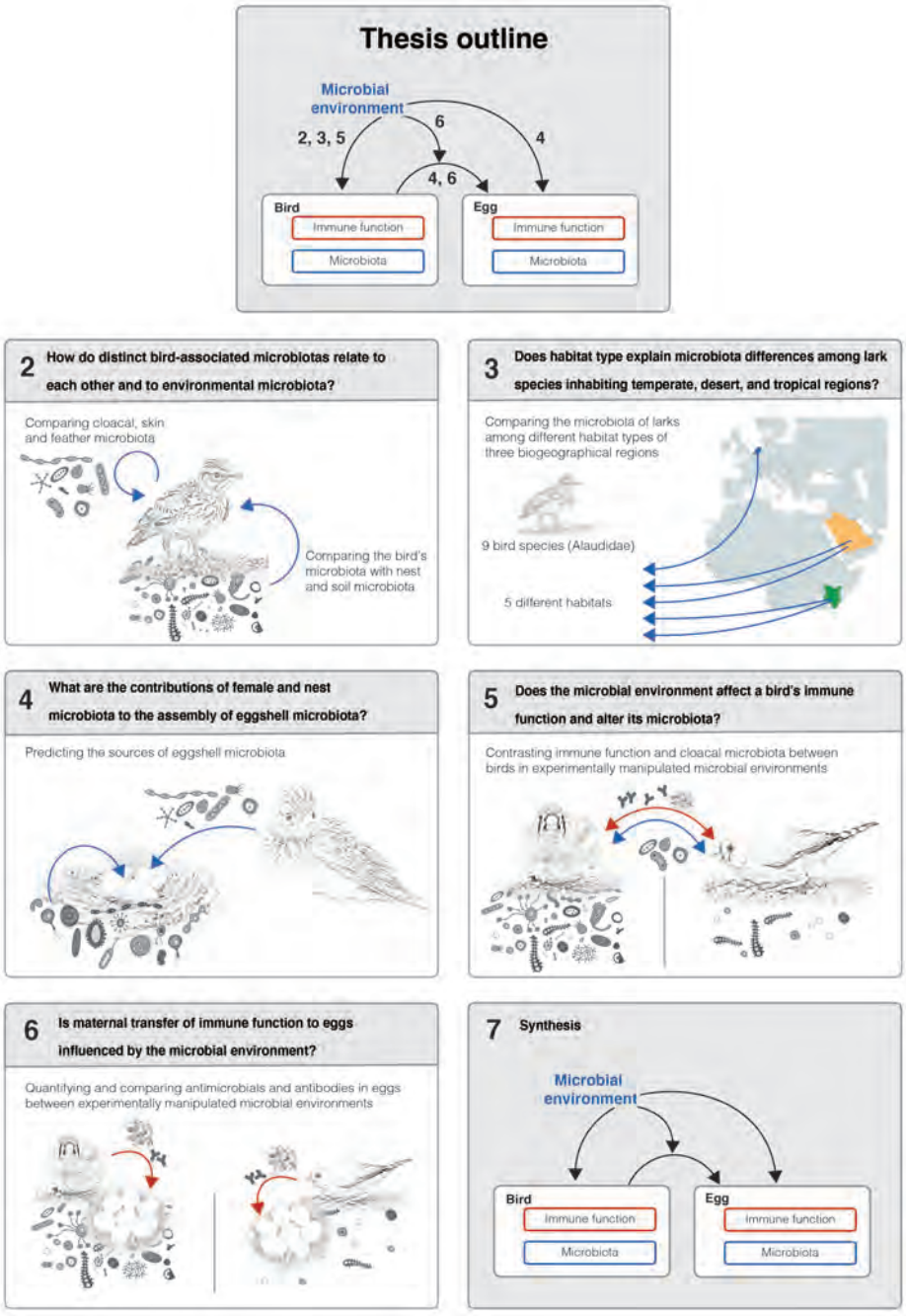

Figure 1.2: Outline of this thesis. The research questions addressed in this work are aimed to elucidate if the microbial environment shapes the microbiota and influences immune function of birds.

To test the antigen-exposure hypothesis it is crucial to experimentally manipulate microbial pressure. By manipulating the microbial environment of zebra finches my colleagues and I simultaneously tested the influence of the environmental microbial pressure on immune function and on the host-associated microbiota. We subjected captive zebra finches to sterilized and non-sterilized soil microbial communities in the bird's environment to test if immune function, the diversity, composition and temporal dynamics of cloacal microbiota, and their covariation differ between these environments. This experimental design also allowed us to investigate if microbial pressure from the environment affects transgenerational transmission of immunity to eggs as a non-genetic maternal effect.

Aim of the thesis

The aim of this thesis is to augment our understanding of underexplored ecological interactions between avian immune function and microbial communities associated with birds and their environment. By making use of the natural distributions of birds, as hosts, as well as using experimental manipulation of the microbial environment of captive birds, my colleagues and I first search for relationships between the microbial environment and host-associated microbiota of birds. We then aim to integrate the gained insights with experimental data of immune function to provide a perspective on the influence of environmental microbial pressure on immune function.

Research questions

This thesis addresses the main research question of whether the microbial environment influences the microbiota and immune function of birds.

Specific research questions asked in this thesis (Fig. 1.2):

- How do different types of bird-associated microbiotas relate to each other and to environmental microbiota?
- Does habitat type explain microbiota differences among lark species inhabiting temperate, desert, and tropical regions?
- What are the contributions of female and nest microbiota to the assembly of eggshell microbiota?

- Does the microbial environment affect a bird's immune function and alter its microbiota?
- Is maternal transfer of immune function to eggs influenced by the microbial environment?

Thesis outline

The conceptual diagram depicting the outline of this thesis (Fig. 1.2) illustrates hypothesized direct and indirect relationships between the microbial environment and (female) birds and their eggs. To answer the research questions, we described host-associated microbiota variation in the context of the natural distribution of birds at local and cross-biome scales (**Chapter 2, 3, 4**). In these chapters, we related the host-associated microbiota (and of their eggs) with the microbial communities that live in the bird's surroundings to gain insight into the influence of the microbial environment. We then applied experimentally-induced differences in the microbial diversity of the environment to test the influence of environmental microbial diversity on host-associated microbiota, immune function (**Chapter 5**) and its transgenerational transmission (**Chapter 6**).

My colleagues and I started with comparing distinct compartments of the host-associated microbiome (i.e. skin, feathers and cloaca) between sympatric bird species that experience the same climatic, biotic and abiotic conditions during a single breeding season (**Chapter 2**). In this initial research chapter, we hypothesized that co-habiting bird species harbour microbial communities with similar characteristics (i.e. taxon richness, dominant taxa and compositions) across their body. To investigate the role horizontal acquisition may play in the assembly of host-associated microbiota, we also compared different components of the bird's microbiota with the microbial communities of the nest environment. We additionally used a null model approach to ask if deterministic or stochastic processes structure of host-associated communities.

In **Chapter 3**, my colleagues and I ask if variation in the host-associated microbiome of nine closely-related bird species distributed across three different biomes can be explained by their geographical segregation. We describe different characteristics at which host-associated microbiotas of different lark species differ among habitats that span temperate, desert and tropical biomes. Similar to **Chapter 2**, but at large geographical

scale, we ask if environmental factors are important in structuring microbiota variation among larks.

In **Chapter 4**, my colleagues and I explored the function of eggshells as carriers of maternal microorganisms to offspring, a hypothesized mechanism of vertical transmission. Vertical symbiont transmission is a common process in insects, but has no unequivocal evidence in mammals, and has not extensively been investigated in oviparous vertebrates such as birds. To explore if eggshells could function as potential carriers of maternal microbiota to offspring, we compared the cloacal microbiota of females with the communities associated with the shells of their eggs before incubation was initiated.

In **Chapter 5**, my colleagues and I tested the antigen-exposure hypothesis which proposes that vertebrate immune function is shaped by the microbial environment that animals experience. We manipulated the diversity of the microbial environment to test how microbial environment affects aspects of innate and adaptive immunity of adult female zebra finches *Taeniopygia guttata*. This experiment simultaneously permitted testing effects of the microbial environment on host-associated microbiota characteristics and their temporal dynamics. We hypothesize that environmental microbial communities shape immune function by upscaling investment in immunity with increasing microbial diversity. We further ask if immune function and host-associated microbiotas correlate at the individual level.

In **Chapter 6**, my colleagues and I utilized the experimental set-up of **Chapter 5** to test the hypothesis that the microbial environment affects egg immune function by influencing maternal transfer of immunity to eggs. We assessed immune function in eggs by quantifying antimicrobial molecules in egg white and antibodies in yolk. We then explored indirect relationships between immune function in eggs and the condition, immune function and microbiota of females using path modelling. This approach allowed us to provide a systems-level perspective on how females may prime offspring for the microbial environment they will face later in life.

In the final **Chapter 7** I present a synthesis in which I present a general discussion on the results presented in the preceding chapters. I first discuss the patterns of host-associated microbiomes in the light of eco-evolutionary factors and processes underlying microbial community assembly of vertebrates. Then, I use these insights in host-microbiome ecology in combination with patterns of immunological variation to discuss and generalize the

role of microorganisms in causing variation in vertebrate immune function. I end with providing my perspective on future research avenues for incorporating microbial ecology in ecological immunology.

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Chapter 2

Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks

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Abstract

Working towards a general framework to understand the role of microbiota in animal biology requires the characterisation of animal-associated microbial communities and identification of the evolutionary and ecological factors shaping their variation. In this study we described the microbiota in the cloaca, brood patch skin and feathers of two species of birds and the microbial communities in their nest environment. We compared patterns of resemblance between these microbial communities at different levels of biological organisation (species, individual, body part), and investigated phylogenetic structure to deduce potential microbial community assembly processes. Using 16S rRNA gene amplicon data of woodlarks *Lullula arborea* and skylarks *Alauda arvensis*, we demonstrated that bird- and nest-associated microbiota showed substantial OTU co-occurrences and shared dominant taxonomic groups, despite variation in OTU richness, diversity and composition. Comparing host species, we uncovered that sympatric woodlarks and skylarks harboured similar microbiota, dominated by Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Acidobacteria. Yet, compared with the nest microbiota that showed little variation, each species' bird-associated microbiota displayed substantial variation. The latter could be partly ($\sim$20%) explained by significant among-individual differences. The various communities of the bird's body (cloaca, brood patch skin and feathers) appeared connected with each other and with the nest microbiota (nest lining material and surface soil). Communities were more similar when the contact between niches was frequent or intense. Finally, bird microbiota showed significant phylogenetic clustering at the tips, but not at deeper branches of the phylogeny. Our interspecific comparison suggested that environment is more important than phylogeny in shaping the bird-associated microbiotas. In addition, variation among individuals and among body parts suggested that intrinsic or behavioural differences among females and spatial heterogeneity among territories contributed to microbiome variation of larks. Modest but significant phylogenetic clustering of cloacal, skin and feather microbiotas suggested weak habitat filtering in these niches. We propose that lark microbiota may be primarily, but not exclusively, shaped by horizontal acquisition from the regional bacterial pool at the breeding site. More generally, we hypothesize that the extent of ecological niche-sharing by avian (or other vertebrate) hosts may predict the convergence of their microbiota.

Introduction

Symbiotic associations between animals and microorganisms are omnipresent, and can play a fundamental role in animal evolution (Bosch & McFall-Ngai, 2011; McFall-Ngai *et al*., 2013; Moran, 2006; Shapira, 2016). Working toward a general framework to understand the role of microbiota in animal biology requires the characterisation of animal-associated microbial communities and identification of the evolutionary and ecological factors shaping their variation. Although establishing a general theory for eco-evolutionary dynamics of animal-microbial interactions has recently received considerable attention (Bennett & Moran, 2015; Bordenstein & Theis, 2015; Douglas & Werren, 2016; Moran & Sloan, 2015; Rosenberg & Zilber-Rosenberg, 2016; Zilber-Rosenberg & Rosenberg, 2008), general conceptualisation has proven to be difficult. This challenge is hampered by fundamental gaps that need to be filled: first, the great variety of animal-microbiota systems encompasses diverse animal ecologies, reproductive modes, and other life history traits that evolved in a wide range of (microbial) environments (Baumann, 2005; Colston & Jackson, 2016; Franzenburg *et al*., 2013; Human Microbiome Project Consortium *et al*., 2012; Kueneman *et al*., 2014; Ley *et al*., 2008; Moeller *et al*., 2013; Waite & Taylor, 2015). Second, host-microbial dynamics may vary among levels of biological organisation, e.g. between host species, among individuals, and across the body. Third, animal hosts acquire their microbial symbionts vertically from parents and horizontally from the environment (Bright & Bulgheresi, 2010; Funkhouser & Bordenstein, 2013; McFall-Ngai, 2002), but the strength of host-microbe associations and the relative contributions of vertical and horizontal transmission acquisition are still unknown for most systems.

Current ideas on the strength of animal-microbe associations range from tight host-symbiont coevolution and interdependence to loose symbiotic interactions (Dale & Moran, 2006; Gilbert *et al*., 2015; Moran, 2006; Moran *et al*., 2008). In vertebrates, the relationships among host-associated communities and connections with environmental communities are understudied (Pascoe *et al*., 2017) despite their alleged role in horizontal acquisition (Bright & Bulgheresi, 2010). Given the strong connection between symbiont transmission modes and the strength of host-microbe associations (Bright & Bulgheresi, 2010; Shapira, 2016), identifying the transmission modes in diverse systems and host lineages is a crucial step toward establishing general concepts. For instance, larger contributions of horizontal acquisition as compared to vertical symbiont transmission

in microbiota assembly might reduce the strength of vertebrate-microbe associations, diminishing their adaptive potential and shaping their eco-evolutionary dynamics.

Integrative microbiota surveys appraising the nested nature of host biological organisation (that is, species, populations, individuals, organs, etc.) could provide improved insights in the relationships among microbial communities at various scales. Starting at host species-level variation, some studies argued that the phylogenetic inertia in gut microbiota variation among animals supports the idea that hosts and microbiota codiversified or coevolved (Avena *et al*., 2016; Brooks *et al*., 2016; Moeller *et al*., 2016; Wang *et al*., 2015). However, host biogeography and behaviour can distinctly structure the bacterial microbiota of intraspecific populations, such as in humans (Yatsunenko *et al*., 2012) and great apes (Moeller *et al*., 2013), and ecological factors such as diet (Delsuc *et al*., 2014; Godoy-Vitorino *et al*., 2012; Kohl *et al*., 2017; Ley *et al*., 2008; Muegge *et al*., 2011) and habitat features have been demonstrated to affect microbiota variation among individuals (Amato *et al*., 2013; Rausch *et al*., 2016; Rawls *et al*., 2006; Ruiz-Rodríguez *et al*., 2014; Seedorf *et al*., 2015), populations (Weigel & Erwin, 2016) and species (Avena *et al*., 2016; Delsuc *et al*., 2014; Muegge *et al*., 2011). Moreover, the majority of vertebrate microbiome studies has focussed on gastrointestinal microbiota (Kohl *et al*., 2017; Ley *et al*., 2008; Moeller *et al*., 2016; Waite & Taylor, 2015), while many other components of vertebrate-microbiota systems (e.g. skin and oral microbiota) remained relatively underexplored in terms of origin, function, specificity, their reciprocal associations, and their relationships with the microbial environment (but see Roggenbuck *et al*., 2014).

The phylogenetic relatedness within bird-associated microbiota could provide insights in the ecological or evolutionary processes that operate during microbiota assembly (Emerson & Gillespie, 2008). From a metacommunity perspective (Costello *et al*., 2012), microbial community assembly at different parts of the bird's body (e.g., the cloaca, skin and feathers) can be viewed as discrete and permanent habitat patches harbouring local communities which are either neutrally assembled or are selected by local conditions and competition. Local communities are considered interconnected at a regional scale, which in this case comprises an individual's body or territory, but could flexibly scale up to a population, a species, or a study site, depending on the question of interest (Costello *et al*., 2012). Four key assembly processes are distinguished in the metacommunity framework: historical contingency, habitat filtering, dispersal-limitation, and random

assembly (Costello *et al*., 2012). When applied to bird microbiota, historically contingent assembly predicts that bird species harbour and maintain distinctive microbiomes (Brooks *et al*., 2016), retaining an ancestral signal in microbiome variation across the host phylogeny (Bordenstein & Theis, 2015; Brooks *et al*., 2016). Baas-Becking's statement "everything is everywhere, but the environment selects" (Baas-Becking, 1934), would predict habitat filtering in which local (a)biotic conditions select for particular microbial traits or members, and thus predicts that microbiota vary among body sites (e.g., Human Microbiome Project Consortium *et al*., 2012). Dispersal-limited assembly would predict differences among body sites, and between body sites and the environment as a result of different dispersal probabilities, spatial segregation (i.e. contact frequency), or barriers to overcome (Shafquat *et al*., 2014). Random (neutral) assembly predicts that local communities (body niches) are randomly assembled from the regional species pool (i.e. an individual microbiome or the bacteria present in a territory), which is expected to result in microbiota differences among individuals rather than that communities are mostly structured by body niche (Costello *et al*., 2012).

We aimed in this study to integrate different levels of comparison in a natural wild bird-microbiota system to evaluate the relationships among different animal-associated microbial communities and their association with environmental microbial communities in two sympatric bird species. We first described the bacterial communities of different body parts (cloaca, brood patch skin, feather) and nest environments (nest lining and surface soil) of sympatric woodlarks *Lullula arborea* and skylarks *Alauda arvensis* (Aves; Alaudidae). Then, to reveal patterns of resemblance at different levels of biological organisation of bird-associated microbiotas, we compared bacterial community diversity and composition between host species, among individual birds, and among distinct body parts along with their nest environments. Finally, we investigated the phylogenetic structure of the microbiota at each body part and used the resulting patterns to speculate about potential assembly processes that contributed to shaping bird-associated microbiota in the wild.

Methods

Study site and species

We studied sympatric breeding lark species at Aekingerzand, the Netherlands (N 52°55'; E 6°18'; described in Hegemann & Voesten, 2011). Woodlarks *Lullula arborea* and skylarks *Alauda arvensis* scrape shallow cups on bare soils to build their nests, primarily composed of dry grass stems and often adjoining heather or grass tussocks. Adult woodlark and skylark diet largely comprises arthropods during the breeding season (~70-80%), complemented by plant material and seeds (Donald, 2004; Glutz von Blotzheim *et al*., 1985). Breeding territories of both larks overlap, but in contrast to skylarks, woodlarks also exploit the area's peripheral forest clearings.

Sample collection

Between March and July 2014, we sampled adult female woodlarks (n = 15) and skylarks (n = 14) and their nest locations, comprising: cloaca, brood patch skin, body feathers, nest lining material, surface soil. We collected a total of 120 samples, including 20 complete sets and incomplete sets for nine females (see details in Table S2.1). We handled and sampled birds exclusively with 70% ethanol-sterilised gloves and equipment. We sampled the external and internal microbial niches of the bird in three ways: first, we swabbed the bare skin of the brood patch with a sterile cotton swab moistened with sterilised PBS solution. We then inserted a sterile cotton swab through the cloaca and sampled the microbiota by gentle rotation. Finally, we clipped the distal half of ~5 brood patch-lining body feathers with scissors and tweezers. After we released the bird, we collected nest lining material (~3 grass stems) from the centre of the nest cup and collected a composite soil sample of the surface within a 50-cm radius facing the nest entrance. All samples were stored in sterilised 2 ml screw-cap vials that we kept on ice in the field (< 12 h post-collection) and then stored at -20 °C.

DNA extraction and 16S rRNA gene amplification and sequencing

We aseptically peeled cloacal and brood patch skin swabs from the stalks to loosen cotton fibers and added all cotton to extraction tubes. We further transferred ~5 brood patch-lining feathers and ~ 3 stems of nest lining material each into sterile 15-ml tubes and

added 978 µl sodium phosphate buffer with 122 µl MT buffer (kit reagents of FastDNA™ SPIN Kit for Soil; MP Biomedicals, Santa Ana, CA, USA). We then vortexed the tubes 10 s using a Vortex-Genie2 (MoBio Laboratories Inc., Carlsbad, CA), sonicated tubes 15 min, and vortexed another 10 min to detach bacterial cells from the source materials. We transferred the cell suspensions to lysis tubes to complete the extraction. On average, we used (± S.E.M.) 0.3 ± 0.01 g soil per surface soil sample for total DNA extraction. We followed the manufacturer's protocol with minor adjustments for all samples: we enhanced cell lysis by three times 1 min bead beating on a mini bead beater (BioSpec Products, Bartsville, OK, USA). We eluted DNA in 100 µl PCR-grade water (Ambion, Austin, TX, USA) and subsequently quantified DNA concentrations using the Quant-it PicoGreen dsDNA kit (Molecular Probes, Invitrogen, Eugene, OR). We then amplified the V4/V5 region of the 16S rRNA gene using the primers 515F and 926R on the following thermal cycling protocol: 5 min at 95 °C, 35 cycles with 40 s at 95 °C, 45 s at 56 °C, 40 s at 72 °C, and finally 10 min at 72 °C. Nine collected samples did not amplify during PCR and could not be included for downstream analysis; see Table S2.1 for details. Finally, at GenoToul (INRA, Toulouse, France) purified amplicons (QIAquick gel extraction Kit, QIAGEN GmbH, Hilden, Germany) were extended with Illumina adapters using PCR and 7 pM of equal amounts of PCR products including adapters was then sequenced using the 2 x 250 bp v2 chemistry on an Illumina MiSeq platform.

Sequence data processing

We processed raw 16S rRNA gene sequence data using QIIME 1.9.0 (Caporaso *et al*., 2010a). The sequencing facility demultiplexed, and quality filtered reads using the default settings in QIIME: quality score ≥ 25, maximal ambiguous base calls = 6, maximum length of homopolymer run = 6, no primer mismatches. We then joined paired-end reads and truncated reverse primers from joined reads. Subsequently, we commenced an open-reference OTU-picking strategy against the Greengenes reference database (v. 13.8) (DeSantis *et al*., 2006) at 97% identity using the *uclust* algorithm (Edgar, 2010), and *de novo* OTU picking of a 0.1% random subset of reads that failed to match the reference set, following the QIIME tutorial (http://qiime.org/tutorials/open_reference_illumina_processing.html; accessed 10 August 2014). Subsequently, we picked representative sequences for all OTUs prior to merging both OTU tables. We removed all singletons to reduce effects of sequencing

errors on alpha diversity estimates. We then annotated taxonomic information against Greengenes (v. 13.8, 97% identity reference set) and subsequently aligned representative sequences using default settings with PyNast (Caporaso *et al*., 2010b). We identified and removed chimeric sequences using the uchime algorithm in the *usearch81* toolkit (Edgar *et al*., 2011), and constructed a phylogenetic tree using FastTree (Price *et al*., 2009). Finally, we filtered OTUs assigned to Archaea, Chloroplast and Mitochondria from the OTU table and retained OTUs with abundances > 0.01% of the total abundance. Rarefaction curves showed that OTU richness had not reached saturation, where Shannon diversity had levelled at 5000 reads per sample for each sample type (Fig. S2.1). Despite moderate coverage and saturation in our data set, the estimated total diversity clearly differed among sample types (Chao1; Fig. S2.1 c) and Shannon diversity estimates are likely to be unaffected at ∼5000 reads per sample. We removed a single low coverage sample (i.e. brood patch skin sample with 1049 reads), and subsequently rarefied all samples to 5000 reads/sample prior to analyses.

Statistical analyses

We analysed bacterial diversity based on rarefied and unrarefied data using the R packages *phyloseq* (v. 1.14.0) (McMurdie & Holmes, 2013) and *vegan* (v. 2.4-0) (Oksanen *et al*., 2016) using R statistical software (v. 3.2.3) (R Core Team, 2015).

Diversity within bacterial communities

We calculated OTU richness (hereafter 'OTU richness') and Shannon diversity from rarefied data, and used ANOVA with Tukey-Kramer post-hoc tests to analyse group differences between lark species ('Woodlark', 'Skylark') and sample types ('Cloaca', 'Brood patch skin', 'Feather', 'Nest lining', 'Surface soil'), with verification of the normality of residual errors (Q-Q plots) and homoscedasticity (fitted values ∼ residuals plot). We report adjusted *P*-values for pairwise Tukey-Kramer contrasts using the default single-step method of the *multcomp* package (Hothorn *et al*., 2016). We evaluated general differences in OTU richness and Shannon diversity among individual females and their nests by modelling Nest ID as a random factor to our initial ANOVA, fitted by restricted maximum likelihood (REML) using the *nlme* package (Pinheiro *et al*., 2016). These models were run on all sample types, and on subsets containing either female-associated or nest-associated samples. We tested the significance of the random Nest ID effect using a likelihood ratio test

comparing the REML fitted mixed-effects model with a REML fitted linear model without the random Nest ID term (Pinheiro & Bates, 2000), and calculated the explained variance proportion by the random term using the *MuMIn* package (Nakagawa & Schielzeth, 2013). We compared and visualised OTU co-occurrences among the different sample types using venn diagrams for woodlarks and skylarks separately using the methods provided by the Hallam Lab (https://github.com/hallamlab/mp_tutorial/wiki/Introduction-to-Downstream-Analysis-in-R). For both host species separately, we identified and described the most abundant OTUs in each bird-associated sample type with a mean abundance threshold of 5% across all samples per sample type using the core_microbiome function provided by David Elliott (https://github.com/davidelliott/core-microbiome/blob/master/core-microbiome.Rmd). In order to identify differential OTU abundance in woodlarks and skylarks for each sample type we performed Analysis of Composition of Microbiomes (ANCOM) (Mandal *et al*., 2015) with a critical false discovery rate (FDR) corrected *q*-value of 0.05.

Pairwise community similarity between hosts and among sample types

We assessed bacterial community composition (beta diversity) using weighted UniFrac (Lozupone & Knight, 2005) and Bray-Curtis dissimilarities to evaluate phylogenetic similarity among groups and group dispersion (i.e. mean distance to the cluster centroid to represent the variation among individuals within a sample type). We performed principal coordinates analysis (PCoA) using *vegan*. In order to test if different sample types and lark species affected community clustering, we modelled weighted UniFrac distances and Bray-Curtis dissimilarities from an OTU-level table using PerMANOVA with 999 permutations ('adonis' function in *vegan*) (Anderson, 2001; McArdle & Anderson, 2001). We used the 'betadisper' function (Anderson, 2006) in *vegan* to evaluate the degree of within-group dispersions among sample types and calculated group differences using Kruskal-Wallis tests with a post-hoc Dunn's test for multiple comparisons (Pohlert, 2016). We reported FDR-corrected *q*-values. This method allowed us to determine whether PCoA clustering of weighted UniFrac distances were due to location effects of dispersion effects.

Among-individual and among-sample type effects on pairwise similarities

In order to test whether overall differences among females could explain additional variation in community clustering, i.e. individuality of female-nest dyads, we added female ID as a predictor to the PerMANOVA, with permutations restricted per lark species. We calculated the phylogenetic similarity (weighted UniFrac) among pairs of sample types and used ANOVA to determine mean pairwise differences among sample types. All effect sizes and their significance were evaluated with post-hoc pairwise Tukey-Kramer contrasts. We additionally evaluated intrinsic structure of the data using portioning around medoids (PAM) using the *cluster* package (Maechler *et al*., 2016) to evaluate how samples would cluster without prior metadata information.

Null model of phylogenetic community structure

We used a null modelling approach of our 97% identity-based community tables to evaluate the phylogenetic structure of OTUs within each community (following Kembel, 2009; Webb *et al*., 2002). We used the *picante* package (v. 1.6-2) (Kembel *et al*., 2010) to calculate the average distance between co-occurring phylogenetic relatives (observed Mean Nearest Taxon Distance; $MNTD_{obs}$), and the mean pairwise phylogenetic distance (observed Mean Phylogenetic Distance; MPD_{obs}) among all pairs of species in each sample (local community). Then, by comparing observed values with a null distribution ($MNTD_{null}$ and MPD_{null}) following Bates *et al*. (2015), we calculated the standardised effect size for every sample and each metric, which is referred to as -1 times Nearest Taxon Index (NTI) or Net Relatedness Index (NRI) (Kembel, 2009; Webb *et al*., 2002), respectively. We generated the null distributions using the 'independent swap' algorithm (Gotelli, 2000; Gotelli & Entsminger, 2003), referred to as 'null model 4' in Kembel (2009), in which species co-occurrences were randomised 1000 times per randomisation, maintaining species richness and occurrence frequencies in each sample type community's phylogenetic tree. We finally inferred whether phylogenetic clustering or phylogenetic evenness was observed in each sample type (which is expected when the average NTI or NRI value is different from the null communities) by testing whether the mean NTI or NRI value of each sample type differed from zero. Tests were performed using ANOVA and post-hoc Tukey-Kramer pairwise contrasts.

Results

Our sequencing effort produced 5 054 382 quality filtered sequences after removal of singletons, clustered in 1148 OTUs with minimum abundance of 0.01%. The coverage range was 5225 – 80815 reads per sample in the analysed samples. The ranges per sample type were as follows: cloaca: 5225-55146; brood patch skin: 8938-80579; feathers: 8198-64463; nest lining: 7511-64091; surface soil: 11275-50995. Rank-abundance plots for the five sample types are similar (Fig. S2.2), indicating dominance of a few types and a long tail of less abundant OTUs. Samples were rarefied to 5000 sequences to avoid biases due to sequencing effort. Of 1148 OTUs, 4.9% could be assigned to species-level, 35.4% to genus-level, and 83.9% to family-level. OTU richness differed among sample types ($F_{4,104} = 18.2$, $P < 0.001$; Fig. 2.1 a, Table 2.1).

Richness and diversity of bird- and nest-associated microbiota of woodlarks and skylarks

Body feathers harboured the richest microbiota compared to cloaca, brood patch skin, nest lining and surface soil communities in both larks. In woodlarks, the mean (± S.E.M.) number of OTUs recovered from feather communities (473 ± 30) were almost double, and in skylarks (478 ± 25) about 1.7 times the number found in their respective cloacal samples. OTU richness did not differ between lark species in any of the sample types (lark species: $F_{1,100} = 0.17$, $P = 0.86$; sample type x lark species: $F_{4,100} = 0.28$, $P = 0.89$). Shannon diversity varied among sample types ($F_{4,104} = 16.65$, $P < 0.001$), but not between the woodlarks and skylarks ($F_{1,104} = 1.07$, $P = 0.30$) (Fig. 2.1 b). Mean (± S.E.M.) Shannon diversity in cloacal communities of woodlarks (3.28 ± 0.36) and skylarks (3.39 ± 0.35) was lower than in other sample types, though in woodlarks, the difference with brood patch skin communities received no statistical support (Table 2.1). An OTU table including reads that did not match the Greengenes reference set produced similar patterns (Fig. S2.3 a, b).

The degree of variation in OTU richness and Shannon diversity within each sample type was largest in the bird-associated sample types compared to the nest environment (Fig. 2.1 c). In an attempt to explain the substantial variation in richness and diversity, we tested whether part of the variation might be due to general differences among females. A random Female ID term substantially improved model support (likelihood ratio test

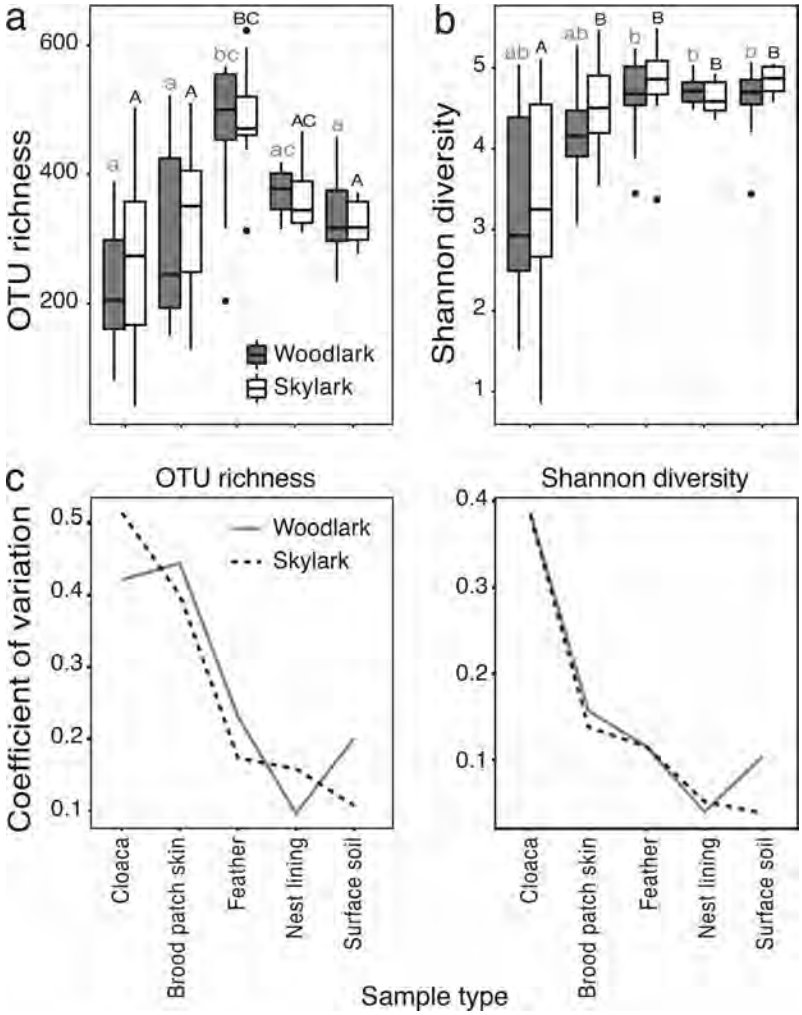

Figure 2.1: Alpha diversity metrics (a-c) and relative abundance (d) of major bacterial taxa across sample types of sympatric lark species. Bacterial OTU richness (a) and Shannon diversity (b) consistently vary within sample types, whereas (c) unbiased estimates of the coefficients of variation show decreasing trends of variability of OTU richness and Shannon diversity of bacterial communities, evaluated for each sample type and ordered from the bird's internal community outward to the surface soil communities. (a, b) Letters represent pairwise contrasts ($P < 0.01$) of sample type means of woodlarks (lower case grey) and skylarks (capital black).

Table 2.1: Pairwise ANOVA statistics of bacterial alpha diversity among sample types.

		OTU richness							
		Pairwise Tukey-Kramer contrasts among sample types							
		Woodlark				Skylark			
Pairwise comparison		Estimate	SE	*t*	P_{adj}	Estimate	SE	*t*	P_{adj}
Cloaca	Brood patch skin	-73.75	40.85	-1.806	0.728	-54.44	40.09	-1.358	0.936
	Feathers	-242.83	39.17	-6.199	<0.001	-212.08	40.09	-5.29	<0.001
	Nest lining	-139.52	39.17	-3.562	0.019	-89.65	45.87	-1.954	0.630
	Surface soil	-106.15	41.89	-2.534	0.262	-51.74	42.43	-1.219	0.967
Brood patch skin	Feathers	-169.08	40.09	-4.218	<0.01	-157.64	41.72	-3.778	<0.01
	Nest lining	-65.78	40.09	1.641	0.824	-35.21	47.31	-0.744	0.999
	Surface soil	-32.4	42.75	0.758	0.999	2.697	43.98	0.061	1.000
Feathers	Nest lining	103.31	38.38	2.692	0.190	122.43	47.31	2.588	0.237
	Surface soil	136.68	41.16	3.321	0.04	160.33	43.98	3.646	0.015
Nest lining	Surface soil	33.37	41.16	0.811	0.998	37.91	49.31	0.769	0.999

		Shannon diversity							
		Pairwise Tukey-Kramer contrasts among sample types							
		Woodlark				Skylark			
Pairwise comparison		Estimate	SE	*t*	P_{adj}	Estimate	SE	*t*	P_{adj}
Cloaca	Brood patch skin	-0.87	0.286	-3.061	0.095	-1.14	0.280	-4.069	<0.001
	Feathers	-1.37	0.274	-5.012	<0.001	-1.41	0.280	-5.041	<0.001
	Nest lining	-1.46	0.274	-5.319	<0.001	-1.27	0.320	-3.971	<0.010
	Surface soil	-1.32	0.293	-4.491	<0.001	-1.48	0.267	-5.009	<0.001
Brood patch skin	Feathers	-0.50	0.280	-1.779	0.819	-0.27	0.292	-0.934	0.993
	Nest lining	-0.58	0.280	-2.079	0.626	-0.13	0.331	-0.403	1.000
	Surface soil	-0.44	0.299	-1.477	0.941	-0.35	0.308	-1.124	0.993
Feathers	Nest lining	-0.08	0.268	-0.313	1.000	0.14	0.331	0.421	1.000
	Surface soil	0.06	0.287	0.199	1.000	-0.07	0.308	-0.238	1.000
Nest lining	Surface soil	0.14	0.288	0.491	1.000	-0.21	0.345	-0.616	1.000

(LRT) = 6.51, $P < 0.05$), and explained 10% of the total variance of OTU richness, while sample type explained 41% of the variance within nests. Female ID did not significantly explain variation in Shannon diversity (LRT = 0.55, $P = 0.46$). We also modelled the random

Female ID term on OTU richness in a data set restricted to the bird-associated samples (i.e. excluding nest lining and surface soil) (LRT = 5.17, $P < 0.05$), and found that the proportion of variance explained by Female ID increased to 18%, with sample type accounting for 43% of variance within females. A similar model that included only nest lining and surface soil communities did not reveal such individual differences (LRT = 0.32, $P = 0.57$), thus demonstrating that Female ID primarily predicted richness variation in bird-associated bacterial communities, but not among nest-associated communities.

OTU co-occurrence patterns between bird- and nest-associated microbiota of woodlarks and skylarks

Analysis of OTU co-occurrence patterns showed that 78%, 80% and 89% of the OTUs identified in cloacal, brood patch skin and feathers, respectively, were shared between woodlarks and skylarks. Comparisons of sample types revealed that the majority of OTUs on the female body are shared among cloacal, skin and feather communities, and in similar proportions for both larks (woodlark: 72%; skylark: 71%; Fig. 2.2 a, b). Cloacal microbiotas of woodlarks and skylarks harboured a small proportion of unique OTUs (woodlark: 5%; skylark: 7%) compared to communities of the nest environment with which they shared 50% and 46%, respectively (Fig. 2.2 c, d). With a majority of OTUs shared by both external body niches (brood patch skin and body feathers) and both environmental communities (woodlark: 51%; skylark: 44%), skin and feathers harboured few unique OTUs (Fig. 2.2 e, f). Our proxy for the microbial environment of breeding larks, i.e. nest material and surface soil around the nest, showed that nest materials and surface soils each harboured a substantial number of unique OTUs, and illuminated the complexity of the microbial environment (Fig. 2.2 g, h).

Relative taxon abundances in the microbiota of woodlarks, skylark and their nests

Of a total of 15 identified bacterial phyla, nine phyla dominated the lark's microbiota (cumulative abundance > 94.3%) and included the bacterial phyla characterised as dominant in avian gut microbiota studies: Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes and Firmicutes. The relative abundances of each of the 15 phyla significantly varied among the sample types (ANCOM, FDR $q < 0.05$) but the general pattern

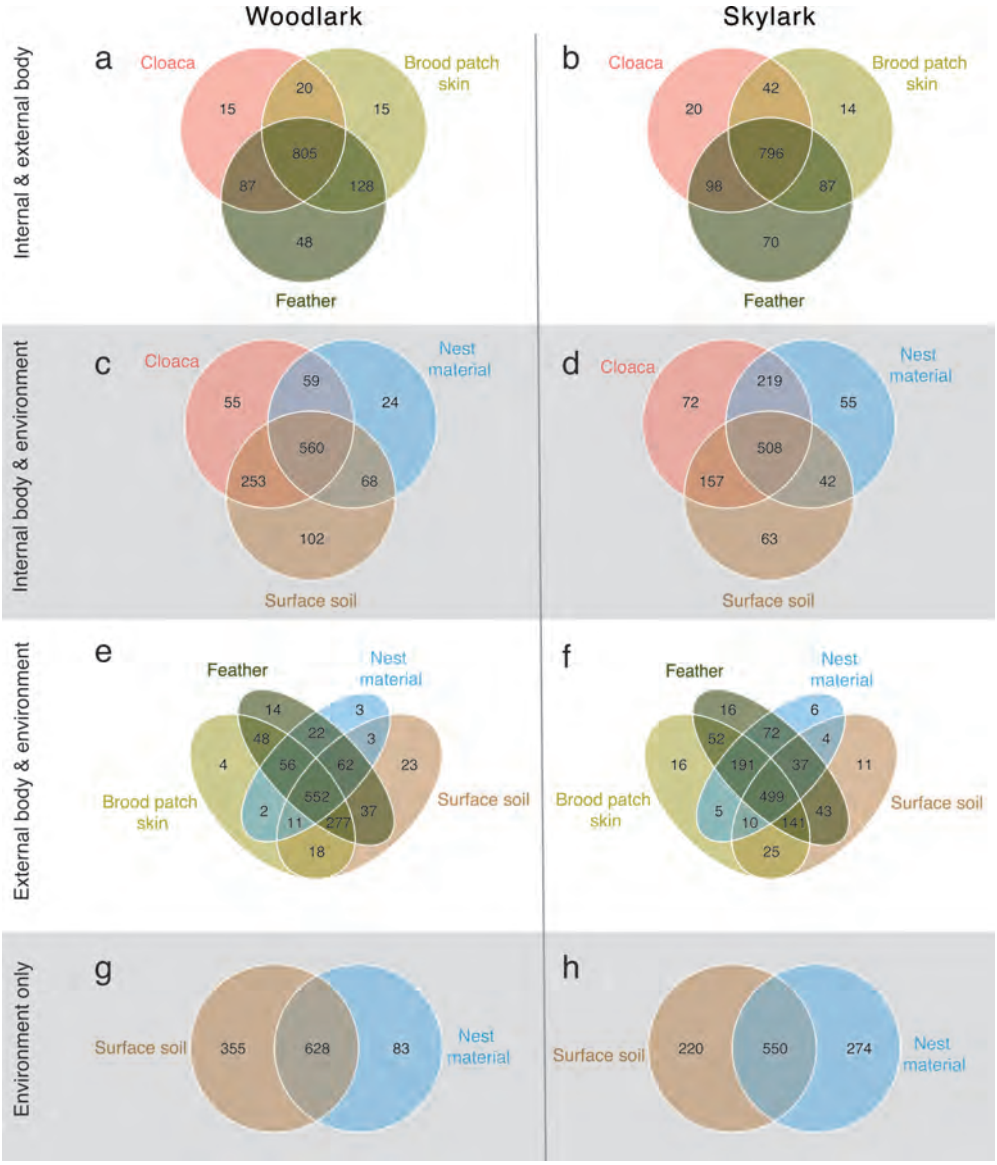

Figure 2.2: Venn diagrams of co-occurring OTUs among sample types for woodlarks and skylarks. (a, b) Comparison of internal (cloaca) and external (brood patch skin and feather) communities, (c, d) internal and environmental (nest material and surface soil) communities, (e, f) external and environmental communities, and (g, h) environmental communities of woodlarks and skylarks, respectively. Numbers in each compartment denote the number of unique and shared OTUs of the (non-) overlapping communities, respectively.

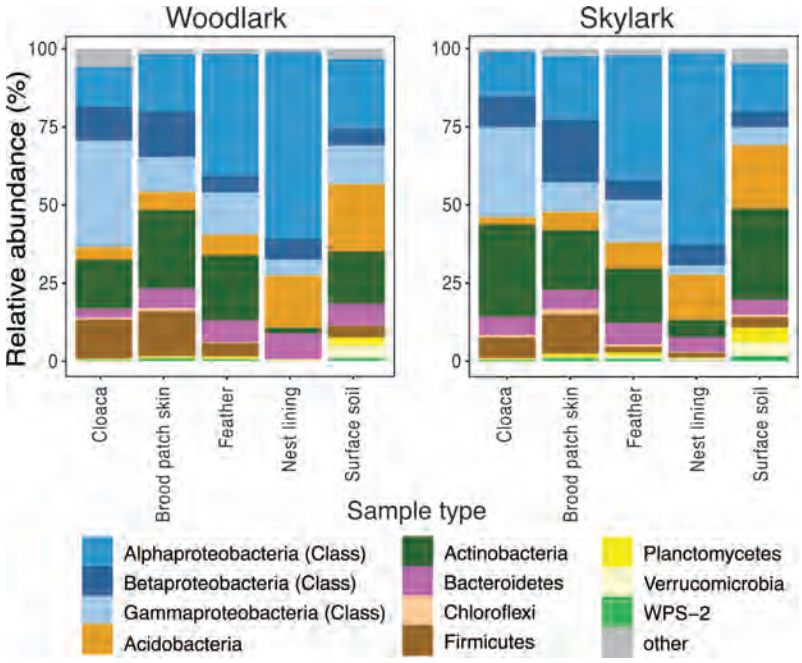

Figure 2.3: Barplots of relative abundances of the most abundant bacterial phyla and Proteobacteria classes in each sample type in woodlarks and skylarks. Calculations were based on rarefied data (5000 reads/sample).

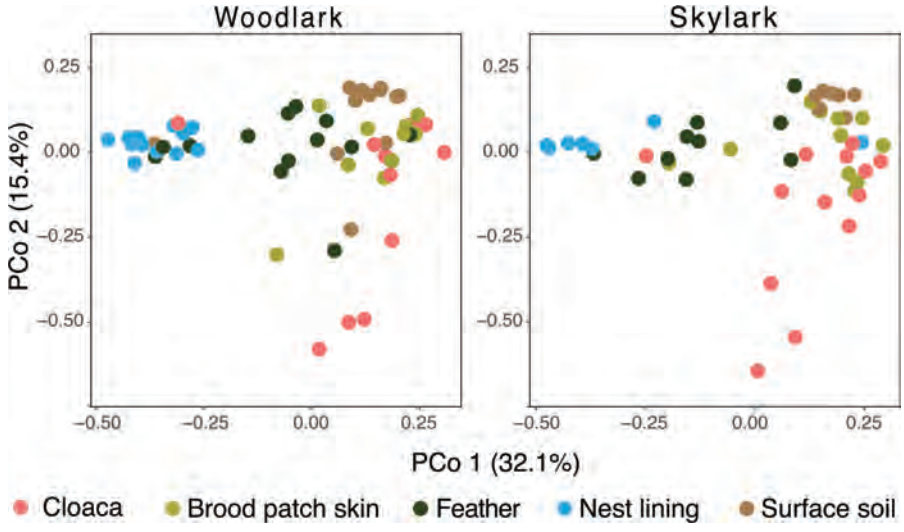

Figure 2.4: Beta diversity of bacterial communities associated with different sample types of sympatric lark species. Principal coordinates analysis (PCoA) on weighted UniFrac distances among sample types is shown along the first two principal coordinate axes, and was calculated on a single rarefied data set and visualised for both species separately. Clustering significance was determined by PerMANOVA. Sample type (33%; $P < 0.001$) and lark species (1%, $P < 0.05$) explained of total variation.

was consistent in both lark species (Fig. 2.3). Proteobacteria comprised the most dominant phylum in the bird-associated microbiota and on nest lining material, but were relatively less abundant in surface soil communities. However, within these Proteobacteria-dominated microbiota, class-level patterns showed that cloacal microbiota harboured on average the largest fraction of Alphaproteobacteria, brood patch skin microbiota the highest proportion of Betaproteobacteria and feathers and nest lining communities predominantly Gammaproteobacteria (Fig. 2.3). Actinobacteria comprised the second dominant phylum in bird microbiota, and in contrast to Proteobacteria, comprised a larger proportion of soil communities than of nest material communities. Acidobacteria were relatively more abundant in the environmental communities than in bird microbiota, and Firmicutes appeared relatively more abundant in cloacal and brood patch skin communities. The patterns were, however, highly variable among individuals and particularly at higher taxonomic resolution (Fig. S2.4 a-f). ANCOM identified only 4 out of 1148 OTUs (Firmicutes: Aerococcaceae OTU1110381, Proteobacteria; Neisseriaceae OTU965048, FBP; OTU224307, Planctomycetes; Gemmataceae OTU1042) that varied significantly in abundance between woodlarks and skylarks, presumably resulting from the large variation among individual birds. These higher-level abundance patterns (Fig. 2.3) were partly the result of a few dominating OTUs (Fig. S2.4). OTUs belonging to Oxalobacteraceae and Enterobacteriaceae were revealed as dominant Proteobacteria OTUs in cloacal communities of both lark species (Fig. S2.4 a, b). Furthermore, the most abundant Actinobacteria OTUs in most cloacal microbiota samples were represented by Intrasporangiaceae in woodlarks and Corynebacteriaceae in skylarks. In the brood patch skin communities of woodlarks and skylarks, OTUs belonging to the families Oxalobacteraceae, Enterobacteriaceae and Methylocystaceae were the most dominant Proteobacteria taxa, and a Pseudonocardiaceae OTU appeared the most abundant OTU from Actinobacteria in both larks, with Intrasporangiaceae also being a dominant taxon on woodlark skin (Fig. S2.4 c, d). An OTU belonging to Solibacteraceae dominantly represented the Acidobacteria, and an OTU belonging to Chitinophagaceae dominantly represented Bacteroidetes in both species. Feather microbiota constituted of the same dominant OTUs as brood patch skin microbiota but with an additional high prevalence of Acetobacteriaceae as a member of Proteobacteria, and an OTU representing Acidobacteriaceae replacing the Solibacteraceae OTU in the Acidobacteria phylum (Fig. S2.4 e, f).

Community resemblance between host species, among individuals and within each sample type

Analysis of beta diversity based on weighted UniFrac distances revealed differential community clustering of sample types (PerMANOVA, pseudo-F = 13.2, df = 4, 104, R^2 = 0.33, P < 0.001; Fig. 2.3), but only weakly supported clustering of woodlarks and skylarks (pseudo-F = 2.19, df = 1, 104, R^2 = 0.01, P < 0.05). In addition to sample type (R^2 = 33%) and lark species (R^2 = 1%), Female ID explained an additional 20% of variation in clustering of weighted UniFrac distances (PerMANOVA, pseudo-F = 1.24, df = 27, 77, R^2 = 0. 20, P < 0.05), suggesting significant bacterial community convergence at the level of individual hosts. Consistent results based on Bray-Curtis are shown in Fig. S2.5. Note that PCoA clustering of samples using a weighted UniFrac matrix based on an OTU table constructed with the full set of reads that did not match the Greengenes reference set were similar to the patterns presented here (Fig. S2.3 c). Sample cluster analysis by partitioning around medoids (PAM) did not reveal an optimal number of K clusters (Fig. S2.6 a), but showed that nest lining and surface soil communities clustered reasonably good when K = 5 (i.e. number of expected clusters; Fig. 2.4) was chosen for ordination (Fig. S2.6 b, c). Female-associated samples were only modestly clustered according to sample type when ordination was based on intrinsic structure of the data. As expected, samples did not cluster in PCoA by individual female since sample type was clearly the strongest driver in community clustering (Fig. 2.4).

Comparing group dispersions in community composition among sample types

As a measure of between-individual variation in PCoA clustering of weighted UniFrac distances, the median distances to the cluster centroid of sample types varied (χ^2 = 34.4, df = 9, P < 0.001). This measure of community variability, also referred to as group dispersion, was highest in cloacal communities (Fig. 2.5 a), but this difference received only statistical support for the comparisons with the dispersion in nest lining communities (woodlark: Kruskal-Wallis H = 4.23, P < 0.001; skylark: H = 2.75, P < 0.05), and in surface soil (woodlark: H = 1.63, P = 0.09; skylark: H = 3.71, P < 0.01). Pairwise Dunn's contrasts revealed that the distances to the cluster centroids of none of the sample type communities

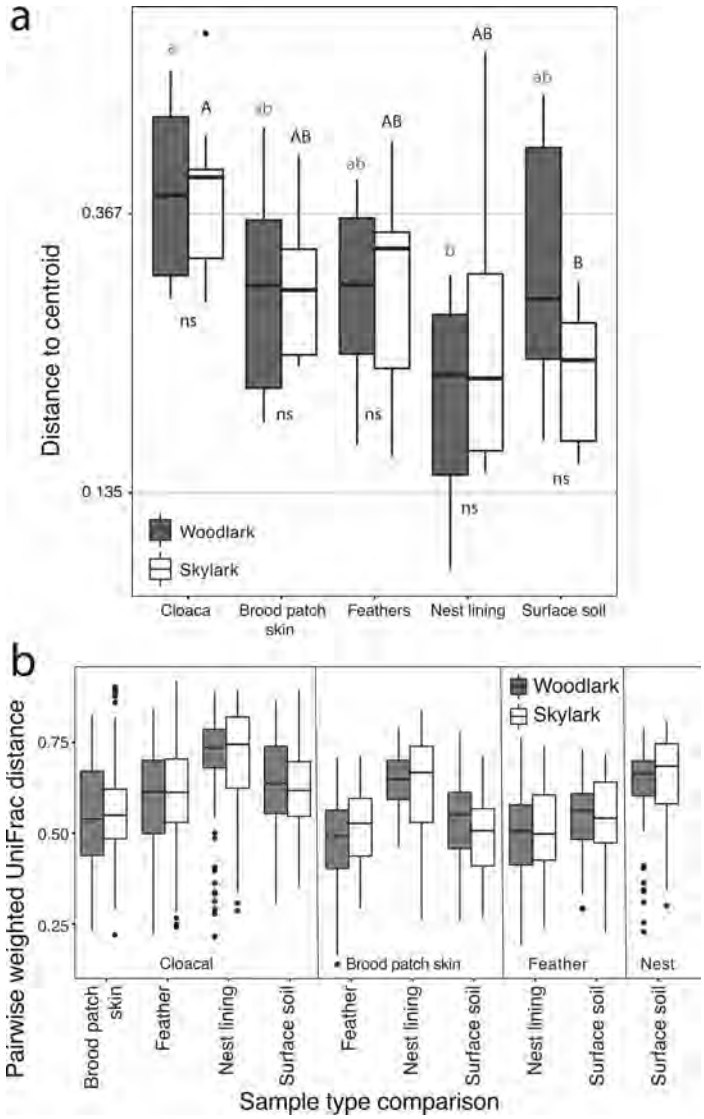

Figure 2.5: Group dispersion within and among microbial niches. Distances to the cluster centroids represent the variation among individuals within sample types and depicts how dispersion varies among among host species and sample types. (a) Group dispersion in PCoA among individuals within each sample type and (b) pairwise weighted UniFrac distances among sample types. Weighted UniFrac distances were calculated based on rarefied data (5000 reads/sample). (a) Letters denote Dunn's contrasts (FDR $q < 0.05$) of median distances between pairs of sample types for woodlarks (lower case grey) and skylarks (capital black). Between-species contrasts of mean distances are expressed below boxes of each sample type (ns = not significant). (b) Rectangular areas denote a base sample type, which weighted UniFrac distance was pairwise compared with the associated sample types labelled along the x-axis. Statistics of between sample type comparisons are reported in Table 2.2.

Table 2.2: ANOVA statistics of phylogenetic dispersion among sample types based on weighted UniFrac.

				Woodlark			**Skylark**		
Comparison I		Comparison II	Estimate (SE)	*t*	FDR *q*-value[a]	Estimate	*t*	FDR *q*-value[a]	
Cloaca	Brood patch skin	Cloaca	Feathers	-0.05 (0.015)	-3.69	**0.050**	-0.04 (0.015)	-2.99	0.35
Cloaca	Brood patch skin	Cloaca	Nest lining	-0.17 (0.015)	-11.62	< **0.001**	-0.14 (0.016)	-8.36	< **0.001**
Cloaca	Brood patch skin	Cloaca	Surface soil	-0.09 (0.016)	-6.08	< **0.001**	-0.06 (0.015)	-4.07	0.013
Cloaca	Feathers	Cloaca	Nest lining	-0.12 (0.014)	-8.29	< **0.001**	-0.09 (0.016)	-5.72	< **0.001**
Cloaca	Feathers	Cloaca	Surface soil	-0.04 (0.015)	-2.73	0.57	-0.02 (0.015)	-1.23	1.00
Cloaca	Nest lining	Cloaca	Surface soil	0.07 (0.015)	5.00	< **0.001**	0.08 (0.017)	4.39	0.004
Cloaca	Brood patch skin	Brood patch skin	Feathers	0.06 (0.015)	3.76	0.041	0.05 (0.015)	3.25	0.18
Cloaca	Brood patch skin	Brood patch skin	Nest lining	-0.11 (0.015)	-7.17	< **0.001**	-0.05 (0.017)	-3.09	0.28
Cloaca	Brood patch skin	Brood patch skin	Surface soil	0.00 (0.016)	-0.15	1.00	0.07 (0.016)	4.49	**0.003**
Cloaca	Feathers	Brood patch skin	Feathers	0.11 (0.014)	7.68	< **0.001**	0.09 (0.015)	6.12	< **0.001**
Cloaca	Feathers	Brood patch skin	Nest lining	-0.05 (0.014)	-3.71	**0.047**	-0.01 (0.017)	-0.59	1
Cloaca	Feathers	Brood patch skin	Surface soil	0.05 (0.015)	3.35	0.14	0.12 (0.016)	7.20	< **0.001**
Cloaca	Nest lining	Brood patch skin	Feathers	0.22 (0.014)	15.79	< **0.001**	0.19 (0.017)	10.98	< **0.001**
Cloaca	Nest lining	Brood patch skin	Nest lining	0.06 (0.014)	4.40	**0.003**	0.08 (0.019)	4.42	**0.003**
Cloaca	Nest lining	Brood patch skin	Surface soil	0.17 (0.015)	10.89	< **0.001**	0.21 (0.018)	11.77	< **0.001**
Cloaca	Surface soil	Brood patch skin	Feathers	0.15 (0.015)	9.86	< **0.001**	0.11 (0.016)	7.01	< **0.001**
Cloaca	Surface soil	Brood patch skin	Nest lining	-0.01 (0.015)	-0.79	1.00	0.01 (0.018)	0.48	1.00
Cloaca	Surface soil	Brood patch skin	Surface soil	0.09 (0.016)	5.67	< **0.001**	0.13 (0.017)	8.02	< **0.001**
Cloaca	Brood patch skin	Feathers	Nest lining	0.04 (0.014)	2.90	0.42	0.05 (0.017)	3.11	0.27
Cloaca	Brood patch skin	Feathers	Surface soil	-0.01 (0.015)	-0.83	1.00	0.03 (0.016)	1.60	1.00
Cloaca	Feathers	Feathers	Nest lining	0.10 (0.014)	6.96	< **0.001**	0.10 (0.017)	5.61	< **0.001**

Cloaca	Feathers	Feathers	Surface soil	0.04 (0.015)	2.81	0.50	0.07 (0.016)	4.31	**0.005**
Cloaca	Nest lining	Feathers	Nest lining	0.21 (0.014)	15.41	< **0.001**	0.19 (0.019)	10.08	< **0.001**
Cloaca	Nest lining	Feathers	Surface soil	0.16 (0.015)	10.71	< **0.001**	0.16 (0.018)	9.16	< **0.001**
Cloaca	Surface soil	Feathers	Nest lining	0.14 (0.015)	9.25	< **0.001**	0.12 (0.018)	6.46	< **0.001**
Cloaca	Surface soil	Feathers	Surface soil	0.08 (0.016)	5.25	< **0.001**	0.09 (0.017)	5.25	< **0.001**
Cloaca	Brood patch skin	Nest lining	Surface soil	-0.10 (0.015)	-6.66	< **0.001**	-0.09 (0.019)	-4.92	< **0.001**
Cloaca	Feathers	Nest lining	Surface soil	-0.05 (0.015)	-3.25	0.19	-0.05 (0.019)	-2.58	0.72
Cloaca	Nest lining	Nest lining	Surface soil	0.07 (0.015)	4.65	< **0.001**	0.05 (0.020)	2.30	0.99
Cloaca	Surface soil	Nest lining	Surface soil	-0.01 (0.016)	-0.44	1.00	-0.03 (0.019)	-1.51	1.00
Brood patch skin	Feathers	Brood patch skin	Nest lining	-0.16 (0.015)	-11.15	< **0.001**	-0.10 (0.018)	-5.75	< **0.001**
Brood patch skin	Feathers	Feathers	Nest lining	-0.01 (0.014)	-1.03	1.00	0.00 (0.018)	0.26	1.00
Brood patch skin	Feathers	Brood patch skin	Surface soil	-0.06 (0.016)	-3.72	**0.046**	0.02 (0.017)	1.37	1.00
Brood patch skin	Feathers	Feathers	Surface soil	-0.07 (0.015)	-4.59	**0.002**	-0.02 (0.017)	-1.42	1.00
Brood patch skin	Feathers	Nest lining	Surface soil	-0.16 (0.015)	-10.53	< **0.001**	-0.14 (0.019)	-7.37	< **0.001**
Brood patch skin	Nest lining	Feathers	Nest lining	0.15 (0.014)	10.58	< **0.001**	0.11 (0.020)	5.44	< **0.001**
Brood patch skin	Nest lining	Brood patch skin	Surface soil	0.10 (0.016)	6.68	< **0.001**	0.13 (0.019)	6.74	< **0.001**
Brood patch skin	Nest lining	Feathers	Surface soil	0.09 (0.015)	6.30	< **0.001**	0.08 (0.019)	4.25	0.006
Brood patch skin	Nest lining	Nest lining	Surface soil	0.01 (0.015)	0.36	1.00	-0.04 (0.021)	-1.81	1.00
Brood patch skin	Surface soil	Feathers	Nest lining	0.04 (0.015)	2.90	0.42	-0.02 (0.019)	-0.97	1.00
Brood patch skin	Surface soil	Feathers	Surface soil	-0.01 (0.016)	-0.65	1.00	-0.05 (0.017)	-2.66	0.64
Brood patch skin	Surface soil	Nest lining	Surface soil	-0.10 (0.016)	-6.20	< **0.001**	-0.16 (0.020)	-8.26	< **0.001**
Feathers	Nest lining	Feathers	Surface soil	-0.05 (0.014)	-3.77	**0.040**	-0.03 (0.019)	-1.52	1.00
Feathers	Nest lining	Nest lining	Surface soil	-0.14 (0.014)	-9.94	< **0.001**	-0.15 (0.021)	-6.97	< **0.001**
Feathers	Surface soil	Nest lining	Surface soil	-0.09 (0.015)	-5.80	< **0.001**	-0.12 (0.020)	-5.92	< **0.001**

[a] Significant mean differences denoted in bold at $q < 0.1$.

differed between woodlarks and skylarks (all pairwise comparisons: FDR $q > 0.05$; Fig. 2.5 a). Since group dispersions differed only between cloacal communities and either nest lining or soil communities, the question remains whether the significance of PCoA clustering arose through location effects or dispersion effects. Because nest lining and surface soil communities each clustered very clearly (Fig. 2.4), and because the group dispersions among bird-associated sample types did not differ (Fig. 2.5 a), significant sample clustering by sample type is likely a true location effect rather than an effect of dispersion. In addition, neither nest lining communities (PerMANOVA, pseudo-$F = 1.43$, df = 1, 18, $R^2 = 0.07$, $P = 0.18$) nor surface soil communities (PerMANOVA, pseudo-$F = 1.57$, df = 1, 17, $R^2 = 0.08$, $P = 0.09$) clustered separately for woodlarks and skylarks (Fig. 2.4).

Comparing community resemblance among sample types

As a measure of phylogenetic similarity among the various sample types, mean pairwise weighted UniFrac distances among sample types varied substantially among all ten pairwise comparisons, but this variation was consistent for woodlarks and skylarks. Across among-sample type comparisons, community variability varied ($F_{9, 2399} = 78.66$, $P < 0.001$; Fig. 2.5 b). Higher mean weighted UniFrac between cloacal and nest lining communities suggests that, on average, these communities least resembled each other (Fig. 2.5 b, Table 2.2). Cloacal communities were least similar to those on feathers and in soils. Instead, cloacal communities mostly resembled skin communities, skin communities were most similar to feather and soil communities, and feather communities mostly resembled skin and nest lining communities. As measures of the nest environment, nest lining and soil communities were markedly different. These resemblance patterns imply that physical contact and spatial proximity among bird- and/or nest-associated bacterial niches influenced the degree of resemblance among them. Here, host species contributed to explaining similarity among sample types, which was demonstrated by a significant 'comparison ID x lark species' interaction ($F_{9, 2399} = 2.37$, $P < 0.05$). The general patterns were similar for both host species and post-hoc pairwise contrasts indicated only small effects (see Table 2.2 for all pairwise statistics).

Phylogenetic clustering in bird- and nest-associated bacterial communities

Analysis of mean NTI values for each sample type (i.e. local community) and lark species separately revealed significant non-random phylogenetic structure at the tips of the phylogenetic trees of each sample type (Fig. 2.6). All sample types were phylogenetically clustered (lower 95% confidence limit > 0). This implies that the taxa found in each sample type were phylogenetically more related than expected in a neutrally assembled community from the same species pool. The mean NTI values did not differ among sample types ($F_{4,100} = 2.27$, $P = 0.07$) or host species ($F_{1,100} = 1.54$, $P = 0.22$). Analysis of mean phylogenetic distances between each pair of taxa, measured as the mean NRI value per sample type, showed a significant deviation from the null distribution in cloacal communities of woodlarks, but not in any other sample type, implying that most of the analysed microbial communities were randomly structured deeper in each sample type's phylogeny (Fig. S2.7).

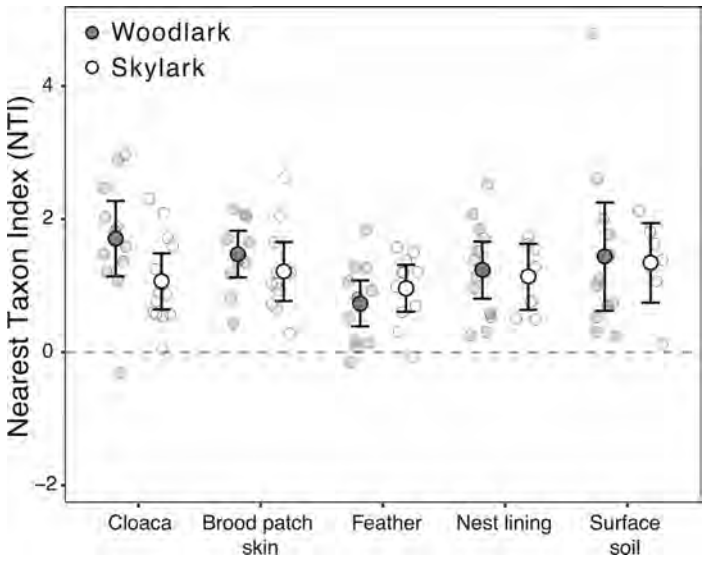

Figure 2.6: Mean NTI of microbiota of sympatric woodlark and skylarks. The Nearest Taxon Index (NTI) describes the standardised effect size of the observed mean distance to the nearest taxon for all taxa in a community compared to a null distribution. NTI is calculated for each sample and is depicted per sample type and for each lark species separately. Mean NTI values that are significantly different from zero (alpha = 0.05) characterise non-random phylogenetic structure where negative values denote significant phylogenetic overdispersion and positive values denote phylogenetic clustering of bacterial OTUs at the tips of the phylogenetic tree. Sample type means (black circles) and 95% confidence intervals (whiskers) are shown per group.

Discussion

Characterising and comparing the microbiotas of sympatric woodlarks *Lullula arborea* and skylarks *Alauda arvensis*, and their nests, we found that the bird-associated microbiotas resembled the environmental microbial communities, and concluded that lark-associated microbiota were shaped more by horizontal acquisition than by habitat filtering or host-microbiota coevolutionary history. Patterns of OTU richness, Shannon diversity, dominant taxonomic groups (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Acidobacteria) and their relative abundances in cloacal, skin, and feather communities did not differ between woodlarks and skylarks. Also ordination analysis of microbiota composition did not separate woodlarks and skylarks in any sample type. Variation in OTU richness and community composition in the three types of bird-associated microbiota (cloaca, brood patch skin and feathers) was partly explained by significant among-individual differences. This was not the case for nest microbiota (nest lining material and surface soil). In addition, the three bird-associated microbiotas harboured few unique and many shared OTUs, of which many were also shared with the nest microbiota. However, using ordination analysis, i.e. also taking into account relative abundances and phylogenetic relationships, we found that samples clustered by sample type, while female identity also had a significant effect. Confirming the effect of individual female, the within-sample type dispersions tended to be higher for the three bird-associated microbiotas than for the nest-associated communities. In all sample types patterns of phylogenetic community structure revealed significant but weak clustering at the OTU-level, not at taxonomic levels deeper in the phylogeny. Here, we first compare our lark microbiota characteristics with microbiota of other birds. We then discuss the implications of our findings at the levels of host species, individual and body part for the evolutionary and ecological factors that shape variation in host-microbe associations. Finally, we discuss the community assembly processes that may govern bird microbiota assembly.

Microbiota of woodlarks and skylarks resemble other avian microbiota

The cloacal microbiotas of woodlark and skylark resembled those of other (passerine) bird species with respect to Shannon diversity (Whittaker *et al*., 2016) and the dominant

bacterial groups (Hird *et al*., 2015; Kreisinger *et al*., 2015; Waite & Taylor, 2014; Whittaker *et al*., 2016). Unfortunately, we cannot compare our OTU richness estimates with other studies, because OTU binning and sequencing / rarefaction depth strongly determine OTU richness estimation. Because our study is the first to describe the avian brood patch skin and because feather microbiotas of wild birds have not been previously characterised based on sequencing data, we cannot compare the results of these body parts to other species. Nevertheless, we showed that the dominant bacterial phyla (Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Acidobacteria) were the same in cloaca, brood patch skin and feathers. These bacterial phyla, with the exception of Acidobacteria, have also been found to dominate the cloacal microbiota of other studied passerines (Hird *et al*., 2015; Kreisinger *et al*., 2015) and non-passerines (Waite & Taylor, 2014). A potential explanation for the dominant presence of Acidobacteria in cloacal, brood patch skin and feather microbiota of our species, is their also dominant occurrence in the larks' nest microbiota.

Interspecific comparison: a large role for the environment in shaping bird microbiota

The small differences between woodlark and skylark in alpha diversity, dominant bacterial taxa and community composition of the microbiota of cloaca, brood patch skin and feathers collectively suggest that the shared environment/ecology is more important than the different host evolutionary histories in shaping these microbiota. Our findings do not support the phylosymbiosis hypothesis (Brooks *et al*., 2016), which postulates that microbiota are host-specific as a result of coevolutionary history between host and microbiota. Phylosymbiosis is supported by studies on passerine birds (Kropáčková *et al*., 2017) and other taxa (Brooks *et al*., 2016; Moeller *et al*., 2016), or partially supported by studies on birds (Hird *et al*., 2015) and mammals (Amato *et al*., 2016) that demonstrate simultaneous effects of ecology and phylogeny on host-microbiota. However, our lark findings are in line with a series of investigations on birds, mammals and reptiles, that also do not find support for phylosymbiosis and instead demonstrate a lack of interspecific microbiota differences among sympatric species (Baxter *et al*., 2015), or strong microbiota convergence due to sympatry (Moeller *et al*., 2013) and dietary similarity (Delsuc *et al*., 2014; Godoy-Vitorino *et al*., 2012). To cover these studies, we propose the "niche-driven microbiota assembly hypothesis" as alternative to the phylosymbiosis-hypothesis, stating

that host-microbiota associations can be shaped by environmental and/or ecological factors instead of coevolutionary history.

Bird-associated microbiota vary among individuals

Differences among individual females explained 18% of the richness, and 20% of the community composition based on the three bird-associated sample types, but had no explanatory power for the Shannon diversity. Variation in nest-associated sample types was not explained by individual for either Shannon diversity, richness, or composition. The among-individual variation in bird-associated microbiota raises the questions whether they are maintained consistently over time, and whether they are caused by genetic or environmental effects. To determine whether differences in host-associated microbiota among individuals are consistently maintained over time requires longitudinal sampling (Hird, 2017). Studies in free-living animals thus far show mixed results: Microbiota of chimpanzees *Pan troglodytes* monitored over eight years (Degnan *et al*., 2012) and of barn swallows *Hirundo rustica* followed during a breeding season (Kreisinger *et al*., 2017) showed individual consistency, while microbiota of deer mice *Peromyscus spp.* were not repeatable over merely one week (Baxter *et al*., 2015). Studies determining whether among-individual differences in host-associated microbiota can be attributed to genetic or environmental effects mainly contributed individual variation to environmental effects (Hird *et al*., 2014; Lucas & Heeb, 2005; Whittaker *et al*., 2016), supporting our niche-driven microbiota assembly hypothesis.

Resemblance of microbiota among body parts and nest environment indicates horizontal transmission

OTU co-occurrence and community resemblance patterns between bird-associated and nest-associated microbiota showed overlap among body parts and with nest samples, suggesting only weak habitat filtering at the level of body part. In humans, the microbes on the body (Hird *et al*., 2014; Lucas & Heeb, 2005; Whittaker *et al*., 2016) demonstrated great overlap with indoor-environment microbiota (Adams *et al*., 2015; Lax *et al*., 2014), but in terrestrial vertebrates only one study has simultaneously measured and compared environmental and animal microbiota (Bisson *et al*., 2007). This study, on wild American redstarts *Setophaga ruticilla*, compared microbial communities on feathers and in soil, and

found that they significantly differed, suggesting that soil plays a minor role in shaping plumage microbiota (Bisson *et al*., 2007). This finding is opposite to our lark results, which may be due to ecological differences between the species: redstarts are arboreal foragers and larks are ground-foragers. In addition, the redstart diversity values, measured using length heterogeneity PCR, were low and probably underestimated as compared to present-day Illumina sequencing results (Claesson *et al*., 2010) applied in our lark study. Mechanisms that might foster transfer between environment and animal include diet (Amato *et al*., 2013; Muegge *et al*., 2011), direct contact with environmental sources such as soil microbiota in ground-foragers (Kent & Burtt, 2016), or inter-individual contact (Kreisinger *et al*., 2015, 2017).

Our finding in both lark species that the microbiota of different body parts (cloaca, brood patch skin, feathers) resembled each other was in contrast with the single other bird study using Next-Generation Sequencing data that compared microbiota among body parts, namely hindgut and facial skin from carcass-eating vultures, and that reported no overlap (Roggenbuck *et al*., 2014). A study of a murine model showed more overlap in composition between lung and vaginal microbiota, than each overlapped with caecal microbiota (Barfod *et al*., 2013), and resemblance among body parts in humans also indicated that differences in habitat filtering and/or varying degrees of horizontal uptake shaped the microbiota of different body parts (Human Microbiome Project Consortium *et al*., 2012), corroborating our findings. The lower diversity and potentially reduced richness in the larks' cloacal microbiota compared with their skin, feather and nest communities may result from more intensive top-down regulation by host genetic factors (Benson *et al*., 2010) or immune function (Thaiss *et al*., 2016) in the intestine/cloaca. Brood patch skin microbiota most strongly resembled feathers and soil, suggesting that horizontal uptake from the surrounding microbiota was profound. The microbiota on feathers were richer than cloacal, brood patch skin and soils, and shared a majority of OTUs with all bird-associated and nest-associated microbiota, suggesting that feathers also horizontally acquired bacterial symbionts from multiple sources. Because OTU richness but not Shannon diversity of feather microbiota exceeded that of other bird-microbiota, we suggest that many taxa on feathers may only be present as low-abundant transient members, which could be expected from horizontal acquisition. For ground-foraging larks it may not be surprising that feathers acquire bacteria from the soil, but the resemblance among these communities also emphasizes that habitat filtering is weak in feathers.

Collectively, we conclude that microbiota of different body parts horizontally acquire microbes from each other and from environmental communities, consistent with our niche-driven microbiota assembly hypothesis.

Phylogenetic community structure in bird-associated samples

Given significant but weak phylogenetic clustering at the OTU-level (NTI) of cloacal, brood patch skin and feather microbiota, we concluded that habitat filtering (Kraft *et al*., 2007; Whittaker *et al*., 2016) plays a role in shaping the bird-associated microbiota of our larks. These results corroborated our findings that the different bird-associated sample types (cloaca, brood patch skin, feather) differed in composition (Fig. 2.4). We did not observe phylogenetic clustering deeper in the phylogenies (NRI) of any of the bird-associated microbiota. However, because our phylogenetic tree was based on the conserved 16S rRNA gene and comprised many (1148) OTUs with much expected functional redundancy (Human Microbiome Project Consortium *et al*., 2012; Kraft *et al*., 2007), we caution that in our study NRI analyses cannot be interpreted as absence of habitat filtering at higher taxonomic levels. The fact that the phylogenetic clustering at the OTU-level was relatively weak compared with NTI values in other bacterial communities (Horner-Devine & Bohannan, 2006; Stegen *et al*., 2012) suggests that there was no strong filtering within the microbial communities present in/on the various body parts. Together with high levels of OTU co-occurrences and strong compositional resemblance among sample types, this weak phylogenetic clustering of bird-associated microbiota provides scope for acquisition of OTUs from the bird's environment onto the bird's body, which would be a prerequisite for our niche-driven microbiota assembly hypothesis.

Conclusion

The sympatric occurrence of two lark species (Alaudidae) enabled us to test, by interspecific comparison of breeding females, if host evolutionary history would generate microbiota differences, while sharing breeding habitat and other resources. Our data showed that the cloacal, skin and feather microbiota did not differ in alpha diversity, community composition, and phylogenetic community structure between woodlarks and skylarks. Based on comparisons of the composition and dominant bacterial taxa of bird- and nest-associated microbiota, we observed associations among the various body

sites and with the nest environment. Patterns of phylogenetic structure of cloacal, skin and feather microbiota suggested weak filtering at each niche. All these patterns were consistent between both lark species, and we therefore suggest that a shared (spatial) environment, and shared ecological factors (e.g. diet), may have avoided these species' microbiotas to differ. These observations raise the hypothesis that sharing an ecological niche among hosts (either species or individuals) leads to convergence of their microbiota. Comparative microbiota studies are typically challenged by the confounding nature of ecological and phylogenetic divergences among hosts, which hampers their use to discern phylogenetic from ecological driving factors. In order to discern evolutionary and ecological effects on interspecific microbiota variation, based on this study, we believe that it is important in future studies either to compare species inhabiting a similar ecological niche to test for effects of host evolutionary history, or to limit the phylogenetic breadth of host species to test ecological factors.

Ethics approval

This study was performed under animal welfare licence DEC6619B/C of the Institutional Animal Care and Use committee of the University of Groningen.

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Supplementary Information

Table S2.1: Overview of collected and successfully sequenced samples.

Woodlark			
	n collected	n sequenced	Remarks
Cloacal gut	14	12	V563807 and V758029 failed PCR amplification
Brood patch skin	13	11	V637773 failed PCR and V573280 removed due to low coverage (1049 reads)
Feathers	13	13	
Nest lining	14	13	Nest B14.140 failed PCR amplification
Surface soil	14	12	Nest B14.11 and B14.54 contained high humic acid content in DNA extract causing PCR amplification failure
Skylark			
Cloacal gut[a]	14	13	V563807 failed PCR amplification
Brood patch skin	11	11	
Feathers	11	11	
Nest lining[b]	7	7	
Surface soil[b]	9	7	Nest V14.16 and V14.42 failed PCR amplification
Total count	**120[c]**	**110**	

[a] Females captured for another study were only sampled for cloacal microbiota assessment.
[b] Nest material and soil samples were not collected for females captured away from their nests, or at replacement nests found after female sampling.
[c] Complete sets of samples for all females (n = 29) would yield 145 samples, but nest failure constrained sample collection.

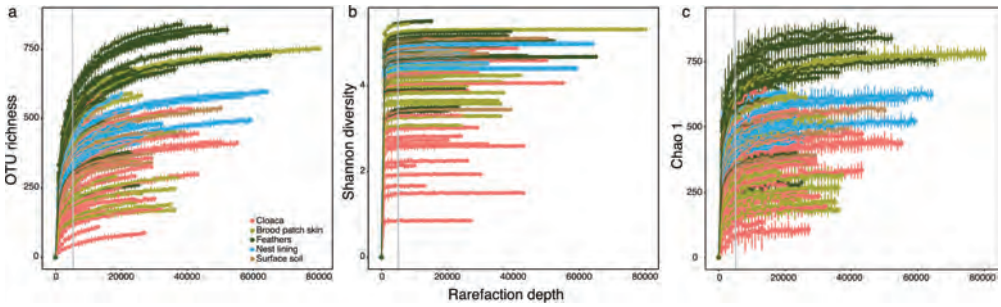

Figure S2.1: Rarefaction curves of samples from different origins. a) Rarefaction curves depict the estimated average a) OTU richness (97% ID), b) Shannon diversity and c) Chao1 (whiskers represent ± 2 SD) which are based on 10 samplings for each rarefaction depth. Samples are coloured by sample type of origin. Despite the maximum OTU richness and predicted total diversity (Chao1) have not reached a plateau at rarefaction depth 5000, Shannon diversity levelled off. Although the current sequencing efforts were unable to capture the total diversity, Chao1 values (c) show some separation of total diversity estimates for different sample types.

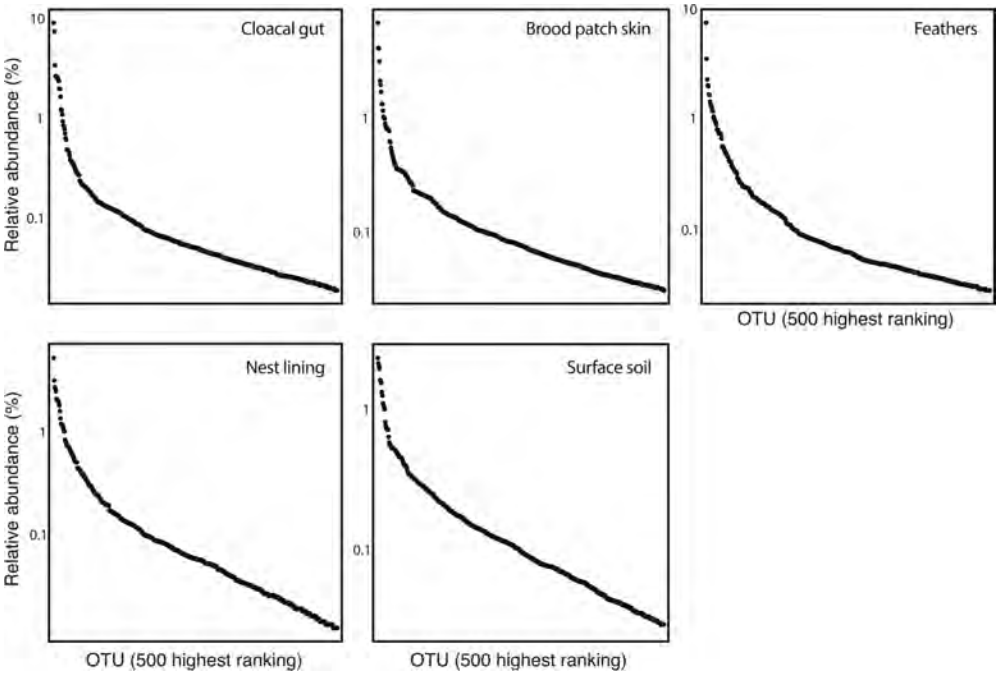

Figure S2.2: Rank-abundance plots for each sample type. Dotplots depict rank-abundance plots for the 500 most abundant OTUs of each sample type. Note that the y-axes are log10 transformed. The patterns are highly skewed with few very abundant and many rare OTUs.

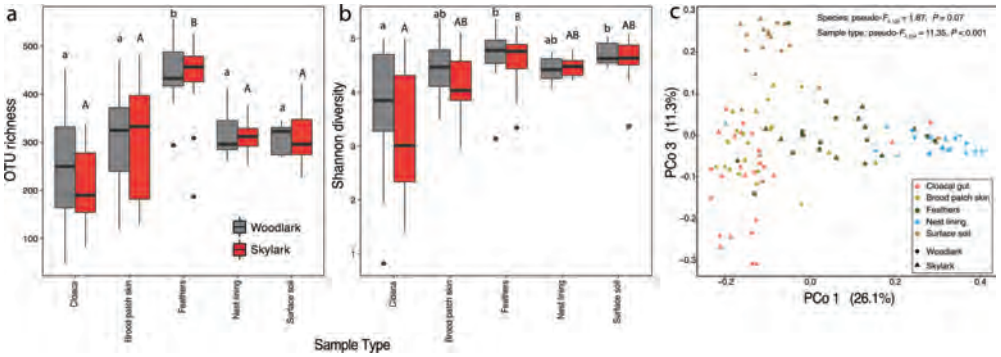

Figure S2.3: Bacterial community characteristics with an OTU table constructed on all high-quality reads. a) Boxplots depict OTU richness (97% ID) and b) Shannon diversity of sample types associated with woodlarks (grey) and skylarks (red). c) Principal coordinates (PCoA) plot of weighted UniFrac shows the bacterial community composition of all samples, coloured by sample type for woodlarks (circles) and skylarks (triangles). a, b) Letters denote group differences determined by Dunn's test for multiple comparisons with FDR $q < 0.05$. PCO axes 1 and 3 are shown for most intuitive cluster visualisation. Group dispersions differ between sample types (betadisper: $F_{4,104} = 5.60$, $P < 0.01$), and thus warrants cautious interpretation of statistical support for differential clustering. Sample type explains about 30% of variation in weighted UniFrac.

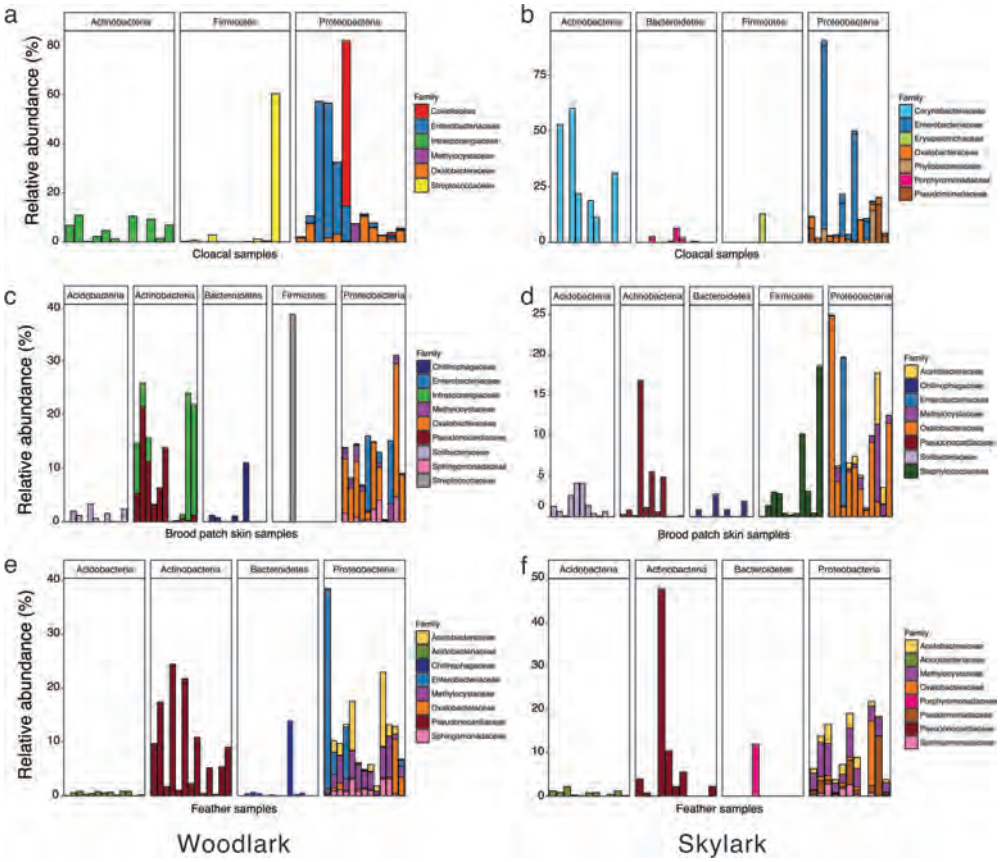

Figure S2.4: Barplots of the most dominant OTUs in body site-specific bird-associated microbiota. OTUs are included when the mean relative abundance of an OTU > 5% across all samples of a sample type, separately evaluated for each lark species. Relative abundances of dominant OTUs are depicted for individual (a, b) cloacal, (c, d) brood patch skin and (e, f) feathers samples from woodlarks and skylarks, respectively. OTU barplots are split by bacterial phylum and coloured by family. Dominant OTUs belong to the same phyla in each body site of both lark species, but the identity (and family) of the dominant OTU varies for Actinobacteria and Firmicutes in cloacae and skin communities, and in Bacteroidetes in feather communities.

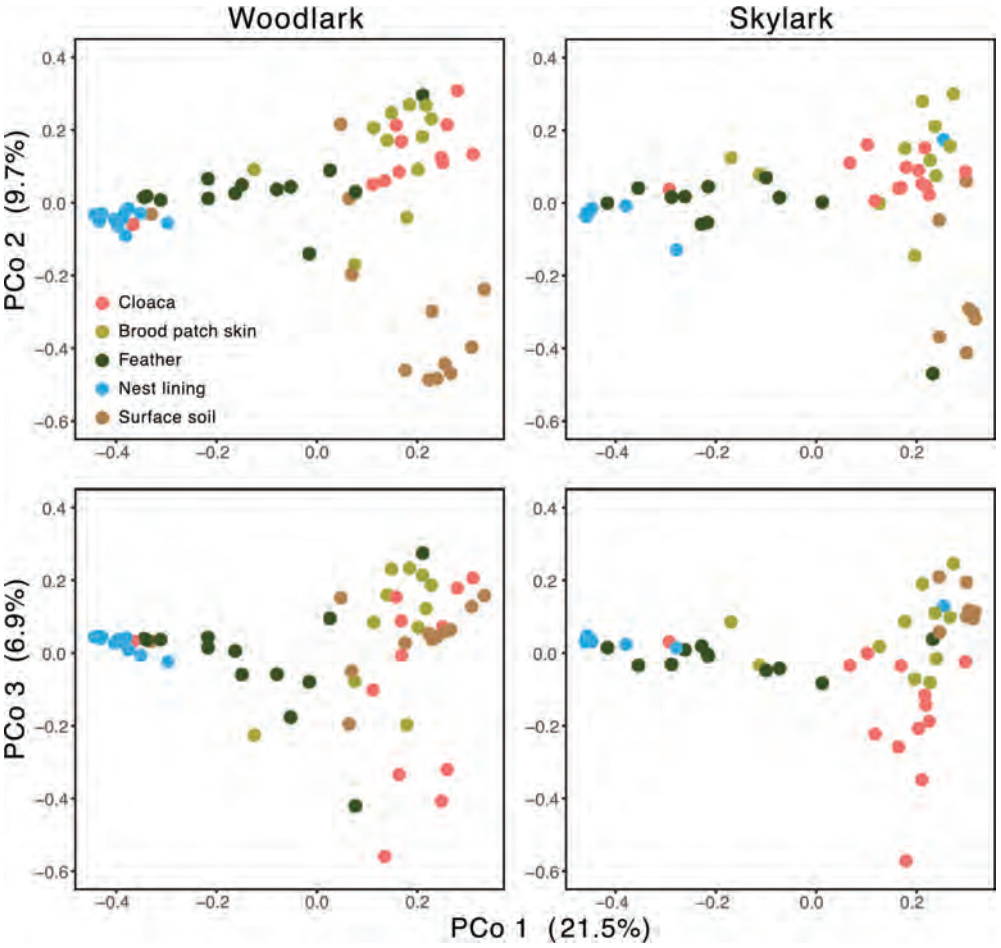

Figure S2.5: Beta diversity of bacterial communities associated with different sample types of sympatric lark species. Principal coordinates analysis on Bray-Curtis dissimilarities between sample types is shown along the first three principal coordinate axes and was calculated on rarefied data (5000 reads/sample). Sample types significantly clustered, explaining 26% of variation (PerMANOVA, pseudo-F = 10.1, df = 4, 104, R^2 = 0.26, P < 0.001) and woodlarks and skylarks only weakly clustering explaining 1% of variation (pseudo-F = 2.13, df = 1, 104, R^2 = 0.01, P < 0.05). Female ID explained 23% of total variation in community clustering (PerMANOVA, pseudo-F = 1.26, df = 27, 77, R^2 = 0. 22, P < 0.01).

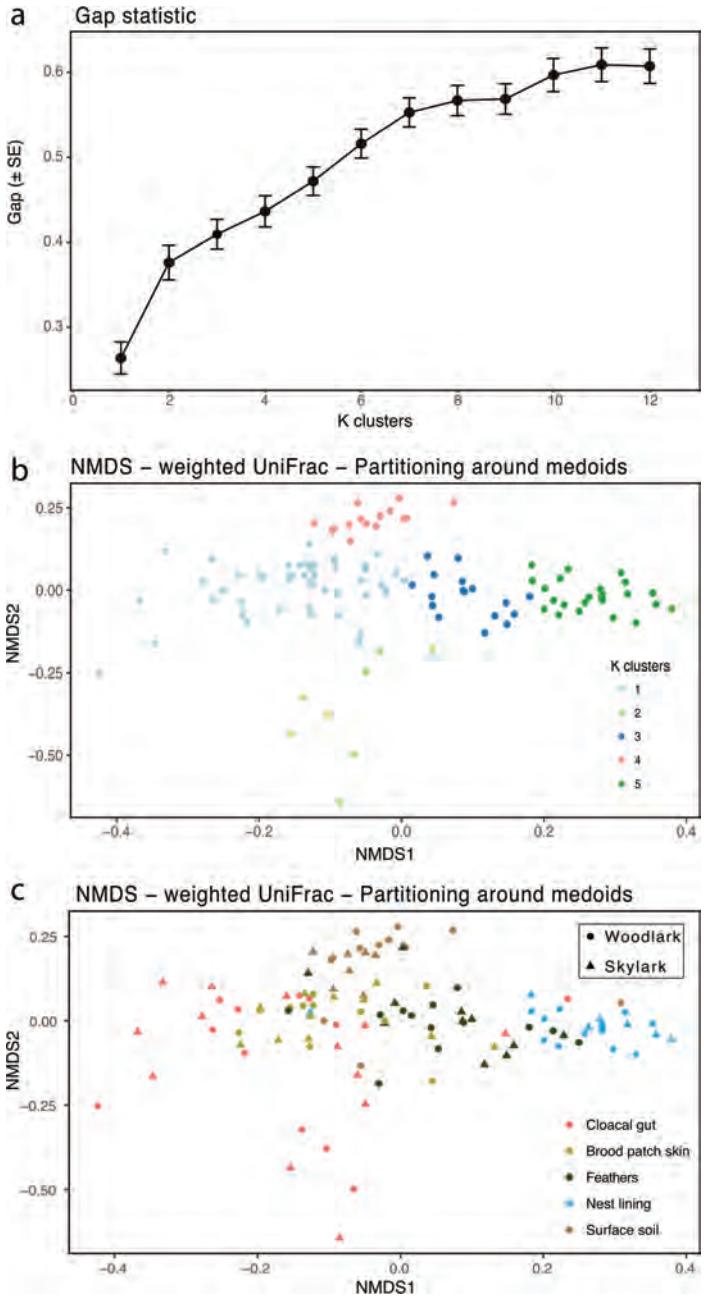

Figure S2.6: Clustering of samples through partitioning around medoids. a) Lineplot shows gap statistic of K clusters as identified using partitioning around medoids (PAM). b) NMDS ordination plot shows samples coloured by K clusters at K=5. c) NMDS ordination plot shows samples clustered by K clusters at K=5 and coloured by sample type for delineated by host species (symbol shape). Stress in both NMDS plots is 0.14.

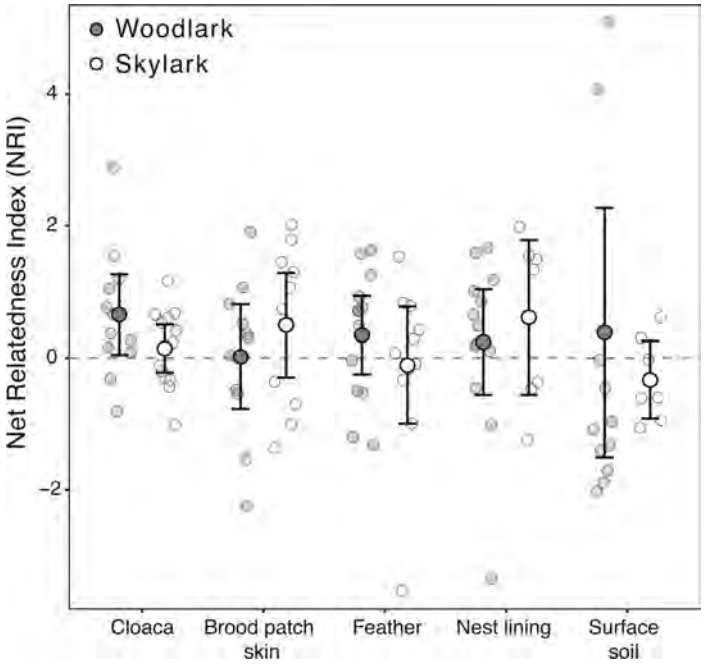

Figure S2.7: Mean NRI across sample types of sympatric lark species and their nest environment. The Net Relatedness Index (NRI) describes the standardised effect size of the observed mean phylogenetic distance among every pair of taxa in a community compared to a null distribution. NRI is calculated for every sample and is depicted per sample type and for each lark species separately. Mean NRI values that are significantly different from zero characterise non-random phylogenetic structure at deep branching of the phylogenetic tree (as opposed to NTI), where negative values denote significant phylogenetic evenness and positive values denote phylogenetic clustering of bacterial lineages. Mean and 95% confidence intervals (whiskers) are shown for woodlarks and skylarks separately for each sample type.

Chapter 3

Cloacal microbiotas are biogeographically structured in larks from desert, tropical and temperate areas

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Abstract

Macroorganisms show strong and consistent biogeographical patterns that are associated with local adaptation of physiology, behavior and life history. Yet, for microbes strong biogeographic patterns have not been found, raising the question of what determines the biogeography of microorganisms. Thus far, large-scale biogeographical patterns have been explored mainly for free-living microbes, paying little attention to host-associated microbes, which play essential roles in physiology, behavior and life history of their hosts. Investigating cloacal gut microbiota of a family of closely-related, ecologically similar songbird species (Alaudidae, larks) inhabiting desert, temperate and tropical regions, we explored influences of geographical location and host species on α-diversity, dominant bacterial taxa and co-occurrence of operational taxonomic units (OTUs), and community composition. We found that geographic location significantly explained 12-16% of the variation in α-diversity measures, whereas species played no significant role. Out of all OTUs identified, 14% were shared by larks from all locations. Considering biogeographic regions, desert larks hold higher numbers of unique OTUs (17%) than temperate zone (12%) and tropical larks (9%). Out of all 29 bacterial phyla identified, five phyla dominated larks' cloacal gut microbiome. We found differences among locations in relative abundance in three of the five dominant phyla, and in six low-abundance phyla. Although the most abundant OTU varied with location, we identified 6 dominant OTUs that made up 40.3% of the total abundance. Bray-Curtis and UniFrac analyses showed significant clustering of community composition with geographic locations and with host species. Taxonomic compositions of microbiomes of desert larks were distinct from those of the tropical and temperate counterparts. We conclude that host-associated microbiota are geographically structured in a group of widespread but closely-related host species, following large-scale macro-ecological patterns (including biogeographical differences in host species diversity, host physiology and life history) and contrasting markedly with previous findings for free-living microbes. Future work should further explore if and to what extent the geographic variation in host-associated microbiota can be explained as result of co-evolution between gut microbes and host adaptive traits, and what the contribution is of acquisition from the environmental pool of bacteria in explaining host-associated communities.

Introduction

For animals and plants, strong and consistent biogeographical patterns of distribution exist and are associated with local adaptation of physiology and life history traits (Hill, 2016; Krebs, 2009). In contrast, for microbes such a consistency in large-scale biogeographical patterns has not been found (e.g., Fierer & Jackson, 2006; Fuhrman *et al*., 2008; Milici *et al*., 2016), fueling a debate about the ecological and evolutionary processes that govern spatial variation in different life forms (Martiny *et al*., 2006; Meyer *et al*., 2018; Thompson *et al*., 2017). Well-established patterns in plants and animals like the greater diversity towards the tropics or the decay of community similarity with geographic distance are often not detected in free-living microbes (Fierer & Jackson, 2006; Fuhrman *et al*., 2008; Hillebrand *et al*., 2001; Milici *et al*., 2016). Several reasons have been proposed to explain this discrepancy including differences in the spatial scales at which dispersal ability or environmental selection affect microbes compared with plants/animals, differences in taxonomic groups between macro (e.g., species) and microorganisms (e.g., Operational Taxonomic Units – OTUs), and methodological issues (e.g., inability to differentiate inactive/dead microorganisms, or under-estimation of microbial diversity) (Martiny *et al*., 2006; Meyer *et al*., 2018). Earlier studies with free-living microbes supported Baas-Becking's paradigm that the local environmental conditions can select and maintain distinctive microbial assemblages (Baas-Becking, 1934; Martiny *et al*., 2006), while the current debate concentrates on whether "everything is everywhere", and on the microbial traits that determine the geographical distribution of microorganisms (Martiny *et al*., 2006; Thompson *et al*., 2017). However, thus far, microbial biogeography has mainly focused on free-living microbial assemblages in aquatic or terrestrial environments, paying little attention to host-associated microbes (Colston & Jackson, 2016) despite the ubiquitous occurrence of host-microbe associations in nature (Bosch & McFall-Ngai, 2011).

In the context of understanding biogeographical patterns and adaptations, host-associated microbes present an especially interesting case. The environment that host-associated microbes inhabit is the host's body, which - for many host taxa including birds and mammals - is generally relatively constant in terms of factors such as pH, temperature and salinity, providing a similar environment for host-associated microbes across different biogeographical areas and despite large geographical distances. Dispersal

of host-associated microbes is not well-understood and may differ from dispersal of environmental microbes, depending on how host-associated microbial communities are formed and maintained. In addition to these unique features of the host-associated microbes' environment and ecology, host-associated microbial communities play fundamental roles in physiology, behavior and life history of their hosts given their key importance for essential functions like food digestion, ontogenetic development or protection against pathogens and parasites (Colston & Jackson, 2016; Dinan *et al*., 2015; Hird, 2017; Kohl & Carey, 2016; McFall-Ngai *et al*., 2013) - the very traits that adapt hosts to their environment. Because of the fundamental roles that host-associated microbes play in animal physiology, behaviour and evolution, and associated coadaptation (Bordenstein & Theis, 2015; Brucker & Bordenstein, 2011; Theis *et al*., 2016), associations between microbes and hosts can be tight (Braendle *et al*., 2003; Brucker & Bordenstein, 2013). Hence, it is currently unclear whether the biogeographical structure of host-associated microbes resembles that often found for free-living microbes ("everything is everywhere") or is determined by host traits. For example, currently unanswered questions include whether the assembly of host-associated microbial communities is driven by the environmental microbial communities or by host physiology and selection. Therefore, studying geographical patterns of the host-associated microbial communities may contribute new perspectives to microbial biogeography.

Current literature on variation of host-associated microbes with geography is limited in scope and offers an equivocal picture (Colston & Jackson, 2016). Some single-species studies on various vertebrates show geographic variation in host-associated microbial communities (Hird *et al*., 2014; Klomp *et al*., 2008; Lankau *et al*., 2012; Linnenbrink *et al*., 2013), partly co-varying with geographic variation in host traits (Hird *et al*., 2014; Klomp *et al*., 2008; Lankau *et al*., 2012), whereas others do not find geographic variation in host-associated microbes (Banks *et al*., 2009; Gaillard, 2014; Llewellyn *et al*., 2015; Perry *et al*., 2017). These single-species studies are constrained by limited environmental variability as most hosts occur over only a small environmental range (e.g., Banks *et al*., 2009; Linnenbrink *et al*., 2013; Perry *et al*., 2017); but see (Llewellyn *et al*., 2015). A multi-species meta-analysis found important roles of both host species and sampling site in shaping bird gut microbiomes, with these factors ranked above others such as diet or captivity status (Waite & Taylor, 2014). Likewise, a recent interspecific study in European birds highlighted the relevance of geographic location in explaining gut microbial diversity

(Kropáčková *et al*., 2017). However, another interspecific comparison found little evidence for a geographical effect on gut microbial communities of 59 Neotropical bird species (Hird *et al*., 2015). Limitations for the interpretive power of these multi-species studies include (i) the small geographical scales (and the associated small environmental variability), and (ii) the confounded elements of variation in ecological niches and evolutionary historical trajectories due to the use of evolutionarily distantly-related hosts. This second limitation is particularly important given the proposed relevance of host evolutionary history in shaping host-microbe associations (Brucker & Bordenstein, 2011, 2013). Studies considering multiple host species covering large environmental variation, while sharing similar ecological niches and evolutionary histories, are required to shed more light on the role of geography in explaining variation in host-microbe associations.

An interesting model system to study biogeographical variation in host-associated microbial communities is the family of larks (Alaudidae) (Hill, 2016; Horrocks *et al*., 2012, 2015; Tieleman *et al*., 2002, 2003a, 2003b, 2004, 2005). Larks comprise a group of globally-distributed, closely-related bird species with fundamentally similar ecologies (e.g., ground-nesting, ground-foraging, social life, diet), despite occurring in very different environments including tropical regions, desert areas and temperate zones (del Hoyo *et al*., 2004; Tieleman *et al*., 2003b). The use of closely-related hosts minimizes historical (co)evolutionary variation, which is an important factor that might affect the biogeography of host-associated microbial communities (Martiny *et al*., 2006). In an early study of the geographic co-variation between culturable free-living and host-associated microbes and the immune system of multiple lark species, Horrocks et al. (Horrocks *et al*., 2012) strongly suggest that geographic location can play an important role in shaping host-microbe interactions. In addition, in a recent study using state-of-the-art sequencing technology van Veelen *et al*. (2017) show that sympatrically living woodlarks and skylarks do not differ in their gut microbial communities. Moreover, these authors suggest that the host-associated microbial communities of skylarks and woodlarks are largely shaped by host filtering of the environmental microbial communities, while providing no support for phylosymbiosis.

Here we have investigated how host-associated microbial communities vary with geography using a unique large geographical scale to study the variation in the gut microbial communities of nine closely related lark (Alaudidae) species from five different

locations encompassing three biogeographical regions (desert, tropics, temperate zone). Using 16S rRNA gene amplicon sequencing, we first explored the influences of geographical location and host species in explaining differences in α-diversity of gut microbial communities of larks. Secondly, we analysed the geographic variation of dominant bacterial taxa and the co-occurrence of operational taxonomic units (OTUs) in the lark-associated microbiota. Finally, we investigated the compositional similarity of bacterial communities (beta diversity) among locations and species.

Methods

Field sampling

We captured 107 individuals of nine lark species at five locations up to 6500 km apart. All locations were sampled during the breeding season for our study species at those sites. One sampling location (Aekingerzand, The Netherlands) was located in a temperate area and corresponds to the Eurasian biogeographical region. The two arid locations (Mahazat as-Sayd and Taif, Saudi Arabia) belong to the Saharo-Arabian biogeographical region, while the other two sampling locations (Kinangop and Kedong, Kenya) were in the tropics within the African biogeographical region. Additional information on the specific environmental conditions of these locations can be found in (Hegemann & Voesten, 2011; Horrocks *et al*., 2015) and (Ndithia *et al*., 2017b). Details of species, sample sizes and year of sampling are provided in Table 3.1. We used the common technique of swabbing the cloaca of birds as proxy for gut microbial communities (e.g., Klomp *et al*., 2008; Lombardo *et al*., 1996; van Veelen *et al*., 2017). We collected swabs by inserting the sterile swab approximately ∼5 mm into the cloaca, and then gently rotated it for 10 s following previous recommendations (Klomp *et al*., 2008; Lombardo *et al*., 1996). The swab was then placed in a sterile Eppendorf tube containing 250 µl of sucrose lysis buffer (Klomp *et al*., 2008) that had been prepared under a sterilized fume hood (wiped clean with 70% ethanol and sterilized with a UV lamp for at least 5 min) and filtered through a sterile filter (0.2 µm) to remove any bacteria present. The swab was kept on ice in the field (< 8 hours) and later frozen at -20°C the same day. Samples remained frozen until they were analysed in the lab.

Table 3.1: Sample size and geographic origin of bird species used in the study.

Species	Latin name	n	Latitude	Longitude	Population	Country	Year
Skylark	*Alauda arvensis*	18	52°56' N	6°18' E	Aekingerzand	Netherlands	2007
Woodlark	*Lullula arborea*	18	52°56' N	6°18' E	Aekingerzand	Netherlands	2007
Greater hoopoe lark	*Alaemon alaudipes*	13	22°20' N	41°44' E	Mahazat as-Sayd	Saudi Arabia	2007
Bar-tailed lark	*Ammomanes cinctura*	3	22°20' N	41°44' E	Mahazat as-Sayd	Saudi Arabia	2007
Arabian lark	*Eremalauda eremodites*	18	22°20' N	41°44' E	Mahazat as-Sayd	Saudi Arabia	2007
Black-crowned Sparrow-lark	*Eremopterix nigriceps*	14	21°15' N	40°42' E	Taif	Saudi Arabia	2007
Crested lark	*Galerida cristata*	4	21°15' N	40°42' E	Taif	Saudi Arabia	2007
Red-capped lark	*Calandrella cinerea*	8	0°34' S	36°28' E	Kinangop	Kenya	2009
Rufous-naped lark	*Mirafra africana*	4	0°34' S	36°28' E	Kinangop	Kenya	2009
Red-capped lark	*Calandrella cinerea*	5	0°52' S	36°23' E	Kedong	Kenya	2009
Rufous-naped lark	*Mirafra africana*	2	0°52' S	36°23' E	Kedong	Kenya	2009

DNA extraction and 16S rRNA gene amplicon sequencing

We extracted DNA from cloacal swabs by aseptically peeling off the cotton from the stalk and placing this in an extraction vial (MoBio PowerSoil-htp 96 well DNA isolation kit, MoBio laboratories, Carlsbad, CA, USA). We performed DNA extractions following the manufacturer's protocol, with addition of 0.25 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) to improve cell disruption during three cycles of 60s bead beating (Mixer Mill MM301, Retsch GmbH & Co, Germany). The V4/V5 region of the 16S rRNA gene was amplified in triplicate using primers 515F and 926R at Argonne National Laboratory, IL, USA, following the Earth Microbiome Project protocol (Gilbert *et al*., 2010), followed by library preparation of pooled triplicates and 2 $\times$ 250 bp paired-end sequencing using V2 chemistry on an Illumina MiSeq platform. In total we sent 25 negative controls for amplification and sequencing. No amplicons and no reads from these controls remained in the final dataset after quality filtering.

Sequence data processing

We processed raw 16S rRNA sequence reads using the QIIME pipeline (v 1.9.1) (Caporaso *et al*., 2010a). Sequence reads were demultiplexed and quality-filtered (quality score ≥ 25). Using an open-reference strategy, we clustered sequences first into OTUs using the *uclust* algorithm (Edgar, 2010) at 97% identity against the Greengenes reference database (v. 13.8) (DeSantis *et al*., 2006), and *de novo* clustered (0.1%) reads that failed to match the reference set. We selected representative sequences per OTU, concatenated both OTU tables, removed singletons to reduce effects of sequencing error on richness estimation, and annotated the reference sequences with taxonomic information from Greengenes. We then aligned representative sequences using PyNast (Caporaso *et al*., 2010b) and identified and removed chimeric sequences using the uchime algorithm in the *usearch81* toolkit (Edgar *et al*., 2011), followed by phylogenetic tree construction using FastTree (Price *et al*., 2009). We filtered OTUs assigned to Archaea, chloroplast and mitochondrial sequences from the dataset, and offset the OTU table to retain OTUs at > 0.001% of the total abundance to reduce table sparsity. Rarefaction curves showed that OTU richness had not reached saturation while Shannon diversity levelled at 4000 reads per sample for each sample type (Fig. S3.1).

Our sequencing effort produced 1442486 quality-filtered sequences after removal of singletons, clustered in 3940 OTUs with a minimum abundance of 0.001%. The coverage range of our analysed samples was 4016-45457 reads per sample. Rank-abundance plots for the five sampling sites were similar (Fig. S3.2), indicating dominance of a few very abundant OTUs and a long tail of less abundant ones. Of the 3940 OTUs, 4.8% could be assigned to species level, 94.6% to genus level and 97.2% to family level.

Statistical analyses I: Diversity within bacterial communities – comparing locations and species

We analysed bacterial diversity using the R packages *phyloseq* (v. 1.20.0) (McMurdie & Holmes, 2013), *vegan* (v. 2.4-4) (Oksanen *et al*., 2017) and *DESeq2* (v. 1.16.1) (Love *et al*., 2014) using R statistical software (v. 3.4.0) (R Core Team, 2017).

We calculated OTU richness and Shannon's diversity index (hereafter 'Shannon diversity') from rarefied data (4000 reads) and created linear mixed models to analyse differences

among sampling locations. We used the *lme4* (v. 1.1-14) (Bates *et al*., 2014) and *lmerTest* packages (v. 2.0-33) (Kuznetsova *et al*., 2016) for these analyses. The *multcomp* package (Hothorn *et al*., 2008) was used for the Tukey-Kramer posthoc tests in order to explore pairwise differences between locations. We systematically verified the normality of residuals errors (Q-Q plots) and homoscedasticity (fitted values $\sim$ residual plot) in our models. We did not use phylogeny in the models given that the species in this study are evenly distributed across the lark family tree (Alström *et al*., 2013), Fig. S3.3) and because phylogenetic corrections are only reliable with at least 20 species (Blomberg *et al*., 2003). However, we used host species identity as a random factor in these analyses to account for the non-independence of birds within host species. We tested the significance of the random factor host species identity using a likelihood ratio test comparing REML-fitted linear model without the random term (Pinheiro & Bates, 2000) and calculated the proportion of explained variance (Nakagawa & Schielzeth, 2013) using the *MuMIn* package (v.1.40) (Bartoń, 2017).

We compared OTU co-occurrence among locations by means of Venn diagrams using the venn package (v. 1.5) (Dusa, 2017). We used a negative binomial model on non-rarefied data to test for differential OTU abundances among different geographic locations as implemented in *DESeq2* (Love *et al*., 2014; McMurdie & Holmes, 2013). We performed pairwise contrasts for exploring differences between locations (False Discovery Rate (FDR)-corrected q-value < 0.1). In order to identify differential relative abundances among locations for different taxonomic levels (Phyla and Classes), we performed analysis of composition of microbiomes (ANCOM v. 1.1-3; Mandal *et al*., 2015) with a critical false discovery rate (FDR)-corrected q-value of 0.05 using the *shiny* interface (v. 1.0.5) (Chang *et al*., 2017).

Statistical analyses II: Community similarity among locations and species

We assessed bacterial community composition (beta diversity) based on the variance-stabilised data to evaluate taxonomic (Bray-Curtis) and phylogenetic (weighted UniFrac) dissimilarities among locations (Lozupone & Knight, 2005). We performed principal coordinates analysis (PCoA) using *vegan*. We modelled Bray-Curtis and weighted UniFrac dissimilarities from an OTU-level table using constrained ordination (distance-based

redundancy analysis with 999 permutations; 'adonis2' function in *vegan*; Anderson, 2001; McArdle & Anderson, 2001). Given the structure of our data indicating partial correlation between host species identity and location, a nested design (host species within location) would have been ideal to test statistically for the effect on community structure. Unfortunately, this is still not possible with the statistical tools currently available. Therefore, we first carried out analyses to test if differences among locations affected community structure. We then ran separate models, this time replacing location with host species identity, to examine the effect of host species on community structure. Both models produced qualitatively the same finding; we present and discuss only the results from the location model in the main text, and for transparency, include results from the host species model in the supplementary material. To explore pairwise differences between populations, we performed Tukey-Kramer *post hoc* tests with the *multcomp* package (v.1.4-8) (Hothorn *et al*., 2008) and using the first and second PCoA axis of the corresponding beta diversity metric (Bray-Curtis or weighted UniFrac) as the response variable. Host species identity was included as a random factor in the models to correct for non-independence of samples due to different host species. We also tested for the homogeneity of within-group dispersions among locations ('betadisper' function in package *vegan*; Anderson, 2006).

Results

Richness and diversity of microbiota

The OTU richness of lark cloacal gut microbiota varied significantly with geographic location ($F_{4,84.1} = 4.12$, $P = 0.042$; Fig. 3.1 a) with desert larks from Saharo-Arabian Taif holding significantly fewer OTUs than those from the two tropical locations (Fig. 3.1 a). Host species did not significantly affect OTU richness (LRT: $\chi^2 = 1.30 \cdot 10^{-7}$, $P = 0.99$; Fig. S3.4 a) and explained no variation in this α-diversity measure, in contrast with the 16% of variance explained by geographic location. Shannon diversity showed a similar pattern (Fig. 3.1 b) with a significant effect of geographic location (Shannon *H'*: $F_{4,844} = 2.93$, $P = 0.026$) that explained 12% of the variance, and no significant effect of host species (LRT: $\chi^2 = 4.39 \cdot 10^{-8}$, $P = 0.99$; Fig. S3.4 b), which explained no variance. *Post hoc* tests

revealed that desert larks from Taif showed a lower Shannon diversity than tropical larks, in this case only significant for the comparison with African Kinangop (Fig. 3.1 b).

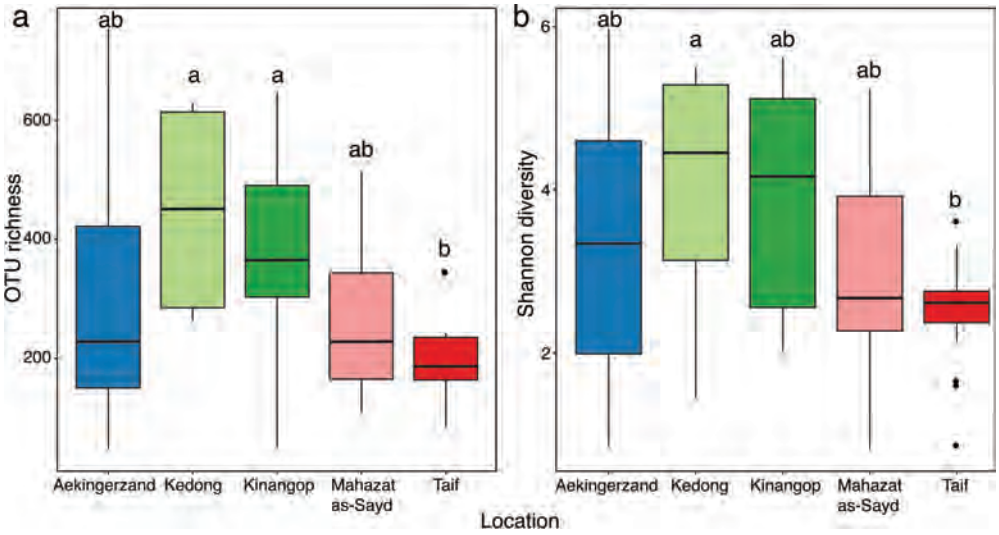

Figure 3.1: OTU richness (a) and Shannon diversity (b) of gut microbiota of larks from the five study locations. Letters indicate significant differences ($p < 0.05$) between locations according to Tukey-Kramer *post hoc* tests.

OTU co-occurrence patterns and relative taxon abundances

We found that 14% of all OTUs identified were shared by larks from all locations (Fig. S3.5). Unique OTUs for each location represented 1%, 2%, 4%, 7% and 12% of the lark gut microbiome for Taif, Kedong, Kinangop, Mahazat as-Sayd and Aekingerzand, respectively, indicating that the gut microbial communities of larks inhabiting the latter location comprised the highest number of unique OTUs. However, when considering biogeographic regions (rather than specific locations), Saharo-Arabian larks hold the highest number of unique OTUs overall (Saharo-Arabian: 17%, Euroasian: 12%, African: 9%; Fig. S3.5). The distinctiveness of the microbiome of the desert larks is also manifested by the results of the pairwise comparisons of OTU abundances obtained using the *DESeq2* package, where pairwise comparisons with temperate and tropical sites indicate that these two Saharo-Arabian locations (Taif and Mahazat as-Sayd) had the highest number of differentially abundant OTUs (Table S3.1).

Out of all 29 bacterial phyla identified, we found that five phyla (Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Acidobacteria) dominated larks' cloacal gut

microbiome (cumulative abundance > 94.1%; Fig. 3.2). The relative abundances of three of these five phyla (Proteobacteria, Bacteroidetes and Acidobacteria) varied significantly among locations (ANCOM, FDR $q < 0.05$). In addition, six low-abundance phyla including AD3, Chloroflexi, Gemmatimonadetes, Planctomycetes, Verrucomicrobia and WPS2 also showed significant differences among locations (ANCOM, FDR $q < 0.05$). Overall, Proteobacteria was the most dominant phylum in larks at Aekingerzand, Kinangop, Mahazat and Taif, but not Kedong which held a microbiome dominated by Tenericutes. Actinobacteria was the second most abundant phylum in larks, except for tropical African larks where Proteobacteria (Kedong) and Firmicutes (Kinangop) took this place. Three of the five most abundant classes in the lark microbiome corresponded to Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, the other two being Actinobacteria and Bacilli (cumulative abundance > 84.2%; Fig. 3.2). Out of these classes only the first two showed significant differences in their relative abundances among locations (ANCOM, FDR $q < 0.05$). Information on the relative abundances for each host species can be found in Fig. S3.6.

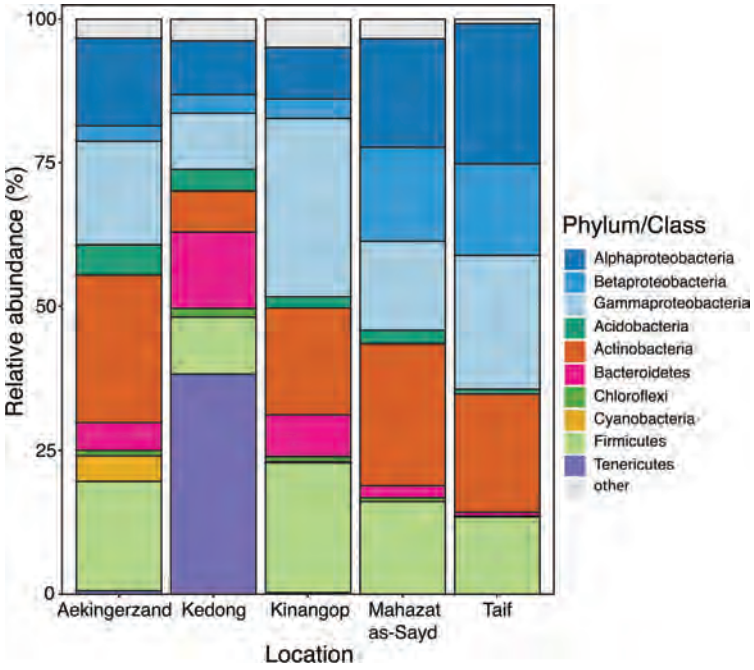

Figure 3.2: Barplots of relative abundances of the most abundant Phyla and Proteobacteria classes per location. Calculations were based on rarefied data (4000 reads/sample).

Considering all lark samples, we identified 6 OTUs that dominated larks' cloacal gut microbiome (cumulative abundance > 40.3%). These OTUs include unassigned bacteria of the genus *Corynebacterium* (*de novo* OTU1096; *de novo* OTU25), *Enterococcus* (1111582), *Ralstonia* (759916) and *Sphingomonas* (4396025) in addition to *Pseudomonas stutzeri* (813945). The most abundant OTU varied with location (Table 3.2). Larks from desert Mahazat as-Sayd and temperate Aekingerzand were dominated by OTUs assigned to *Corynebacterium spp.*, while *Pseudomonas stutzeri* was the most abundant in desert Taif and different OTUs dominated the host-associated microbiome of tropical African larks (Kinangop: family Enterobacteriaceae OTU 922761; Kedong: family Mycoplasmataceae *de novo* OTU256).

Community similarity

Analysis of beta diversity based on Bray-Curtis distances revealed that the taxonomic composition of gut microbiomes differed among geographic locations (PerMANOVA, pseudo-$F = 5.68$, df = 4, 88, $R^2 = 0.21$, $P = 0.001$; Fig. 3.3 a). *Post hoc* tests revealed significant differences between bacterial communities from desert larks (Mahazat as-Sayd and Taif) and those from the other three locations (PCoA Axis 1: $P < 0.0001$ for all pairwise comparisons). No significant differences were found between the two desert locations ($P = 0.21$), indicating consistency in community composition for host-associated microbial communities of desert larks. No significant differences were found among Aekingerzand, Kinangop and Kedong either (P > 0.77 in all comparisons), suggesting that the significant location effect in the PerMANOVA is driven by the distinctiveness of desert larks. Bray-Curtis analysis also showed a significant effect of host species (PerMANOVA, pseudo-$F = 3.58$, df = 8, 88, $R^2 = 0.26$, $P < 0.001$; Fig. S3.7 a). Results obtained using weighted UniFrac distances showed a similar effect of geographic location on community clustering of larks' cloacal gut microbiome (PerMANOVA, pseudo-$F = 6.59$, df = 4, 88, $R^2 = 0.24$, $P = 0.001$; Fig. 3 b), and again significant community clustering depending on host species (PerMANOVA, pseudo-$F = 4.40$, df = 8, 88, $R^2 = 0.31$, $P < 0.001$; Fig. S3.7 b). Significant differences were found between temperate Aekingerzand and the other four locations (PCoA Axis 2: $P < 0.007$ for all pairwise comparisons). Within-group dispersion for locations can be found in supplementary material (Fig. S3.8).

Table 3.2: Relative abundance (%) and taxonomic association of the most common OTUs in each geographic location. In bold are highlighted the OTUs that represent a cumulative proportion of up to 40% of the bacterial community for that location. Those OTUs absent from a location are shown with the symbol (-).

OTU	Phylum	Class	Genus	Aekingerzand	Kedong	Kinangop	Mahazat	Taif
1011712	Actinobacteria	Actinobacteria	*Corynebacterium*	1.98	0.01	**1.04**	0.07	0.50
De novo OTU1096	Actinobacteria	Actinobacteria	*Corynebacterium*	0.09	-	**1.43**	**13.86**	7.32
De novo OTU25	Actinobacteria	Actinobacteria	*Corynebacterium*	**14.69**	0.04	**5.60**	3.01	6.45
1095073	Actinobacteria	Actinobacteria	*Propionibacterium*	0.37	0.11	**0.93**	0.32	0.05
208315	Cyanobacteria	Oscillatoriophycideae	*Phormidium*	**3.45**	-	0.13	-	-
1111582	Firmicutes	Bacilli	*Enterococcus*	2.04	2.11	**4.93**	**9.88**	**9.70**
716006	Firmicutes	Bacilli	*Lactococcus*	**2.86**	0.06	0.05	0.03	<0.01
1084045	Firmicutes	Bacilli	*Sporosarcina*	**3.75**	0.30	0.26	0.62	0.07
222433	Firmicutes	Clostridia	*Veillonella*	**3.41**	-	**1.85**	0.03	0.84
1107243	Proteobacteria	Alphaproteobacteria	*Rhizobium*	0.01	<0.01	0.03	2.38	5.91
4396025	Proteobacteria	Alphaproteobacteria	*Sphingomonas*	0.05	0.25	0.06	**5.21**	**12.00**
573413	Proteobacteria	Alphaproteobacteria	*Sphingomonas*	**3.13**	0.81	0.63	1.40	0.60
759916	Proteobacteria	Betaproteobacteria	*Ralstonia*	0.15	0.63	0.52	**10.53**	**11.76**
512485	Proteobacteria	Gammaproteobacteria	*Acinetobacter*	**5.39**	0.01	0.34	2.96	0.82
1097359	Proteobacteria	Gammproteobacteria	*Acinetobacter*	**4.47**	0.42	**4.78**	1.29	2.29
922761	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae (f)	1.10	**4.19**	**13.64**	0.85	0.02
797229	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae (f)	0.13	0.18	**3.51**	0.29	0.01
687940	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae (f)	0.04	0.04	**2.94**	1.28	0.01
813945	Proteobacteria	Gammaproteobacteria	*Pseudomonas*	0.01	-	0.01	**4.62**	**16.03**
De novo OTU256	Tenericutes	Mollicutes	Mycoplasmataceae (f)	0.56	**37.85**	-	-	<0.01

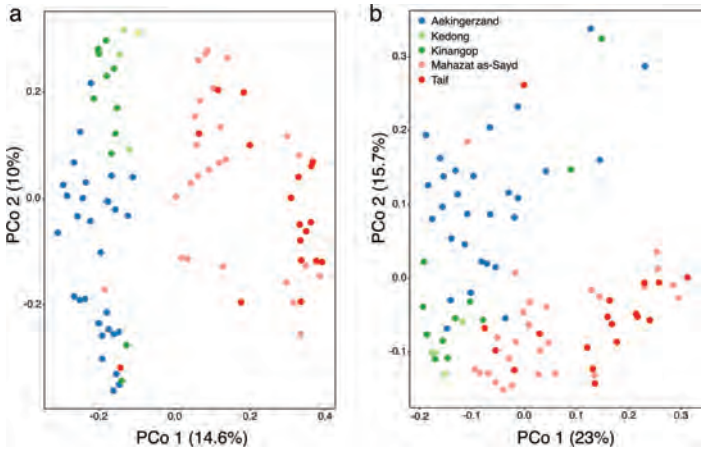

Figure 3.3: Principal coordinates analysis (PCoA) of Bray-Curtis (a) and weighted UniFrac (b) distances among locations, showing the first two principal coordinates axes.

Discussion

This study is the first to explore large-scale patterns of geographic variation in gut microbial communities of wild birds using a multispecies comparison and across three biogeographical regions. Our results reveal substantial geographical structure in bird-associated microbial communities, despite the generally relatively constant environment provided by different birds' bodies, and contrary to the "everything is everywhere" hypothesis. This geographical structure is evident with respect to all three aspects of cloacal gut microbial communities analysed: (i) patterns of OTU richness and Shannon diversity (Fig. 3.1); (ii) dominant taxonomic groups and relative abundances (Fig. 3.2); and community composition (Fig. 3.3). Our geographic patterns of host-associated microbial communities resemble biogeographic patterns found in higher taxonomic groups (e.g., vertebrates) including lower taxonomic diversity in deserts compared to tropical areas, and environment-dependent adaptations of host physiological and life history traits. The geographic differences and commonalities raise questions about the role of environmental microbial communities as source for host-associated microbiota, about codiversification of microbial lineages with hosts, and about the potentially functional relationships between host-associated microbes and host-adaptive traits.

Our finding that host-associated microbial diversity varied with geographic location provides strong evidence that different selective forces might determine the distribution

of free-living and host-associated microbial communities, and raises questions about the connections between free-living and host-associated microbial communities. This finding is relevant in the current debate on whether all life forms are equally affected by biogeography (Martiny *et al*., 2006; Meyer *et al*., 2018) and has important implications for the evolutionary processes shaping both macro and microorganisms (Colston & Jackson, 2016; Hird, 2017). Several studies on free-living microbial communities have found support for the "everything is everywhere" hypothesis (Fierer & Jackson, 2006; Fuhrman *et al*., 2008; Milici *et al*., 2016). In this study, we found a significant effect of geographic location on α-diversity metrics and co-occurrence of taxonomic groups, strongly suggesting that geography influences how many and which bacterial types can be found in lark-associated gut microbiota (Fig. 3.1, Fig. 3.2), despite the generally relatively constant environment provided by larks' bodies. Other local or regional studies focused on these symbiotic relationships have similarly found a geographical effect on several α-diversity metrics in microbiota of birds (Klomp *et al*., 2008; Perry *et al*., 2017) and other vertebrates (Lankau *et al*., 2012; Linnenbrink *et al*., 2013; Llewellyn *et al*., 2015). However, our study provides the first large-scale evidence that host-associated microbes do not fit the "everything is everywhere" hypothesis. We hypothesize that some causes used to explain the "everything is everywhere" hypothesis for free-living microbes (e.g., high dispersal abilities (Finlay, 2002; Hillebrand *et al*., 2001; Martiny *et al*., 2006) could be modified due to the association with hosts. For example, processes such as host selection of host-associated communities by filtering from the pool of environmental microbial communities, could be (at least partially) responsible for cloacal gut microbial assemblages. Previous studies that show that culturable free-living and host-associated bacteria of larks are less abundant in the desert compared to less arid areas (Horrocks *et al*., 2012), mimicking the results of this study, and that the environmental microbial communities play a large role in the acquisition of gut microbes in two temperate larks (van Veelen *et al*., 2017), are also in line with the hypothesis that gut microbial assemblages are impacted by free-living environmental bacterial communities. Multi-species, large-scale studies comparing both free-living and host-associated microbes simultaneously would be a first step towards further testing of this hypothesis.

Our results on beta diversity, notably the different geographic effects in the Bray-Curtis and weighted UniFrac analyses, also shed light on the processes that might shape geographical differences in lark-associated microbial assemblages, particularly codiversification

of microbial lineages with hosts and uptake of host-associated microbes from the environmental pool. The Bray-Curtis analyses highlight the distinctiveness of desert locations (Taif and Mahazat) regarding taxonomic community composition. In the weighted UniFrac analyses, the desert locations also cluster together regarding phylogenetic community composition, but here temperate Aekingerzand appears to be the main driver of the geographic differences (Fig. 3.3 b and *post hoc* differences of weighted UniFrac). The phylogenetic differences are partially illustrated by the dominant OTUs for each location (Table 3.2) as temperate Aekingerzand holds several unique genera (e.g., *Sporosarcina*) among their most abundant OTUs not shared with tropical or desert larks. These results might signify that gut microbes in Aekingerzand are phylogenetically more distant from those of the other locations, potentially indicating different co-evolutionary historical processes of host species at different locations or, alternatively, phylogenetically different environmental bacterial pools at different locations. Overall, the geographic effects in our Bray-Curtis and weighted UniFrac analyses match another recent multi-species comparative analysis of gut microbial assemblages in a group of temperate-zone phylogenetically-distant birds, as well as partially match (Bray-Curtis results) studies with other birds (Hird *et al*., 2014, 2015) and vertebrates (Linnenbrink *et al*., 2013). This gives support for the generality of our findings. However, additional multi-species comparative studies controlling for the co-evolutionary history of hosts (e.g., restricting to closely-related species, or taking into account phylogenetic relationships among hosts), using large-scale geographic comparisons, and potentially using other vertebrate host taxa (e.g., fishes) or host-associated materials (e.g., nesting materials) would be required before making further conclusions on the contribution of co-evolutionary historical processes in explaining geographic variation in host-microbe associations.

In addition to demonstrating that host-associated microbes do not follow a distribution compatible with the "everything is everywhere" hypothesis, a key finding of our study is that host-associated microbes can follow large-scale macro-ecological patterns. One such well-known pattern is that of lower species richness in arid areas (Currie, 1991) compared to tropical regions (Hillebrand, 2004; Jetz *et al*., 2012). In our study, the main difference in cloacal gut OTU richness was detected between lark species from the two tropical African locations and the desert larks from Taif, who harboured the least rich and diverse cloacal gut communities. Investigating why biogeographic rules might affect host-associated

microorganisms similarly to macro-organisms and differently from free-living microbes is essential for understanding the processes that shape microbial assemblages (Colston & Jackson, 2016). Based on the differences with free-living microbes, it is possible that the host is playing an intermediate role, either through codiversification of hosts and specific microbes or through functional links of specific microbes with host adaptive traits, favouring the influence of large-scale biogeographic patterns in microbes.

Geographic variation in host-associated microbial communities could result if these host-associated microbial communities have functional relationships with adaptive traits of hosts, such as adjustments in physiology and life history to live in different environments (Hird, 2017; del Hoyo *et al*., 2004). Previous investigations of physiologies and life histories of the lark species from the same locations as used in this study have highlighted differences among desert, tropical and temperate zone larks. Desert larks have lower immune response, slower growth rates, smaller and fewer clutches per year, as well as lower basal metabolic rate compared with temperate larks, while they also differ from tropical larks with respect to immune function and reproductive strategy (Horrocks *et al*., 2012, 2015; Ndithia *et al*., 2017a; Tieleman *et al*., 2003a,b, 2004). Interestingly, our results also highlight the uniqueness of the cloacal gut microbial communities of desert larks. For instance, lark gut microbial communities in the desert showed the lowest α-diversity while at the same time containing the highest number of unique OTUs (Fig. S3.5). This pattern was confirmed using pairwise comparisons between locations (comparisons including Mahazat and Taif at Table S3.1). These results in addition to those regarding dominant bacterial groups at different taxonomic levels (Fig. 3.2 and Table 3.2) demonstrate the similarity in cloacal gut microbial communities of lark species at the two desert locations. Furthermore, our beta diversity analysis indicates that the geographic differences in gut microbiome composition of larks are mainly due to bacterial communities of desert larks (Fig. 3 a and *post hoc* differences of Bray-Curtis dissimilarities). These pieces of evidence, together with previous studies on the physiology of larks (Horrocks *et al*., 2012, 2015; Ndithia *et al*., 2017a; Tieleman *et al*., 2003a,b, 2004), strongly illustrate the co-variation between gut microbes and the physiological and life history traits that adapt hosts to their environment. Whether these lark-associated bacteria provide their hosts with specific functions or are simply the by-product of unique environmental OTUs incorporated into their gastrointestinal tract by different processes (e.g., via ingestion with food; Muegge *et al*., 2011) remains

unknown. However, given the importance of gut microbes for some key functions of their hosts (Dinan *et al*., 2015; Hird, 2017; Kohl & Carey, 2016; McFall-Ngai *et al*., 2013) including those previously analysed for larks (e.g., immune function, metabolism and growth; Horrocks *et al*., 2012, 2015; Tieleman *et al*., 2004), we hypothesize that there may be functional associations between the cloacal gut microbes and the adaptations of larks to their respective environments (Horrocks *et al*., 2015). To investigate this intriguing possibility, additional studies are required to further explore these potential functional relationships and to what extent gut microbes could contribute to the adaptive values of these host traits, which is an important gap in current microbiology and animal ecology (Kohl & Carey, 2016). In general, future studies should confirm the generality of our findings by also including different animals and different body parts, paying special attention to integrate hosts from arid areas into their comparisons. Overall, our study provides a novel example of the importance of integrating host-associated microbes into the field of microbial biogeography in order to advance not only our understanding on key biogeographic questions but also on the evolution of host-microbe interactions.

Ethics approval

All birds were sampled under license from the relevant authorities in the various countries. This study was performed under animal welfare licence D4743A and DEC6619B of the Institutional Animal Care and Use committee of the University of Groningen obeying the Dutch Law. The National Commission for Wildlife Conservation and Development of Saudi Arabia and the National Wildlife Research Center approved this research, as well as the Human Resource Training and Development Committee of the National Museum of Kenya (Ref: NMK/TRN/PF/177015/045).

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Supplementary Information

Table S3.1: Significant differences in normalized read counts of OTUs among sites based on pairwise analyses using *DESeq2* and an adjusted $P < 0.1$. Positive values indicate the number of OTUs with higher read counts in the first location compared to the second, and negative values indicate the opposite.

Comparison		**OTUs**	
Aekingerzand	Kedong	+23	-9
	Kinangop	+15	-122
	Mahazat as-Sayd	+89	-258
	Taif	+41	-190
Kedong	Kinangop	+2	-12
	Mahazat as-Sayd	+5	-44
	Taif	+10	-66
Kinangop	Mahazat as-Sayd	+178	-90
	Taif	+100	-93
Mahazat as-Sayd	Taif	+3	0

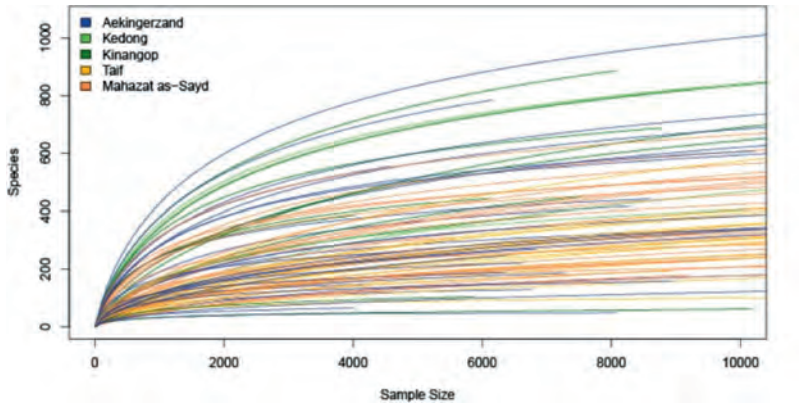

Figure S3.1: Rarefaction curves of samples from the different locations included in the study.

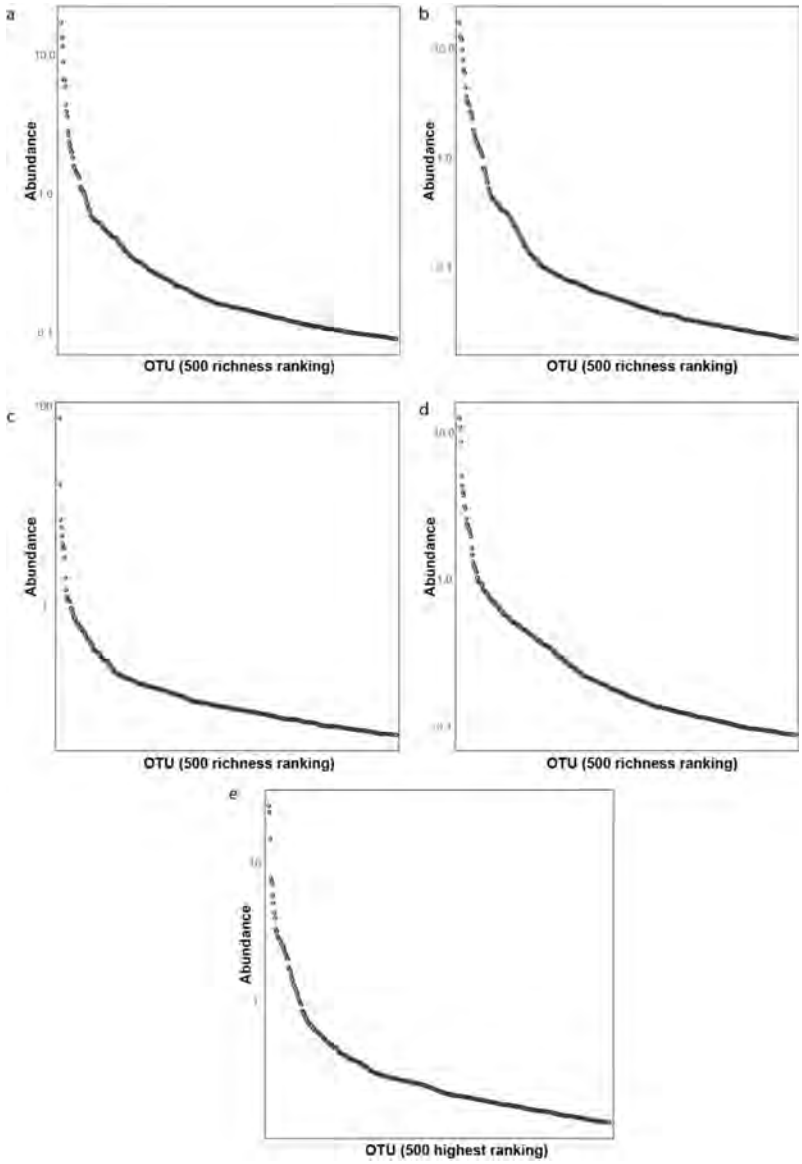

Figure S3.2: Rank-abundance plots of the 500 most abundant OTUs for desert (a) Mahazat as-Sayd and (b) Taif, (c) tropical Kedong and (d) Kinangop, and temperate (e) Aekingerzand. Each dot represents an OTU. Abundance is represented in percentage. Note that the y-axes are log10 transformed.

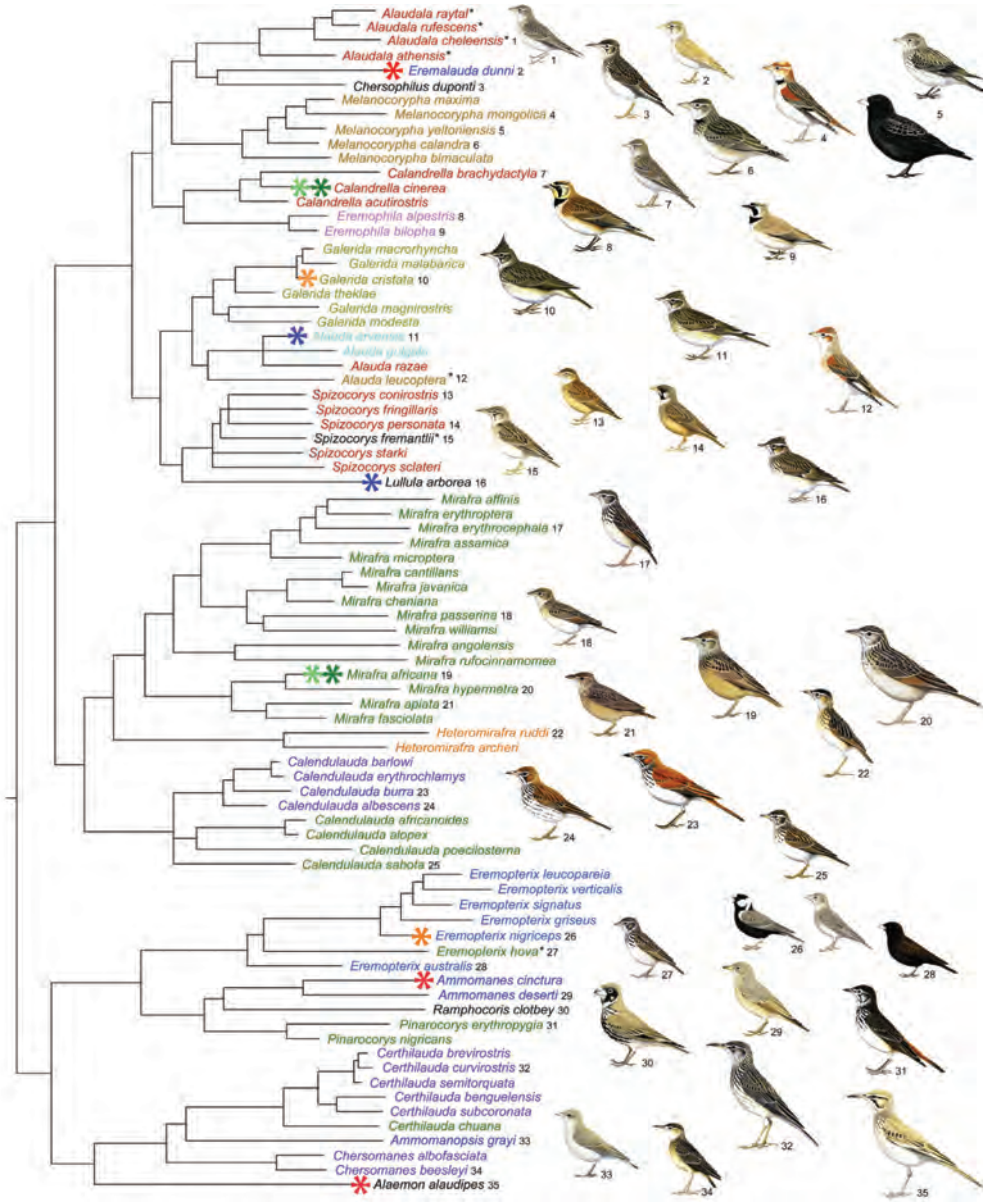

Figure S3.3: Phylogenetic relationship of the Alaudidae family. The figure is adjusted from Alström *et al*., 2013, used with permission from the authors. Lark species included in our study are marked with an asterisk (blue: Aekingerzand, light green: Kedong, dark green: Kinangop, red: Mahazat, orange: Taif). *Eremalauda dunni* has been split and renamed as *Eremalauda eremodites* (Arabian lark)

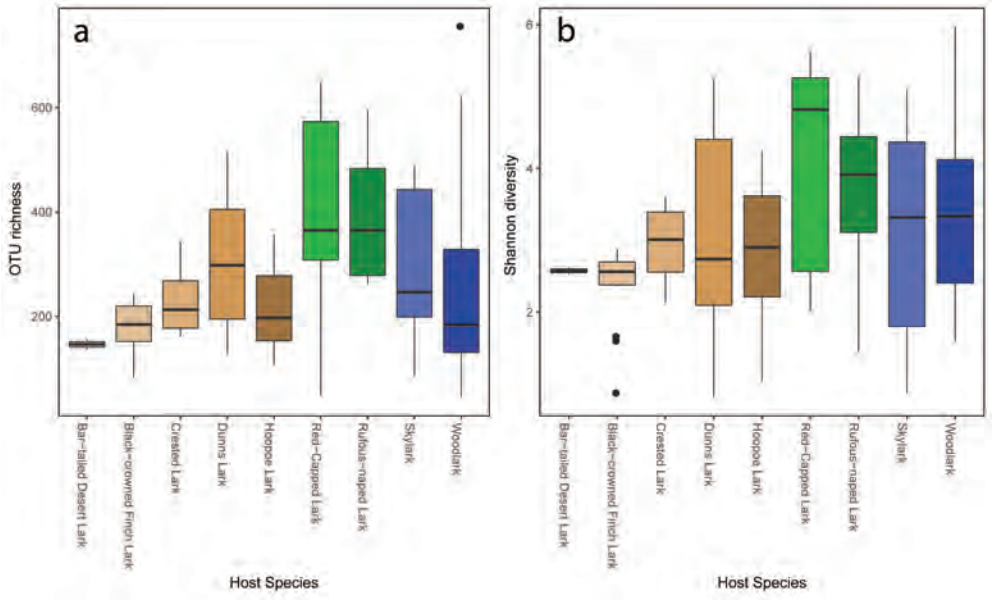

Figure S3.4: OTU richness (a) and Shannon diversity (b) of gut microbiota of the nine species of larks included in this study. Results of a linear model including geographic location as a fixed factor showed a significant effect of this variable on OTU richness ($F_{8,79}$ = 2.39, P = 0.023) but not on Shannon diversity ($F_{8,79}$ = 1.65, P = 0.125). Pairwise comparisons using Tukey *post hoc* tests showed that only Bar-tailed desert larks and Red-capped larks differed significantly in the OTU richness of their gut microbial communities (P = 0.019). Colours of bars depict the species location: temperate Aekingerzand (blue), tropical Kinangop and Kedong (green), and desert Mahazat and Taif (brown).

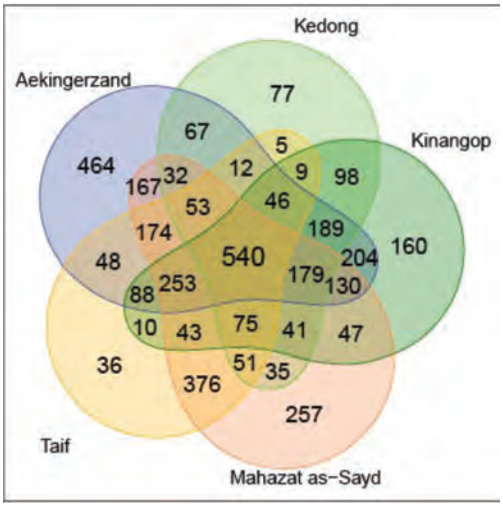

Figure S3.5: Venn diagram showing the co-occurrence of OTUs detected in our analyses among locations. Colours depict the different locations.

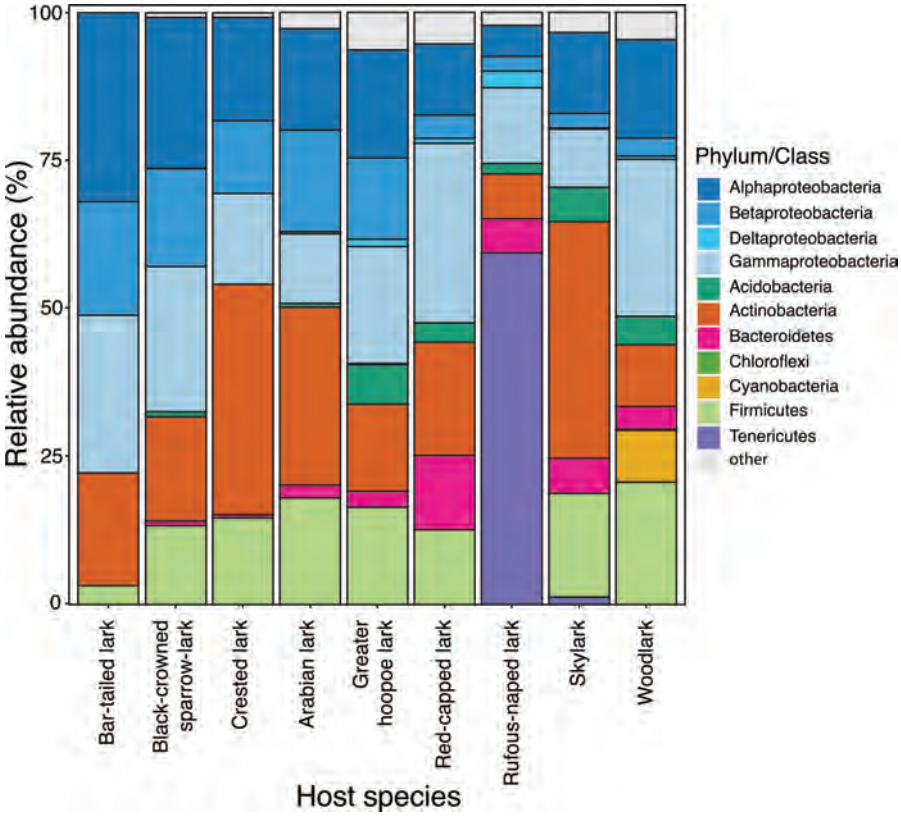

Figure S3.6: Barplots of mean relative abundances of the most abundant Phyla and Proteobacteria classes for every host species. Calculations are based on rarefied data (4000 reads/sample).

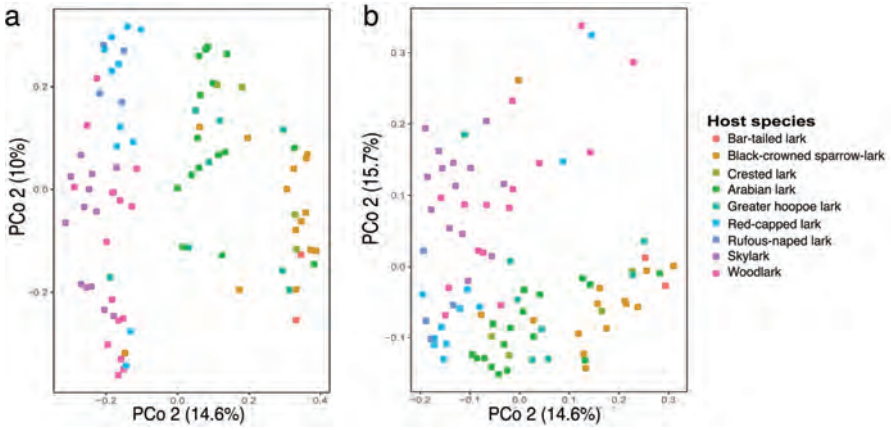

Figure S3.7: Principal coordinates analysis (PCoA) of Bray-Curtis (a) or weighted UniFrac (b) distances among host species for the first two principal coordinate axes. This figure illustrates variation in cloacal gut microbial community composition among the lark species considered in the study. Note that the patterns shown are qualitatively similar to those presented in Fig. 3.3 for geographic location.

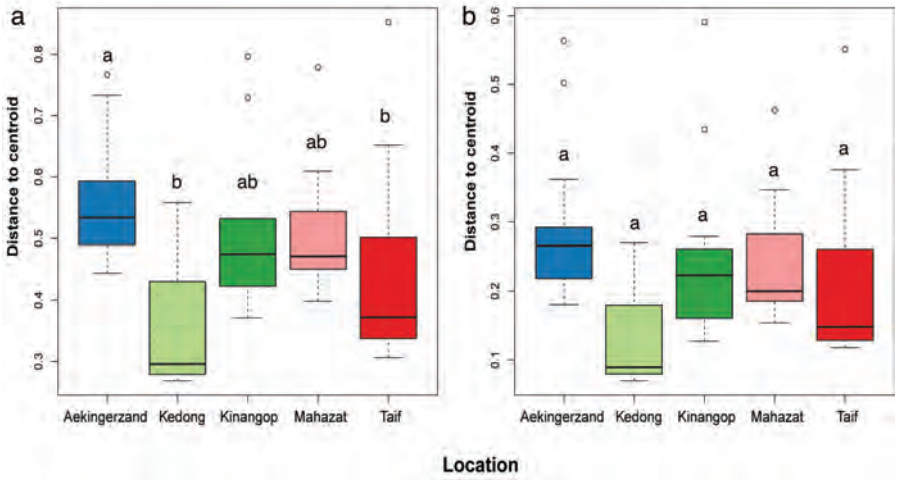

Figure S3.8: Group dispersion in community composition of lark gut microbiota within each location: (a) Bray-Curtis and (b) weighted Unifrac dissimilarities calculated with principal coordinates analyses. The test for the homogeneity of within-group dispersion indicated significant differences (Bray-Curtis: pseudo-F = 4.90, df = 4, 84, P = 0.004; weighted Unifrac: pseudo-F = 2.79, df = 4, 84, P = 0.04). Letters denote significant *post hoc* differences among locations.

Chapter 4

Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota

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Abstract

The microbiome is essential for development, health, and homeostasis throughout an animal's life. Yet, the origins and transmission processes governing animal microbiomes remain elusive for non-human vertebrates, oviparous vertebrates in particular. Eggs may function as transgenerational carriers of the maternal microbiome, warranting characterisation of egg microbiome assembly. Here, we investigated maternal and environmental contributions to avian eggshell microbiota in wild passerine birds: woodlark *Lullula arborea* and skylark *Alauda arvensis*. Using 16S rRNA gene sequencing, we demonstrated in both lark species, at the population and within-nest levels, that bacterial communities of freshly laid eggs were distinct from the female cloacal microbiome. Instead, soil-borne bacteria appeared to thrive on freshly-laid eggs, and eggshell microbiota composition strongly resembled maternal skin, body feather and nest material communities, sources in direct contact with laid eggs. Finally, phylogenetic structure analysis and microbial source tracking underscored species sorting from directly contacting sources rather than *in vivo* transferred symbionts. The female-egg-nest system allowed an integrative assessment of avian egg microbiome assembly, revealing mixed modes of symbiont acquisition not previously documented for vertebrate eggs. Our findings illuminated egg microbiome origins, which suggested a limited potential of eggshells for transgenerational transmission, encouraging further investigation of eggshell microbiome functions in vertebrates.

Introduction

Host-microbe associations are universal to all animals (McFall-Ngai *et al*., 2013) and increasingly recognised as a host trait in evolutionary biology (Hird, 2017). An emerging paradigm concentrates on identification of the proximate mechanisms underlying natural microbiome variation and prediction of its fitness consequences when under selection (Hird, 2017; Kohl *et al*., 2017). Understanding the origin, maintenance and transience of microbial symbionts across generations of animal hosts is crucial to predict potential host fitness consequences of microbiome variation (Funkhouser & Bordenstein, 2013; Shapira, 2016). Although natural animal-microbiota investigations are mounting (Kueneman *et al*., 2014; Ley *et al*., 2008; Waite & Taylor, 2015), enhanced coverage of animal lineages across diverse spatiotemporal scales, between and within host species, is essential to find general and differential features in the ecology and evolution of animal microbiomes.

A few studies already demonstrated that the establishment of a healthy microbiota can constitute a key aspect of vertebrate host fitness, e.g. affecting later-life disease risk (Knutie *et al*., 2017) and offspring growth rate (Jacob *et al*., 2015). Microbiome variation resulting from non-random transmission of symbionts from one generation to the next provides opportunities for selection through effects on host fitness (van Opstal *et al*., 2015). Yet, non-random transgenerational transmission has only been documented in some animal clades and ranges from obligate vertical transmission in insects (Douglas, 1989; Moran & Telang, 1998), mixed vertical and horizontal acquisition in sponges (Reveillaud *et al*., 2014), lizards (Kohl *et al*., 2017) and humans (Funkhouser & Bordenstein, 2013), to highly selective horizontal symbiont acquisition in squids (Koch *et al*., 2014; McFall-Ngai & Ruby, 1991). This variety of transmission routes (reviewed in Bright & Bulgheresi, 2010) calls for systematic investigations in a broad array of animal species - an effort that is crucial for identifying general patterns and developing general concepts of animal-microbiome dynamics (Colston & Jackson, 2016; Shapira, 2016). Since vertical transmission sensu stricto refers to maternal transmission through the germ line (Douglas, 1989), it is increasingly considered more broadly by including environmental maternal effects, hereafter referred to as 'transgenerational transmission' (Funkhouser & Bordenstein, 2013). As transgenerational transmission is understudied in non-human vertebrate systems (Colston & Jackson, 2016; Funkhouser & Bordenstein, 2013) compared to

humans (e.g. Dominguez-Bello *et al*., 2010), its investigation is fundamental to assess its meaning in evolutionary biology.

Symbiont transmission routes may have evolved alongside the different vertebrate reproductive modes (i.e. oviparity, ovoviviparity and viviparity). In humans, birth mode and early-life maternal effects are important factors driving newborn microbiome assembly (Dominguez-Bello *et al*., 2010; Gilbert, 2014), which might prove universal to most or all viviparous vertebrates (Kohl *et al*., 2017). Offspring of oviparous vertebrates, however, hatch (mostly) *ex vivo* in the exterior environment (e.g. oviparous fish and amphibians in water, and reptiles and birds in nests). The lack of maternal-offspring inoculation upon hatching from an egg, especially in the absence of parental care, raises the question of how oviparous vertebrates acquire their (symbiotic) microbiome after hatching.

Considering the microbiome as a host trait (Hird, 2017; Kohl, 2012) and benefitting from birds as benchmark models in evolutionary biology, we argue that studying associations between avian hosts and their microbial communities could aid in uncovering microbiome effects on vertebrate host fitness. Identifying transmission routes and avian microbiome assembly may be a first step towards this goal. As fecundity of oviparous vertebrates heavily relies on egg survival and subsequent survival of offspring, we hypothesize that eggs could have evolved to function as transgenerational carriers of an initial inoculum to hatching offspring, and/or potentially providing a protective shield to invaders causing egg infection (Sarmiento-Ramírez *et al*., 2014). At laying, eggs pass through the distal intestine and then through the cloaca (hereafter 'cloacal gut'), which serves to expel both faeces and eggs. Yet, healthy eggs of wild birds are thought to be internally sterile, but trans-shell and internal egg infection negatively affect hatchability and offspring survival probability (Cook *et al*., 2003, 2005b; Godard *et al*., 2007; Hansen *et al*., 2015). Conversely, egg incubation (Cook *et al*., 2005a; Grizard *et al*., 2014; Lee *et al*., 2014) and eggshell smearing with symbiont-containing secretion induce compositional changes that increase hatchability (Martín-Vivaldi *et al*., 2014). Additional to such maternal effects, associations between nests and egg microbiota have been identified (Goodenough *et al*., 2017; Martínez-García *et al*., 2016), though high-throughput sequencing data integrating the multi-faceted maternal, nest and eggshell microbiota assembly are lacking. We previously demonstrated significant inter-individual variation among adult

females in our lark study system (van Veelen *et al*., 2017), providing potential for non-random transgenerational microbiota transmission. If eggs function as carriers of maternal microbiota, we hypothesized similarity among eggshell and maternal cloacal microbiotas.

Here we used 16S rRNA gene sequencing to identify sources of avian eggshell microbiota in woodlarks *Lullula arborea* and skylarks *Alauda arvensis*. We evaluated alpha diversity and beta diversity of eggs and potential source communities to map egg-source microbiome associations by testing effects of host species, (individual) nests, and resemblance within nests between the first and second laid eggs of each clutch. We assessed phylogenetic structure of eggshell communities to evaluate how selective eggshells are in bacterial community assembly, and used cross-sectional and within-nest analyses of phylogenetic beta diversity to evaluate similarities between eggshell and source communities. Finally, we applied predictive modelling to estimate the proportional contribution of each source to eggshell communities.

Methods

Study species and sample collection

We monitored ground-nesting woodlarks *Lullula arborea* and skylarks *Alauda arvensis* at Aekingerzand, National Park Drents-Friese Wold, the Netherlands (N 52°55'; E 6°18') between March and July 2014. Both species start incubation after completion of a clutch (4-6 eggs for woodlarks and 3-4 eggs for skylarks). Females contact their eggs while sitting on the nest for protection against rain, heat and nest predation, as well as during overnight resting. Without handling eggs, we marked each first laid egg with a small dot using a water-resistant marker at the blunt end to distinguish it from second eggs. We collected both eggs when the second egg had been laid (first eggs within 36 h and second eggs within 12 h post-laying), minimising egg age and incubation effects (Grizard *et al*., 2015). We aseptically stored eggs individually in sterile plastic bags (Whirl-Pak®, Nasco, Fort Atkinson, WI, USA), and replaced them by ethanol-sterilized homemade self-hardening clay eggs to encourage clutch completion. Females (96%) continued egg laying after sampling. We captured females as soon as possible after egg collection (mean ± s.e.: 10.3 ± 4.3 days, n = 19). We collected a cloacal swab, a swab sample (moistened with sterile PBS) of exposed

brood patch skin, ~5 brood patch-aligning feathers, ~3 grass stems from the centre of the nest cup and surface soil within a 50cm radius from the nest entrance. We handled birds and samples using new, clean latex gloves while handling and sampling birds, nests and eggs (changing gloves between samples), which we sterilised using 70% ethanol as an extra precaution to prevent sample contamination. In total, we collected 38 woodlark (19 nests) and 38 skylark eggs (19 nests). High nest predation (Praus *et al*., 2014) reduced our capture success of females, resulting in fewer female and nest samples than collected eggs (for full details see Table 4.1). Samples were kept on ice in the field (< 12 h) and stored at -20 °C.

Table 4.1: Summary of collected eggshell and source community samples of woodlarks and skylarks.

	Woodlark		Skylark	
	n samples	**n nests (with eggs[a])**	**n samples**	**n nests (with eggs[a])**
Egg	38	19 (19)	38	19 (19)
Cloacal gut	12	12 (10)	15	15 (8)
Brood patch skin	13	13 (10)	11	11 (8)
Feather	13	13 (10)	11	11 (8)
Nest material	14	14 (11)	7	7 (7)
Surface soil	14	14 (11)	9	9 (7)

[a]Number between brackets denotes the number of nests per sample type category for which the first two eggs of a clutch were successfully collected.

DNA extraction and 16S rRNA gene amplicon sequencing

We aseptically isolated eggshells from randomly picked frozen eggs following Grizard *et al*. (2014) and powdered eggshells using liquid nitrogen and autoclaved-sterilized mortar and pestle. We aseptically peeled all cotton from swabs and moved it to extraction tubes. We transferred all brood patch-lining feathers and all nest lining grasses into individual sterile 15-ml tubes, added 978 µl sodium phosphate buffer and 122 µl MT buffer (kit reagents), vortexed tubes 10 s using a Vortex-Genie2 (MoBio Laboratories Inc., Carlsbad, CA), sonicated tubes for 15 min, and then vortexed 10 min to detach bacterial cells from the sources. We transferred the resulting suspensions to extraction tubes. We used (mean ± S.E.M.) 0.3 ± 0.01 g soil per sample for DNA extraction, and completed extractions of all samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocol with minor adjustments: cell lysis was

achieved by three times 1 min bead-beating and DNA elution in 100 µl PCR-grade water. We quantified DNA concentrations using the Quant-it PicoGreen dsDNA kit (Molecular Probes, Invitrogen, Eugene, OR, USA), normalised the concentrations to 1 ng template DNA per 25 µl reaction (triplicates per sample) and amplified the V4/V5 region of the 16S rRNA gene using primers 515F and 926R with partial Illumina adapters and thermal cycler protocol: 95 °C for 5 min, 35 cycles at 95 °C for 40 s, 56 °C for 45 s, 72 °C for 40 s, and 10 min at 72 °C. The use of negative controls (no template) the during the amplification ensured no contaminations in the PCR reagents. Eleven eggshell samples failed PCR trials, leaving 65 eggshell samples for further analysis. We sent purified (QIAquick gel extraction Kit, QIAGEN GmbH, Hilden, Germany) pooled triplicates to GenoToul (INRA, Toulouse, France) for library preparation and Illumina MiSeq sequencing using 2 $\times$ 250 bp paired-end v2 chemistry.

Sequence data processing

We processed raw 16S rRNA gene sequence data using QIIME (v. 1.9.0) (Caporaso *et al*., 2010a). We truncated reverse primers from demultiplexed, quality filtered reads (Phred score $\geq$ 25, maximum bad run length = 3, no primer mismatches), and used open-reference OTU-picking (default QIIME settings using Greengenes reference set (v. 13.8) (McDonald *et al*., 2012) with *de novo* clustering of non-matching sequences; 0.01%) using 97% identity in UCLUST (Edgar, 2010). After removal of singletons, assigning taxonomy with UCLUST (Greengenes v. 13.8, 97%), aligning representative sequences using PyNast (Caporaso *et al*., 2010b), and removing chimeric sequences identified by UCHIME (Edgar *et al*., 2011), we generated a phylogenetic tree using FastTree (Price *et al*., 2009). We removed OTUs belonging to Archaea, chloroplasts and mitochondria and filtered OTUs representing < 0.01% of the total abundance.

Statistical analyses

We analysed bacterial community structure and diversity using rarefied data (5000 reads/sample) using *phyloseq* (v. 1.14.0) (McMurdie & Holmes, 2013), *picante* (Kembel *et al*., 2010), and *vegan* (v. 2.4-0) (Oksanen *et al*., 2016), and non-rarefied data using *DESeq2* (v. 1.10.1) (Love *et al*., 2014) and a SourceTracker script (Knights *et al*., 2011) for R statistical software (v. 3.2.3) (R Core Team, 2015). We analysed variation in alpha

diversity metrics and beta diversity distances with ANOVA and Tukey's *post-hoc* contrasts using *multcomp* (Hothorn *et al*., 2008) or Mann-Whitney U tests.

We used ANOVA to compare alpha diversity of eggshell communities with cloacal gut, brood patch skin, body feathers, nest material and surface soil communities. To explore the effect of post-laying time (categorical: <12 h and 24 – 36 h) on eggshell communities, we tested for differential abundance of OTUs between first and second eggs, corrected for host species, using negative binomial models and Wald tests in *DESeq2* (critical FDR *q*-value 0.1). We determined relative abundances of differentially abundant OTUs between first and second eggs in each potential source type. We then explored the prevalence of these OTUs in maternal and nest-associated communities using indicator values for each differentially abundant OTU (Dufrêne & Legendre, 1997). Indicator values represent the strength of associations between taxa and community (sample) types, where larger values indicate greater sample type specificity and fidelity. To test correspondence of estimated alpha diversity metrics between first and second eggs within nests, we estimated the repeatability *r* of OTU richness and Shannon diversity using linear mixed models (LMM) in rpt*R* (Nakagawa & Schielzeth, 2010). To determine whether eggshells form a selective habitat for bacterial settlement, we analysed the degree of non-random phylogenetic community structure using the nearest taxon index (NTI) based on null modelling of mean nearest taxon distances (MNTD) with 999 iterations of taxon label randomisations of our phylogenetic tree (Stegen *et al*., 2012; van Veelen *et al*., 2017; Webb *et al*., 2002).

We analysed taxonomic (Bray-Curtis) and phylogenetic similarity (weighted UniFrac) of eggshell bacterial community composition in three ways: first, we analysed community clustering at the population-level by partitioning variation by lark species, by first versus second eggs, and by nest using constrained distance-based redundancy analysis (Legendre & Anderson, 1999). Then, we compared phylogenetic composition of eggshell communities with potential source types (cloacal gut, brood patch skin, body feathers, nest material and surface soil communities) using principal coordinate analysis (PCoA) and PerMANOVA (Anderson, 2001) at different taxonomic depths: phylum, family and OTU level, using agglomerated OTU tables using *tax_glom* in *phyloseq*. Then, within each nest, we calculated pairwise weighted UniFrac distances to assess the phylogenetic similarities between eggs and potential sources and tested for differential egg-source similarities with LMM, using Nest ID as a random factor to prevent pseudo-replication

bias of eggs from the same nest using *nlme* (Pinheiro *et al*., 2016). We reported *post-hoc* Tukey's contrasts with FDR-corrected *q*-value s (Benjamini & Hochberg, 1995). Finally, we employed SourceTracker (Knights *et al*., 2011) in QIIME as a Bayesian approach to predict the origins of eggshell microbiota, where we designated all maternal and environmental communities as 'source' pool and eggshell communities as 'sink' pool, independently for woodlark and skylark. We used unrarefied data as input for the SourceTracker model, which predicted source contributions using default model parameters. We tested differential proportional contributions of potential source community types using a Kruskal-Wallis test and a Dunn's test for multiple group comparisons (Dinno, 2017). We visualised the relative contributions of maternal sources (cloacal gut, brood patch skin and feathers), and the three dominant sources (nest material, brood patch skin and feathers), separately, illustrated with ternary plots using *ggtern* (v. 2.2.0) (Hamilton, 2016).

Results

The constructed OTU table contained 1148 OTUs and sample coverage ranged between 5225 and 111376 sequences per sample. Rarefaction curves showed that Shannon diversity had levelled at 5000 reads (Fig. S4.1). We rarefied the OTU table to 5000 reads per sample.

Alpha diversity of eggshell bacterial communities

Eggshell communities of woodlarks and skylarks harboured on average 67% ($t = 4.62$, $P < 0.01$) and 50% ($t = 4.25$, $P < 0.01$) more OTUs than cloacal gut communities, respectively, but the number of OTUs found on eggs was not different from other sample types (Fig. 4.1 a). Similarly, Shannon diversity of eggshell communities was 29% higher in eggshell communities of both woodlarks ($t = 5.84$, $P < 0.001$) and skylarks ($t = 6.07$, $P < 0.001$) compared to cloacal gut communities (Fig. 4.1 b). OTU richness ($F_{1,62} = 1.36$, $P = 0.25$) and Shannon diversity ($W = 441$, $P = 0.26$) of eggshells communities differed neither between woodlarks and skylarks (Fig. 4.1 a, b), nor between freshly laid eggs ('Second egg') and eggs laid a day earlier ('First egg') (OTU richness: woodlark,

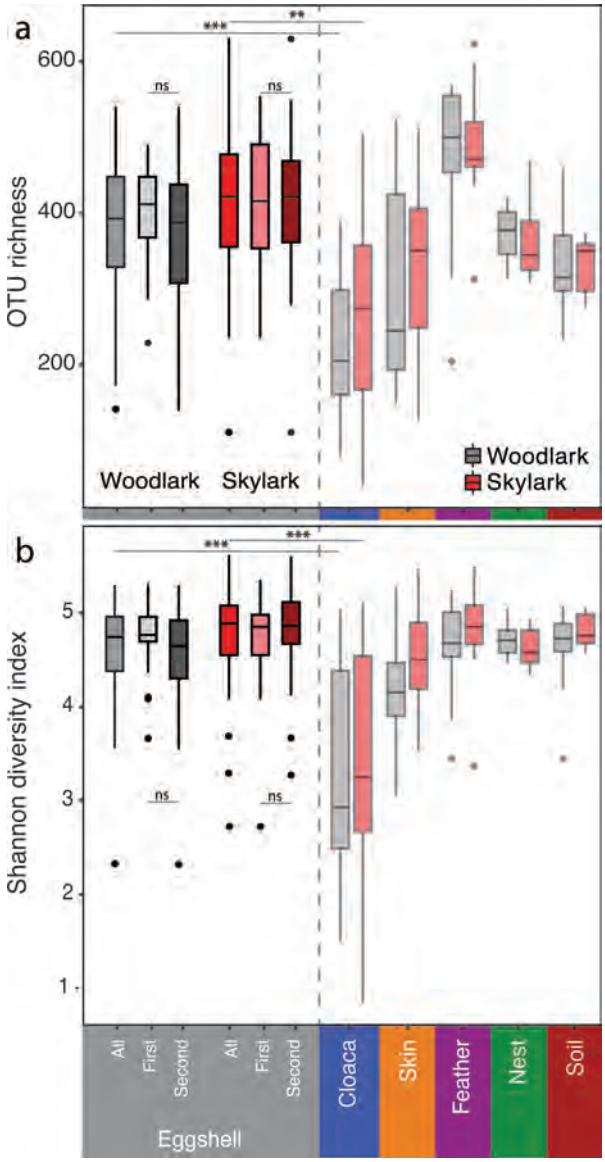

Figure 4.1: Alpha diversity of eggshells of wild woodlarks and skylarks. OTU richness (a) and Shannon diversity (b) of eggshell bacterial communities, stratified by lark species and laying sequence. Alpha diversity metrics are compared to data from maternal and environmental communities (faded boxes) (van Veelen et al., 2017). Significance of comparisons among first and second eggs, and between eggshells and other communities are shown (** $P < 0.01$, *** $P < 0.001$, ns = not significant, other non-significant pairwise differences are not highlighted).

t = 0.54, $P = 0.95$; skylark, t = 0.37, $P = 0.98$; Shannon diversity: woodlark, $W = 163$, $P = 0.34$; skylark, $W = 112$, $P = 0.56$; Fig. 4.1 a, b).

Relative taxon abundances of eggshell bacterial communities

Eggshells of woodlarks and skylarks each harboured six unique OTUs that were not found in the in maternal or nest-associated sources associated with each host species (Table S4.1). One of these OTUs was identified in eggshells of both woodlarks and skylarks. However, all of these eggshell-specific OTUs were low in abundance and infrequently present in eggshell communities (Table S4.1). Further analysis of bacterial abundance in eggshell communities revealed that the abundance of 111 taxa differed between first and second laid eggs, of which 109 taxa were more abundant in first laid eggs, which were present in the nest a day longer than second laid eggs at the moment of sampling (Fig. 4.2 a). Relative abundances of these 111 OTUs in potential sources showed that they were variably abundant in maternal and environmental communities (Fig. 4.2 a). Our efforts to uncover potential links between eggshell and source communities revealed that 58 (52%) of these OTUs were designated significant indicator taxa for one or more potential source community types (FDR $q < 0.1$; Fig. 4.2 b). These indicator taxa were in all cases most indicative for either maternal or environmental communities, and particularly often for soil communities (Fig. 4.2 b). Specifically, bacterial taxa that significantly proliferated on eggshells shortly after laying (< 36 h) were not specific to the eggshell niche, but rather indicators of communities associated with females or the nest environment. Only two taxa that were most indicative for cloacal microbiota of larks, belonging to *Ralstonia* and Caulobacteriaceae, respectively, increased on eggshells (Fig. 4.2 b), whereas the remaining proliferating eggshell-associated taxa were top indicators for skin (n = 2), feather (n = 4), nest material (n = 2) and soil (n = 46). These results suggest that the bacterial taxa thriving on eggshells shortly after they have been laid originate from alternative sources to the maternal cloacal community.

Phylogenetic community structure analysis of eggshell bacterial communities

Of the OTUs found in source samples, 99.4% of taxa was identified in at least one eggshell sample. This high fraction raised the question whether eggshells either form a

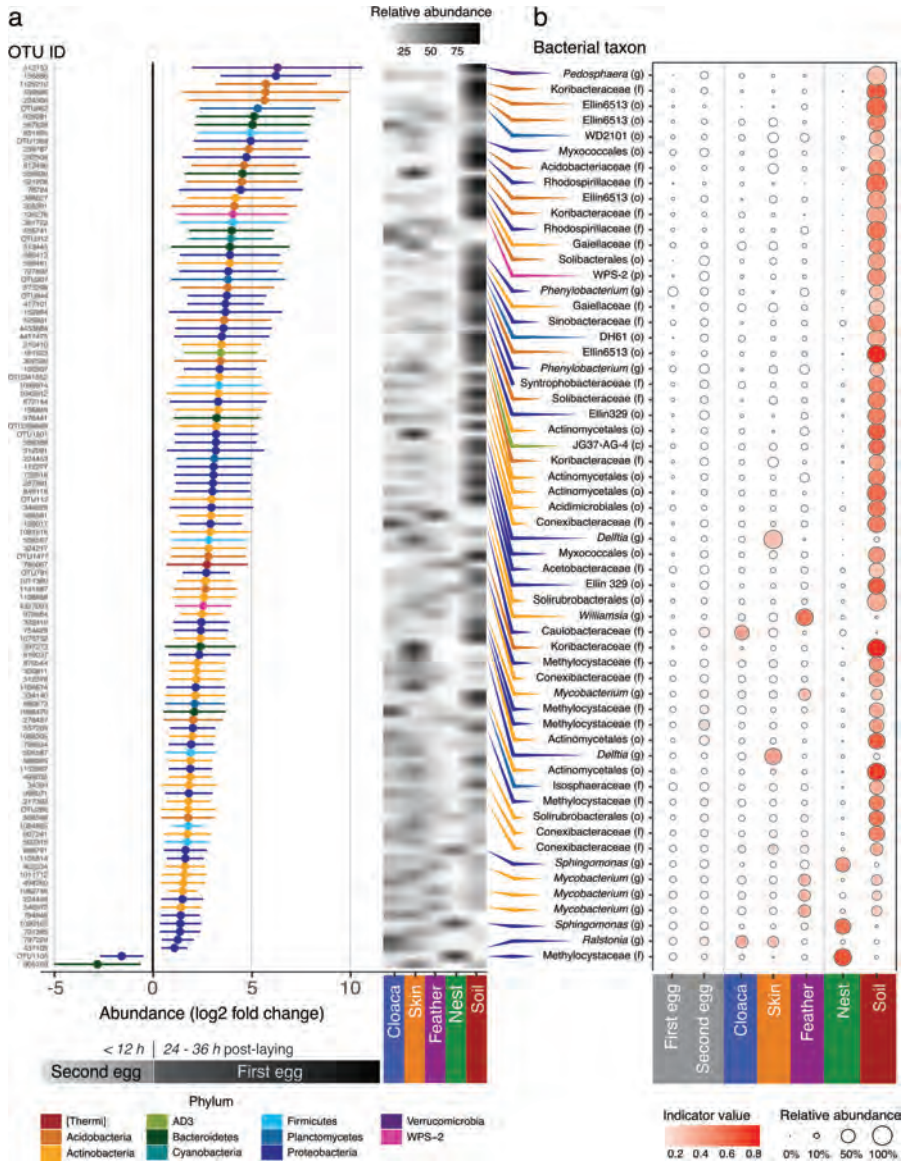

Figure 4.2: OTU abundance between first and second laid eggs, in comparison to maternal and environmental communities. (a) Differential abundance of 111 OTUs between first and second eggs and abundance of each OTU in maternal and nest environmental communities (grey scale). First and second eggs of the laying sequence are exposed to nest environmental conditions 24-36 h, and < 12 h prior to collection, respectively. Mean (circles) and whiskers (95% CI bars) denote log2 fold change of OTU abundance between first and second eggs, coloured by bacterial Phylum. (b) A subset of OTUs (n = 58) was designated significant indicator species (FDR $q < 0.1$) for one or more community types that potentially contribute to eggshell microbiota assembly. Relative abundances in (a) are calculated on sources only (i.e. excluding eggshells). In (b), the average indicator value (colour intensity) and relative abundance (bubble size) were calculated using the cumulative abundance across all communities (i.e. including eggshell communities) as the denominator. Phylum-coloured connectors link OTU IDs in (a) to each indicator OTU in (b), which are labelled by the lowest taxonomic information available.

selective niche or primarily assemble through random processes e.g. random dispersal or ecological drift. Our null modelling analysis of phylogenetic community structure revealed significant deviations of observed mean nearest taxon distance (MNTD) distributions from the permutated null distribution in each eggshell subgroup (Fig. 4.3 a) with average nearest taxon indices (NTI) > +2 in 86% of the eggshell communities (Fig. 4.3 b), indicating predominantly phylogenetically clustered eggshell microbiota.

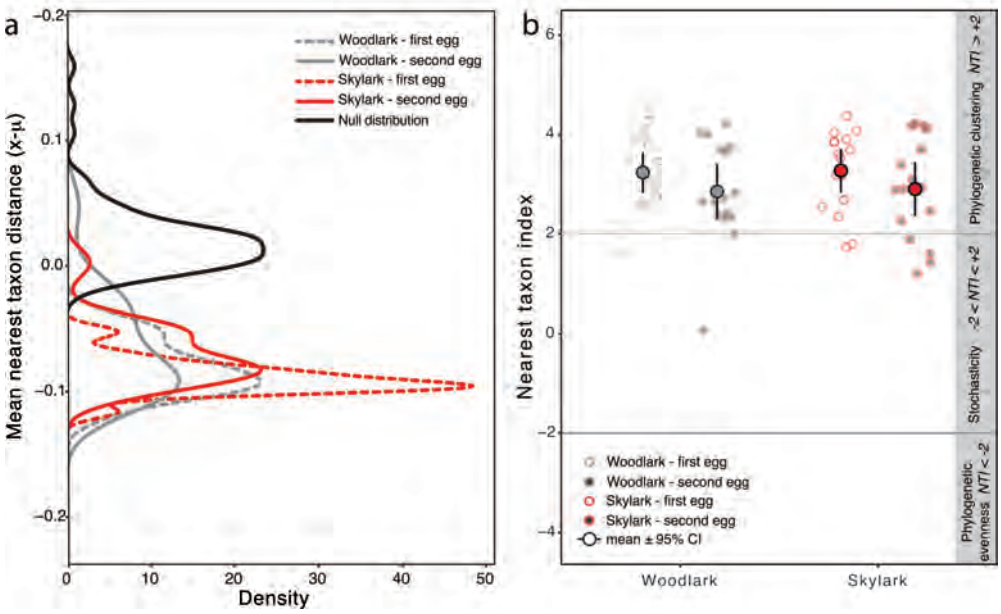

Figure 4.3: Phylogenetic structure in eggshell microbial communities. Mean nearest taxon distance (MNTD) centred around zero (a) and nearest taxon index (NTI) (b) for first and second eggs of woodlarks and skylarks. (a) The area under the curve is determined by the number of pairwise taxon distances (999 for the null distribution), where peak height (density) is negatively related to peak width, together representing the variation of MNTD in a given eggshell subgroup.

Within-nest and between-nest variation of community resemblance of eggshells

The lack of repeatability of OTU richness ($r \pm se = 0 \pm 0.157$) and Shannon diversity ($r \pm se = 0 \pm 0.148$) of eggshell microbiota within nests indicated that alpha diversity of bacterial communities of two consecutive eggs, originating from the same mother, were statistically not more similar than compared to a randomly selected egg from the population. Analysis of taxonomic (Bray-Curtis) and phylogenetic (weighted UniFrac)

community structure showed that host species or egg age did not structure eggshell microbiota (Fig. S4.1 a, b; Table 4.2). Instead, nest identity explained 63% of phylogenetic and taxonomic clustering of eggshell communities based on weighted UniFrac (Table 4.2) and Bray-Curtis dissimilarities, respectively (Fig. S4.1 c; Table 4.2). Notably, PCoA showed very high similarity (clustering) for some nests, but not others (Fig. S4.2), indicating substantial variation in how similar eggs originating from the same nest are. Nonetheless, the significant effect of nest identity on PCoA clustering was at least partly maintained at higher taxonomic levels (Table 4.2).

Table 4.2: Taxonomic (Bray-Curtis) and phylogenetic (weighted UniFrac) beta diversity of eggshell bacterial communities analysed across lark species, laying sequence and nests, at the bacterial OTU, family and phylum levels using ANOVA on constrained ordination (capscale analysis).

	Bray-Curtis dissimilarity				**weighted UniFrac**			
				OTU				
	Df	*F*	R^2	*P*	Df	*F*	R^2	*P*
Lark species	1	1.34	0.02	0.131	1	0.88	0.01	0.5
Egg 1 vs. egg 2	1	1.28	0.02	0.184	1	1.19	0.02	0.282
Nest	35	1.45	0.63	**<0.001**	35	1.41	0.63	**0.013**
Residual	27		0.34		27		0.34	
				Family				
Lark species	1	0.84	0.01	0.562	1	0.7	0.01	0.633
Egg 1 vs. egg 2	1	1.43	0.02	0.158	1	1.16	0.01	0.287
Nest	35	1.48	0.64	**0.004**	35	1.56	0.65	**0.006**
Residual	27		0.33		27		0.32	
				Phylum				
Lark species	1	0.71	0.01	0. 474	1	0.84	0.01	0.485
Egg 1 vs. egg 2	1	2.02	0.03	0.121	1	1.54	0.02	0.19
Nest	35	1.05	0.55	0.417	35	1.42	0.63	**0.041**
Residual	27		0.41		27		0.34	

P-values < 0.05 are represented bold.

Eggshell community composition in relation to potential sources

Our female-egg-nest triad system allowed comparisons between eggshell microbiota and maternal and nest-associated sources. Separate beta diversity analysis between eggs and potential sources showed that eggshell communities least resembled cloacal and soil communities but were taxonomically and phylogenetically most similar to nest

material, and maternal skin and feather communities (Fig. 4.4 a, b, Table 4.3). Within nests, phylogenetic distances (weighted UniFrac) between eggs and potential sources showed that average distances between eggshell communities and each potential source varied significantly (Fig. 4.4 c; LMM, $F_{5, 105} = 12.6$, $P < 0.0001$). Within-nest patterns showing that eggshell communities were more similar to feather and nest material communities than to cloacal gut, brood patch skin and soil communities were mostly concordant with population-level patterns (Fig. 4.4 c, Table S4.2).

Predictive source tracking of eggshell communities

Our population-level SourceTracker model predicted that both maternal and environmental sources contributed to eggshell community assembly (Fig. 4.5). The model estimated a minor role for cloacal gut communities in contributing to eggshell communities (Fig. 4.5 a, b), corroborating patterns derived from beta diversity analyses. Regardless of the significant compositional dissimilarity between eggshell microbiota and the considered source communities (Table 4.3), SourceTracker identified skin, feathers and nest material communities as dominant sources to eggshell community assembly (Fig. 4.5, Table 4.4). Visualisation of predictions at the individual egg level revealed substantial variation among eggs with respect to the relative contributions of the three dominant sources (Fig. 4.5). This variation among eggs complements population-level variation of beta diversity where eggshell communities spread widely in ordination space, indicating variable similarities to each (potential) source community (Fig. 4.4). The estimated proportional contribution of potential sources differed neither between woodlarks and skylarks ($t < 0.70$, $df = 1, 305$, $P > 0.90$; Fig. 4.5 a), nor between first and second eggs of the laying sequence ($t < 0.70$, $df = 1, 305$, $P > 0.90$).

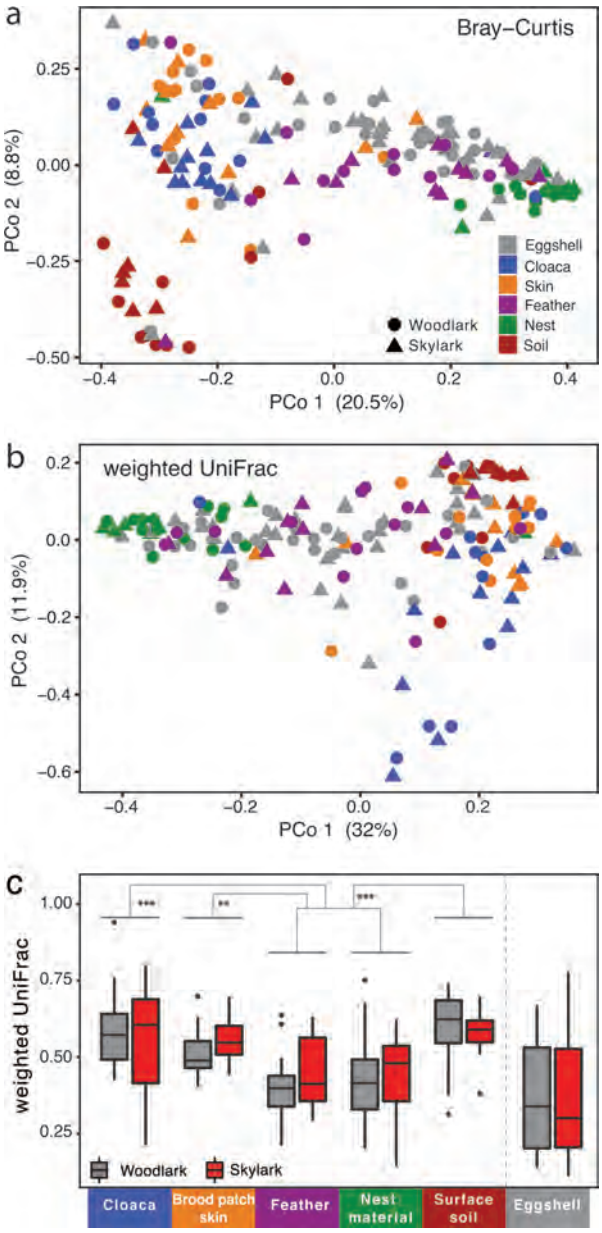

Figure 4.4: Beta diversity of eggshell, maternal and nest environmental communities. (a) PCoA of taxonomic community composition based on Bray-Curtis, and (b) phylogenetic community composition based on weighted UniFrac. (c) Within-nest pairwise weighted UniFrac distances between eggshells and other community types, and among two eggs in the same nest (two rightmost boxes). (a, b) Population-level PerMANOVA statistics are detailed in Table 4.3. (c) Eggshells communities were on average phylogenetically more similar to feathers and nest material than to cloacal gut, brood patch skin or surface soil communities (*** FDR $q < 0.001$; ** $q < 0.01$), full statistical details are in Table S4.2.

Table 4.3: Population-level statistics of comparisons between eggshell communities and maternal and environmental source communities using PerMANOVA.

		Bray-Curtis dissimilarity			weighted UniFrac		
Sample Type	df	pseudo-*F*	R^2	*P*	pseudo-*F*	R^2	*P*
Cloacal gut	1, 88	9.63	0.1	**< 0.001**	13.2	0.13	**< 0.001**
Brood patch skin	1, 85	7.87	0.08	**< 0.001**	11.2	0.12	**< 0.001**
Feather	1, 87	4.82	0.05	**< 0.001**	3.32	0.04	**< 0.01**
Nest material	1, 83	9.53	0.1	**< 0.001**	12.3	0.13	**< 0.001**
Surface soil	1, 82	13.7	0.14	**< 0.001**	16.6	0.17	**< 0.001**

P-values < 0.05 are represented bold.

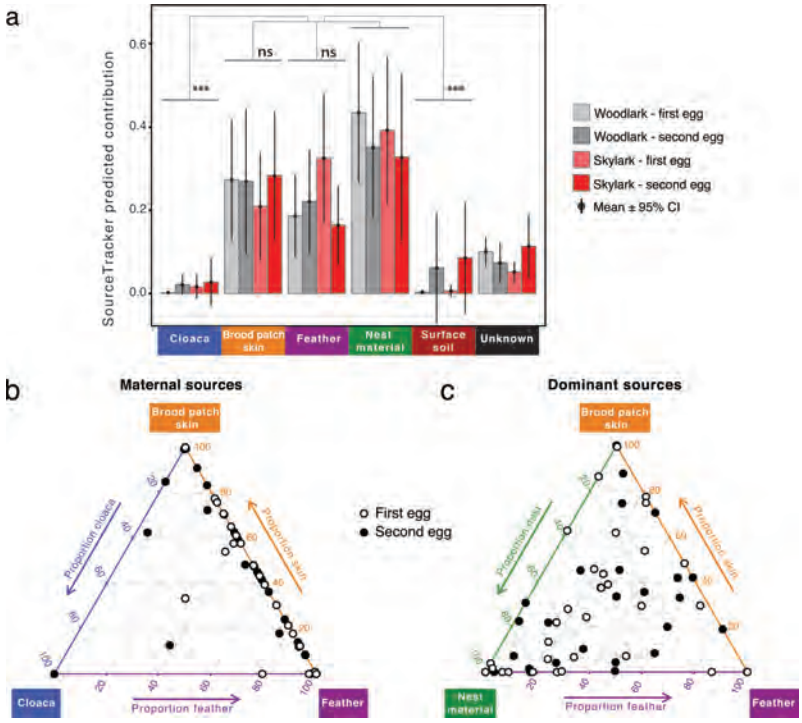

Figure 4.5: Predicted origins of bacterial communities of lark eggshells. (a) Mean (± 95% CI) predicted contribution of bacterial source communities to eggshell communities for woodlarks and skylarks, separately presented for first and second laid eggs. (b) Predictions for individual eggs are plotted in three-axes ternary plots indicating the proportion of OTUs originating from maternal sources (indicated by the triangle vertices) and (c) from each of the three dominant sources identified in (a): nest material, brood patch skin and feathers. Each point represents a single eggshell community and its position represents the relative proportions of the potential source communities indicated at the triangle tips. (a) Dunn's z statistics are detailed in Table 4.4.

Table 4.4: Pairwise contrasts of SourceTracker predicted proportional source contributions to eggshell microbiota using Dunn's z statistic.

	Cloacal gut	Brood patch skin	Feather	Nest material
Brood patch skin	-6.48 (**< 0.001**)			
Feather	-6.32 (**< 0.001**)	0.16 (0.48)		
Nest material	-7.70 (**< 0.001**)	-1.22 (0.14)	-1.38 (0.12)	
Surface soil	0.002 (0.50)	6.49 (**< 0.001**)	6.32 (**< 0.001**)	7.71 (**< 0.001**)

Dunn's z for pairwise contrasts (FDR q-value; bold = $q < 0.1$)
Kruskal-Wallis Chi-squared (adjusted for ties) = 114.51, df = 4, $P < 0.001$

Discussion

Eggshell (bacterial) microbiomes of wild woodlarks *Lullula arborea* and skylarks *Alauda arvensis* were shaped through horizontal uptake from nest material-associated communities and transgenerational transmission from body feathers and brood patch skin of breeding females. The prominent differences in OTU richness, Shannon diversity and phylogenetic beta diversity between eggshell and cloacal gut microbiota, and the negligible predicted contribution of maternal gut microbiomes to eggshell community assembly indicate that avian eggshells did not carry maternal gut communities shortly after laying. This suggests a limited potential for transgenerational gut symbiont transmission via the eggshell. Our results were congruent both at the population-level and within-nest level, and similar in both host species. Future studies in a wider range of bird species and habitats should prove the generality of these findings across the avian clade.

Our findings did not support the hypothesis that eggshells function as potent carriers of maternal gut symbionts, challenging the role of eggshells in transgenerational offspring inoculation. Higher alpha diversity of eggshell communities compared with maternal and nest environmental communities suggests that colonisation of eggshells continued after the eggs had been laid. Predicted contributions of nest material communities and proliferation of soil-borne bacteria corroborated these findings. Hence, we speculate that maternal gut bacteria that initially cover an eggshell *in vivo* are unable to survive and thrive *ex vivo* and create niche space for immigrants after experiencing inferior competitiveness in the novel (aerobic) niche. Our alpha diversity estimates of eggshell microbiomes contrast earlier egg microbiome studies, which concluded that egg communities were not richer than cloacal gut and were markedly less rich than nest material communities,

respectively. Note that these patterns were based on low-resolution ARISA data (Brandl *et al*., 2014; Martínez-García *et al*., 2016). The lack of within-nest repeatability of alpha diversity metrics for first and second eggs could imply that colonisation occurred from multiple sources simultaneously, leading to composite communities from available sources, which could be expected when high metacommunity diversity and dispersal drove this early successional phase of community assembly (Dini-Andreote *et al*., 2015). Nevertheless, egg microbiota richness and diversity seemed not to be strictly pre-set by the maternal cloacal gut microbiome. One could wonder if amplification of relic DNA extracted from our samples may have caused the high variability in alpha diversity estimation (Carini *et al*., 2016). However, if the drastic environmental change from *in vivo* to *ex vivo* impaired gut symbiont survival, and our DNA came from dead cells, we would expect higher similarities between cloacal and egg communities than we found, and hence we do not believe that this issue qualitatively confounded our results. Despite the limitations of DNA-based approaches (Carini *et al*., 2016), we believe that DNA-based surveys can provide informative insights into ecological interactions and processes in natural host-microbiome systems, and create avenues for further hypothesis-driven investigations. For our specific goal of assessing the transfer of microbes from parents to offspring, the next step would be to select specific taxa from our DNA-based community descriptions for tracking, e.g. with in situ hybridisation techniques (Amann & Fuchs, 2008).

By evaluating taxon abundances as a proxy for live bacterial dynamics we pursued a biologically informative understanding of bacterial taxon performance of first and second laid eggs. In two lark species, horizontally-derived (i.e. through direct contact) bacterial taxa appeared to outperform gut symbionts on freshly-laid unincubated eggs under natural conditions, showing that eggshells form a particularly suitable niche for free-living bacteria. Changes in bacterial abundances between second laid eggs (<12 h post-laying) and first laid eggs (24-36 h post-laying), as a means of taxon-specific growth or thriving survivors, revealed that proliferating taxa on eggshells were not indicative for maternal cloacal microbiomes but for soil communities, and occasionally for nest material, feather and skin communities. The minimal success of gut symbionts on eggshells adds to the current understanding of egg microbiome dynamics: incubation induces an increase of bacterial abundance but a decrease of diversity of eggshell communities (Giraudeau *et al*., 2014; Grizard *et al*., 2014, 2015; Lee *et al*., 2014) enhancing egg viability through reduction

of the probability of trans-shell infection (Cook *et al*., 2005a,b). Shawkey *et al*. (2009) showed that mainly potential pathogenic bacteria were inhibited by incubation. Other studies also suggested incubation as a mechanism for preventing horizontally acquired (potentially pathogenic) microbes to thrive on eggshells and subsequently infect egg contents (Brandl *et al*., 2014; Cook *et al*., 2003, but see Walls *et al*., 2011; Wang *et al*., 2011). Determining the identity and origin of bacterial taxa that thrive also during egg incubation up to the time of hatching, and subsequent monitoring of hatchling microbiome assembly will be essential and fertile ground to further explore the function of eggshells in transgenerational transmission.

Since phylogenetic community structure in egg microbiomes has not previously been described, we evaluated the phylogenetic structure of eggshell bacterial communities. Our data showed significant phylogenetic clustering of eggshell communities within two days after laying. This suggests bacterial species sorting (i.e. some bacterial clades are more successful than others) through either environmental selection (niche-based selection) of the eggshell niche (Webb *et al*., 2002), or alternatively but not mutually exclusive, the outcome of competition among bacterial taxa. As eggshells harboured richer and more diverse communities than the average female cloaca, we hypothesized that multiple sources were involved in sourcing eggshell microbiomes, with some bacterial communities being more dominant than others. Compositional similarity among eggs and skin and feather communities also indicated that horizontal transmission contributed to shape the egg microbiome. As a technical note, the time lag between egg collection and sampling of females varied among nests, an aspect of our study that in theory may have reduced the resemblance between egg and cloacal gut communities. However, we do not expect major shifts in cloacal gut microbiota during the sampling timespan of this study (Benskin *et al*., 2010; Hird *et al*., 2015), but we acknowledge that testing this time lag effect warrants experimental investigation. Nevertheless, our results from population and within-nest level analyses were consistent and indicate substantially robust patterns.

Additionally, we sought to quantify the source contributions to eggshell microbiomes from the set of potential maternal and nest environmental bacterial communities sampled. Egg-source beta diversity comparisons and model-based source predictions for eggshell communities revealed that the phylogenetic composition of eggshell communities was not associated with cloacal gut microbiomes of breeding females. Cross-sectional

(population-level) and within-nest analyses of phylogenetic composition between eggshells and the potential sources were congruent, except that eggshell and brood patch skin communities were not statistically more similar than eggshells with gut and soil communities in the within-nest analysis. Conversely, and most notably, the bare brood patch skin that females use to incubate eggs, the body feathers surrounding the bare skin, and the nest material adjoining the eggs were overall phylogenetically most similar to eggshell microbiomes. With respect to earlier egg-source investigations, it has been suggested that the cloacae of Eurasian hoopoes *Upupa epops* (Martínez-García *et al*., 2016) and pied flycatchers *Ficedula hypoleuca* (Ruiz-de-Castañeda *et al*., 2011) might source their eggshell communities, although pairwise comparisons revealed mainly shared OTUs between hoopoe eggshells, beak and brood patch skin, whereas associations between uropygial secretion and eggshells were unexpectedly lacking (Martínez-García *et al*., 2015). Inferences from predicted contributions of potential sources underscored that eggshell microbiomes were largely derived through direct contact with sources, supported by beta-diversity patterns. As parental birds typically incubate their eggs, and with our findings indicating that skin, feathers and nest environment were dominant sources for eggshell microbiome assembly, we hypothesize that direct contact may be universally driving avian egg microbiome assembly. We refer to this idea as the 'direct-contact inoculation hypothesis'. Because birds inhabit all biomes on Earth, we expect that egg microbiome compositions vary across large ecological scales, e.g. seabirds versus terrestrial birds (Dewar *et al*., 2014; Hird *et al*., 2015), and along climate gradients (Wang *et al*., 2011). However, within each large-scale spatial setting, we expect that host-dependent ecological factors, such as nest type and nest materials (Godard *et al*., 2007; Mennerat *et al*., 2009; Peralta-Sánchez *et al*., 2010; Peralta-Sánchez *et al*., 2012) may delimit the availability and composition of potential sources for eggshell microbiomes within a female-egg-nest triad context.

In light of symbiont transmission modes, one could argue that horizontal transmission appeared to be the main mode of eggshell community assembly, because bacterial inoculation of eggs seemed to occur through direct contact. However, because two out of three dominant sources were represented by maternal skin and feathers (∼40-50% cumulative contribution), one could similarly justify dominance of (broad-sense) transgenerational transmission. Shifts in avian eggshell microbiota during the incubation phase have been reported (Grizard *et al*., 2014) and could potentially

lead to convergence of egg microbiota to more cloaca-like communities. Regardless, testing of our direct-contact inoculation hypothesis in more avian lineages may be a fruitful avenue, especially with experimental application of artificially assembled sources, and comparing eggshell microbiomes across different nest types and materials during the course of incubation.

Alternative symbiont acquisition by avian progeny expectedly occurs as proposed for lizards (Colston, 2017): direct parent-offspring contact such as during food provisioning (only in altricial birds), horizontal transmission among individuals, ingestion of diet-associated microbes, passive environmental uptake, and coprophagy. If these alternative routes constitute the dominant factors, contrary to vertical transmission during egg formation, it then implies (partial) build-up of avian microbiomes every generation anew, which would limit prospects for adaptive evolution of the microbiome through natural selection acting on microbiome variation (Byler *et al*., 2013). Zooming out, egg(shell) microbiomes of other oviparous vertebrates are also only beginning to be elucidated, but community dynamics, forces shaping them and subsequent implications for development and fitness remain poorly resolved. Rooted in food production or public health frameworks, studies on fish and reptilian eggs identified egg microbiota effects on (mitigating) egg diseases (Liu *et al*., 2014; Sarmiento-Ramírez *et al*., 2014), offering insights in early-life microbiome acquisition (Llewellyn *et al*., 2014). Salmon egg surface communities associated more strongly with female gut microbiota than found here in birds (Romero & Navarrete, 2006), questioning generality of our direct-contact inoculation hypothesis to other oviparous vertebrate classes. While direct-contact inoculation of bird eggs, as we suggested here, needs experimental validation, our eggshell microbiome analysis in a natural context provides future avenues for developing and testing hypotheses regarding the functions of the egg microbiome and its consequential role in vertebrate biology.

Ethics statement

This study was carried out obeying the Dutch Law on animal experimentation under licence DEC6619B/C of the Institutional Animal Care and Use Committee of the University of Groningen.

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Supplementary Information

Table S4.1: Unique bacterial OTUs present on eggshells of woodlarks and skylarks.

OTU ID	Phylum	Class	Order	Family	Genus	Relative abundance in eggshell communities
			Woodlark eggshells only			
OTU819	unassigned					0.02% in 1/33 eggs
202454	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Dysgomonas*	0.02% in 1/33 eggs
568143	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae		0.96% in 1/33 eggs
580411	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae		1.9%, 0.98% and 0.02% in 3/33 eggs
173726	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*	0.22, 0.16 and 0.06 in 3/33 eggs
			Woodlark and Skylark eggshells			
1142554	Firmicutes	Bacilli	Lactobacillales	Streptococaceae	*Streptococcus*	1.12%, 5.6% and 0.06% in 3/65 eggs
			Skylark eggshells only			
641481	Proteobacteria	Deltaproteobacteria	Myxococcales			0.04%, 0.08%, 0.02%, 0.04%, 0.02%, 0.26%, and 0.02% in 7/32 eggs
172804	Firmicutes	Bacilli	Lactobacillales	Streptococaceae	*Streptococcus*	1.42% in 1/32 eggs
105552	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae		0.1% and 0.82% in 2/32 eggs
591986	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	*Dechloromonas*	0.04% and 0.04% in 2/32 eggs
OTU92538	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	*Opitutus*	0.02% and 0.36% in 2/32 eggs

Relative abundances of unique OTUs are shown in the rightmost column for each egg with abundance > 0

Table S4.2: Mixed-effects model statistics of within-nest phylogenetic beta diversity between woodlark and skylark eggshell bacterial communities and their potential sources.

Eggshells compared with *i* vs. with *j*[a]		**Estimate (se)**	***z* statistic**	**FDR *q*[b]**
Comparison *i*	**Comparison *j***			
Cloacal gut	Brood patch skin	0.05 (0.04)	1.39	0.19
	Feather	0.16 (0.04)	4.32	**< 0.001**
	Nest material	0.15 (0.04)	4.24	**< 0.001**
	Surface soil	0.00 (0.04)	0.00	1.00
Brood patch skin	Feather	0.10 (0.04)	2.89	**0.007**
	Nest material	0.10 (0.04)	2.85	**0.007**
	Surface soil	0.05 (0.04)	1.45	0.19
Feather	Nest material	0.00 (0.04)	0.04	1.00
	Surface soil	0.16 (0.03)	4.50	**< 0.001**
Nest material	Surface soil	0.15 (0.03)	4.47	**< 0.001**

[a]First column indicates the comparison of eggshells with community type *i*, and second column with community type *j*, where statistics of differential weighted UniFrac distances between column *i* and *j* are represented in each row.
[b]Significant FDR-corrected *q*-values are represented bold.

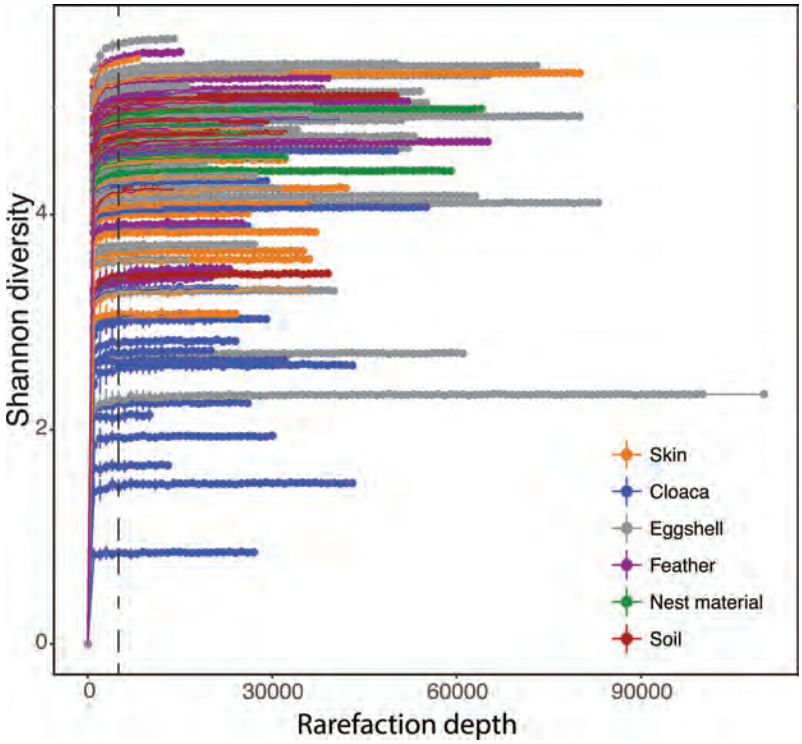

Figure S4.1: Rarefaction curves for Shannon diversity of all samples. Samples of eggshells and potential sources are coloured by sample type. Shannon diversity reached a plateau in all samples at 5000 reads per sample (dashed line).

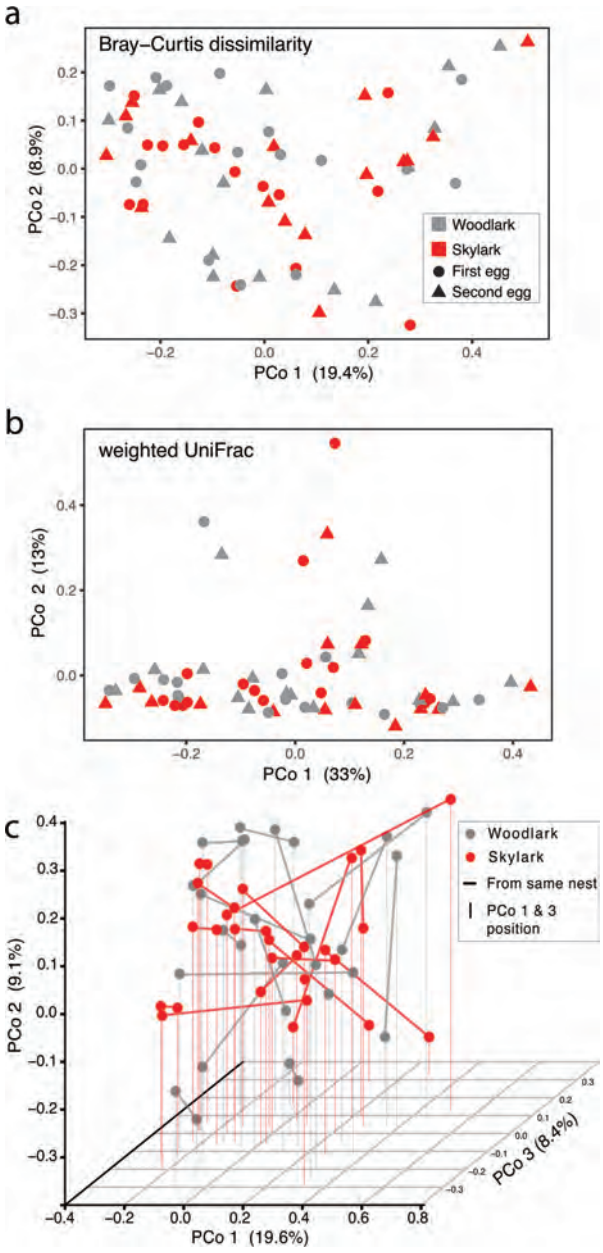

Figure S4.2: Eggshell bacterial community resemblance among hosts and within nests. (a) Principal coordinates analysis (PCoA) calculated from Bray-Curtis dissimilarities and (b) weighted UniFrac coloured by lark species: woodlark grey, skylark red, and shaped by egg age: first egg circle, second egg triangle. The first three PCo axes of the data presented in (a) are represented in (c), where eggs within nests are connected by bold lines; only eggs from nests with data available for two eggs are depicted. Eggshell communities do not cluster by lark species (Table 4.2 in Main Text).

Chapter 5

Microbial environment shapes immune function and cloacal microbiota dynamics in zebra finches *Taeniopygia guttata*

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Abstract

The relevance of the host microbiota to host ecology and evolution is well acknowledged. However, the effect of the microbial environment on host immune function and host microbiota dynamics is understudied in terrestrial vertebrates. Using a novel experimental approach centered on the manipulation of the microbial environment of zebra finches *Taeniopygia guttata*, we carried out a study to investigate effects of the host's microbial environment on: 1) constitutive immune function, 2) the resilience of the host cloacal microbiota; and 3) the degree to which immune function and host microbiota covary in microbial environments that differ in diversity. We explored immune indices (hemagglutination, hemolysis, IgY levels and haptoglobin concentration) and host-associated microbiota (diversity and composition) in birds exposed to two experimental microbial environments differing in microbial diversity. According to our expectations, exposure to experimental microbial environments led to differences related to specific antibodies: IgY levels were elevated in the high diversity treatment, whereas we found no effects for the other immune indices. Furthermore, according to predictions, we found significantly increased richness of dominant OTUs for cloacal microbiota of birds of the high diversity compared with the low diversity group. In addition, cloacal microbiota of individual females approached their baseline state sooner in the low diversity environment than females in the high diversity environment. This result supported a direct phenotypically plastic response of host microbiota, and suggests that its resilience depends on environmental microbial diversity. Finally, immune indices and cloacal microbiota composition tend to covary within treatment groups, while at the same time, individuals exhibited consistent differences of immune indices and microbiota characteristics. We show that microbes in the surroundings of terrestrial vertebrates can influence immune function and host-associated microbiota dynamics over relatively short time scales. We suggest that covariation between immune indices and cloacal microbiota, in addition to large and consistent differences among individuals, provides potential for evolutionary adaptation. Ultimately, our study highlights that linking environmental and host microbiotas may help unravelling immunological variation within and potentially among species, and together these efforts will advance the integration of microbial ecology and ecological immunology.

Introduction

Diverse microbial communities are ubiquitous components of animals and the aquatic and terrestrial ecosystems that they inhabit (McFall-Ngai *et al*., 2013). The immune systems of animals invariably deal with numerous microbial organisms at any given place and time, and have consequently evolved to prevent microbial over-exploitation, infection and disease (i.e. parasitism) and to allow beneficial (i.e. mutualism) and neutral host-microbe interactions (i.e. commensalism). Studies in a relatively new research domain, ecological immunology, have begun to reveal some sources of immunological variation across species (Horrocks *et al*., 2012, 2015; Martin *et al*., 2001; Tella *et al*., 2002; Versteegh *et al*., 2012), among individuals (Ardia, 2007; Hegemann *et al*., 2013; Martin *et al*., 2004), and during life cycles (Buehler *et al*., 2008a; Hegemann *et al*., 2012). However, a large part of this work has collectively demonstrated that immunological variation is poorly aligned with life history strategies among species (e.g. pace-of-life) (e.g. Horrocks *et al*., 2012; Versteegh *et al*., 2012). Likewise, immunological variation within individuals frequently does not follow predictions based on life-history trade-offs (Ardia, 2007; Hegemann *et al*., 2012; Pigeon *et al*., 2013). Instead, immunological variation often is better correlated with environmental variability (Buehler *et al*., 2008b; Horrocks *et al*., 2015; Martin *et al*., 2004; Matson, 2006), supporting ideas that animals optimize immune defenses to fit their environment, on both evolutionary and ecological time scales (Buehler *et al*., 2008a; Horrocks *et al*., 2011; Tieleman, 2018). The pathogenic and nonpathogenic effects of microbial life on wildlife health and fitness and the origins, maintenance, and disturbance of animal-microbe interactions represent major frontiers in contemporary biology (Hird, 2017; Kohl & Carey, 2016; Shapira, 2016). One important unresolved issue is whether the environmental microbial communities encountered by an animal affect the immune function, and ultimately survival, of that animal (Horrocks *et al*., 2011; Tieleman, 2018).

Another component of the interface between a host and its environment is the host-associated microbiota, the sum of the microbial communities residing in and on an animal's body. Like immune function, host-associated microbiotas show tremendous variation among species and individuals and through time and space (Aivelo *et al*., 2016; Colston & Jackson, 2016; Ley *et al*., 2008; Schmitt *et al*., 2011; Springer *et al*., 2017). The status of host-associated microbiotas is currently debated: some view the host-associated microbiota as a phenotypic trait of its host; others see the microbiota and the host as a

meta-organism (Bordenstein & Theis, 2015; Douglas & Werren, 2016; Moran & Sloan, 2015; Zilber-Rosenberg & Rosenberg, 2008). Regardless, several fundamental questions in this debate remain to be addressed, including whether the host-associated microbiota is determined by inheritance or by the environment, and whether the host's microbiota acts as a phenotypically plastic trait for quickly responding to versatile environments (Apprill, 2017; Tieleman, 2018). Understanding the latter requires concomitant measurement of host-associated and environmental microbial communities; however, this type of work is just beginning to be carried out in terrestrial nonhuman vertebrates. Irrespective of whether the microbiota should be defined as a host trait or not, the conceptual distinction between an animal's microbiota and its (microbial) environment fades as a result of weak host-microbe partner fidelity (Douglas & Werren, 2016), common host-environment microbial exchange (Lemieux-Labonté *et al*., 2016; van Veelen *et al*., 2017), or both. Ideally, testing effects of the microbial environment on host-associated microbiota diversity, composition and dynamics should be done while controlling for factors known to shape animal microbiota (Apprill, 2017; Evans *et al*., 2017; Grond *et al*., 2018; Kohl, 2012; Tasnim *et al*., 2017; Trevelline *et al*., 2018), such as diet or sex (Muegge *et al*., 2011; Pearce *et al*., 2017).

Individual animals routinely experience very different environments within their lifetimes, for example when migrating or when seasons change (reviewed in Shaw & Couzin, 2013). As a prerequisite for investigating how microbial environments shape host immunological phenotypes via host-associated microbiota, quantifying the resilience of host-associated microbiota to shifts in environmental microbial communities may prove vital. Tracking how the host-associated microbiotas of individuals respond to novel microbial environments (e.g. Risely *et al*., 2017a) will offer insights into the individuality, flexibility and resilience of microbiota traits, and into the time span at which responses to novel microbial environments occur. Earlier attempts at this type of tracking did not control for important confounding factors, e.g., dietary effects on gut microbiota variation (Lewis *et al*., 2017; Risely *et al*., 2017b). Hence, experimental approaches that subject animals to novel microbial environments while limiting confounding effects are needed, and need also consider the individuality of responses. Widely used indices of immune function can fluctuate temporally within individuals; simultaneously, individuals can consistently differ, i.e., be repeatable (Matson *et al*., 2012; Tieleman *et al*., 2010). Host-associated microbiota can similarly show signs of individuality (Benskin *et al*.,

2010; but see Ren *et al*., 2015). Accordingly, questions about individual-level connections between host immune function and host-associated microbiota have emerged (Horrocks *et al*., 2011; Tieleman, 2018), and call for simultaneous assessment of immune function and host-associated microbiota.

While not investigated in an ecological immunology framework, studies constitutive immunity in humans and rodent models implicated that levels of specific antibodies (Fadlallah *et al*., 2019; Slack *et al*., 2009), polyclonal natural antibodies (Magri *et al*., 2017), and complement activity (Yoshiya *et al*., 2011) were positively associated with gut microbiota diversity. Here, we describe an experiment in which we manipulated the microbial environment to test its influence on innate and adaptive aspects of immune function and on the diversity and resilience of host-associated microbiota of captive zebra finches *Taeniopygia guttata*. 1) We explored temporal patterns of immunity and cloacal microbiota characteristics over eight weeks in birds that were continuously exposed to one of two experimental environments that differed in microbial diversity and composition. Based on the literature, we predicted that, if constitutive levels of antigen-specific IgY, natural antibodies and complement-like factors are influenced by the diversity of environmental microbial communities, their concentration would increase in response to high environmental microbial diversity. In addition, if infection incidence increases with microbial diversity, we predicted elevated levels of haptoglobin, a marker of inflammation (Matson *et al*., 2012), under high environmental microbial diversity. We accordingly predicted decreasing or a lack of patterns under conditions with low environmental microbial diversity. 2) We also investigated whether microbial environments with different diversities affected the diversity and resilience (i.e. degree and time to recovery) of the cloacal microbiota. We minimized dietary influences on the microbiota by supplying sterilized food and water. we then predicted that a more diverse microbial environment would increase the diversity and slow the recovery of cloacal microbiota. 3) Finally, we examined correlations between immune indices and host-associated microbiota characteristics, where correlations may suggest that vertebrate immune function responds to environmental microbiota within eight weeks. Our longitudinal study design additionally allowed us to quantify repeatability of immune indices and host-associated microbiota characteristics.

Methods

Experimental soils

We divided 2.5 m^3 soil in two equal fractions and applied three cycles of 25 kGy gamma irradiation (Synergy Health Ede B.V, the Netherlands) to one fraction to generate a highly reduced microbial environment ('low diversity' soil; Fig. S5.1). The remaining fraction constituted a high diversity microbial environment ('high diversity' soil). We applied in all cages either low or high diversity soil as a $\sim$2 cm deep bedding layer, which we replaced every two weeks (mean $\pm$ S.E.M.: 15 $\pm$ 1 days, n = 4). High diversity soils were stored at 4 °C enabling soil respiration while limiting bacterial activity to reduce temporal variation. Low diversity soils remained sealed and were stored under outdoor storage conditions (mean $\pm$ S.E.M.) 4.7 $\pm$ 0.41 °C. We maintained soil moisture content by spraying $\sim$30 ml autoclaved water per cage per day. We monitored the temporal stability of soil communities by sampling soils every 3rd (n = 20), 10th (n = 20) and 14th (n = 18) day after soil was (re)placed in the cages. Soil samples were stored immediately at -20 °C. Nine additional samples (high diversity n = 5, low diversity n = 4) were collected from stored bags to monitor changes during storage. A detailed description is provided as Supplementary information.

Zebra finch husbandry

Experiments were approved by the Animal Experimentation committee of the University of Groningen (license DEC61314A), in accord with the Dutch Law on Animal Experimentation, and standard protocols. Indoor ambient temperature was kept constant at 20 °C $\pm$ 1, relative humidity at 55% $\pm$ 15 with a 12:12 h light-dark (L:D) cycle. In the current experiment we restricted ourselves to sampling of females for practical considerations regarding sampling schemes (Table 5.1 for a summary of collected samples per female). Details on handling, sample processing and storage are provided as Supplementary information.

Laboratory analysis of immune function

Non-specific antibody titers and complement-like lytic activity of blood plasma was assessed using the hemolysis-hemagglutination assay and rabbit erythrocyte antigens

(Envigo, Leicester, UK) (Matson *et al*., 2005). Total plasma IgY concentration was quantified in duplicate using enzyme-linked immunosorbent assays (ELISAs) with rabbit anti-chicken IgG antigens (Sigma-Aldrich, St Louis, MO, USA) (Demas & Nelson, 1996; Grindstaff *et al*., 2005). Haptoglobin concentration was quantified using a commercial haem-binding assay (Tri-delta Diagnostics Inc., Morris Plains, NJ, USA) (Matson *et al*., 2012).

DNA extraction, 16S rRNA gene sequencing

DNA was extracted from 250 mg of homogenised soil samples and cloacal swabs. Swab fibers were aseptically peeled from swab stalks, placed in MoBio PowerSoil DNA extraction vials (MoBio laboratories, Carlsbad, CA, USA) and DNA was isolated following the manufacturer's protocol with addition of 0.25 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) to improve cell disruption during 3 cycles of 60 s bead beating (Mini-bead beater, BioSpec Products, Bartlesville, OK, USA). Samples were characterized by (triplicate) PCR of 16S rRNA gene (V4/V5) using 515F and 926R primers, library preparation of pooled triplicates and 250 bp paired-end sequencing on an Illumina MiSeq (V2) at Argonne National Laboratory, IL, USA, following Earth Microbiota Project protocols (http://press.igsb.anl.gov/earthmicrobiota/protocols-and-standards/16s/) (Gilbert *et al*., 2010). Seven no-sample technical negative controls for each batch of DNA extraction were included. None of the negative controls detectably produced reads in the quality-filtered sequence data set.

Bioinformatic processing of sequence reads

Sequence reads were quality filtered and assembled using QIIME (1.9.0) (Caporaso *et al*., 2010a) retaining reads lengths ranging 368-382 bp and discarding reads ($\sim$ 267 bp) identified as zebra finch 12S rRNA gene (99% identity) using BLAST. A final 4.2 million high quality sequences were obtained (51% of raw data). OTUs were defined by 97% sequence identity with an open-reference strategy using UCLUST (Edgar, 2010) and the Greengenes reference set (13.8) (DeSantis *et al*., 2006). After removal of singletons, taxonomy was assigned to representative sequences based on the Greengenes reference set (97% identity). Representative sequences were then aligned using PyNast (Caporaso *et al*., 2010b) and chimeric sequences were removed using UCHIME from the USEARCH81 toolkit (Edgar *et al*., 2011) before construction of a phylogenetic tree using FastTree (Price *et al*., 2009). OTUs originating from Archaea, Chloroplast and Mitochondria were filtered

from the data and the OTU table was offset to retain only OTUs that account for 0.001% of the total abundance.

Statistical analysis of immune function

Linear mixed-effects models (LMMs) to analyse immune indices included fixed effects for experimental group and sampling moment (0, 2, 4 and 8 weeks), as well as their interaction, and individual identity and replicate room as random effects. The probability of lytic activity was modelled using a generalized linear mixed-effects model (GLMM) with a logit link function and the same set of independent variables. ANOVA was then performed using *LmerTest* (Kuznetsova *et al*., 2016) with a two-tailed test. Distance-based redundancy analysis (db-RDA) in *vegan* (Oksanen *et al*., 2017) was used as a multivariate approach to test for immunological segregation of treatment groups. Repeatability *R* was calculated with a two-tailed test controlling for fixed effects using (G)LMM models with *rptR* package (Stoffel & Nakagawa, 2017). Confidence intervals for R were estimated by parametric bootstrapping and significance was inferred from two-tailed permutation tests. A detailed description is provided as Supplementary information.

Statistical analysis of soil communities

To analyse bacterial community characteristics *vegan* (Oksanen *et al*., 2017), *phyloseq* (McMurdie & Holmes, 2013), and *lme4* (Bates *et al*., 2015) for R Statistical Software (R Core Team, 2016) were used. We rarefied soil samples to 1115 reads for alpha diversity estimation and then examined variation in OTU richness and Shannon diversity using LMMs with experimental treatment and time point (3, 10 and 14 days; categorical) as fixed predictors and replicate room as random effect in all models (Zuur *et al*., 2009). Treatment by time-interactions were not significant and removed before parameter estimation with REML. ANOVA was used with *lmerTest* (Kuznetsova *et al*., 2016) to estimate marginal effects (two-tailed), and *P*-values were adjusted for multiple comparisons using *multcomp* (Hothorn *et al*., 2016). Variance-stabilizing transformation based on the fitted mean-variance relationship was applied to coverage-normalized counts (Anders & Huber, 2010) was performed on a non-rarefied OTU table of soil communities (Love *et al*., 2014; Weiss *et al*., 2017), which was then used for PCoA based on the weighted UniFrac distance metric. We tested experimental treatment and temporal effects using unconstrained ordination and marginal effect estimation using two-tailed *adonis* and

adonis2 (Anderson, 2001; McArdle & Anderson, 2001), respectively, with permutations stratified by replicate room and 999 permutations. A detailed description is provided as Supplementary information.

Statistical analysis of host-associated microbiota

Cloacal microbiota variation was analysed similar to soil communities. Based on rarefaction curves of Shannon diversity (Fig. S5.2), a minimum of ~1200 reads per sample was decided as sufficient to analyse within-sample diversity. The lack of plateau for OTU richness implicated that rare OTUs were missed at the reached sampling depths. We therefore interpreted OTU richness as the dominant fraction of the microbiota. The OTU table was subset to retain the upper 80% of the coverage distribution (min: 1240 reads per sample, n = 145), as some cloacal samples had a low coverage (median: 3214, range: 52-88999 reads per sample). Alpha diversity metrics were log-transformed to fulfil normality assumptions. LMMs were used to estimate effects of experimental treatment and sampling moment and included individual identity and replicate room as random effects. Pairwise contrasts of the experimental treatment factor at each sampling moment were calculated (two-tailed) using phia (De Rosario-Martinez, 2015), and FDR-corrected *q*-values (critical *q*-value = 0.1) were reported. Temporal shifts were examined by calculating the difference of OTU richness and Shannon diversity between sampling moment ti and ti-1 within each individual. LMMs were used to test (two-tailed) treatment and temporal shift effects. Beta diversity was calculated similarly to soil communities on a subset comprising the upper 90% of the coverage distribution of cloacal samples (n = 204; minimum coverage: 545 reads per sample). Within-individual shifts in the phylogenetic composition were calculated from the weighted UniFrac distance matrix and analysed using LMM including bird identity and room as random effects and evaluated using *post hoc* contrasts. Negative binomial GLMs implemented in *DESeq2* (Love *et al*., 2014) were used to identify differentially abundant taxa (McMurdie & Holmes, 2014; Weiss *et al*., 2017) across sampling moments during the experiment. A detailed description is provided as Supplementary information.

Statistical analysis of associations between immune function and microbiota

PCoA of a Bray-Curtis distance matrix of all immune indices and of (unweighted and weighted) UniFrac distance matrices of the cloacal microbiota were created using cmdscale function of stats (R Core Team, 2016). A Procrustes superimposition was then applied to test whether immune function covaried with host-associated microbiota composition (Peres-Neto & Jackson, 2001). The protest function (Peres-Neto & Jackson, 2001) was subsequently used to test (two-tailed) the significance of the Procrustean fit M^2 with 10 000 permutations. Univariate regression (LMM) was applied to test associations between the first Procrustean axes of immune function and the microbiota, including sampling moment, individual identity and replicate room as random terms. Additional (G)LMMs were used to test relationships between each immune index and OTU richness, Shannon diversity, taxon occurrence (unweighted UniFrac; PCoA axis 1 and 2). A detailed description is provided as Supplementary information.

Results

Microbial environment affects IgY concentration but not innate immune indices

To experimentally test if microbial environments (Fig. S5.1) affect indices of immunity, we moved 53 adult females and 54 adult males from single-sex outdoor aviaries to indoor cages (50 × 50 × 40 cm), each of which housed two birds of the same sex. We supplied all cages with bedding materials comprising soils with bacterial communities of high (Shannon $H' \pm$ SE = 5.6 ± 0.05) or low bacterial diversity (3.9 ± 0.05) and different community compositions (Fig. S5.1). Each of the two replicate rooms per experimental microbial environment contained 12 cages arranged in a 3 × 4 grid with alternating male and female cages. Birds were randomly assigned to a room and a sex-specific cage (see Supplementary information for more details on experimental procedure and housing conditions). We provided a standardized diet of *ad libitum* gamma-irradiated seed mixture and autoclave-sterilized water to all birds. The water was supplemented with 4 g · l^{-1} of a micropore-filtered multivitamin-amino acid solution (Omni-vit, Oropharma

N.V., Deinz, Belgium) to compensate potential irradiation-induced vitamin degradation in seed. We measured indices of innate (agglutination titer of natural antibodies, complement-mediated lysis titer, and haptoglobin concentration (Matson *et al*., 2012, 2005)) and adaptive immune function (total plasma concentration of immunoglobulin Y (IgY), i.e. the avian equivalent of IgG (Demas & Nelson, 1996; Grindstaff *et al*., 2005)), in females at four time points: < 1 day before the experiment (i.e. baseline) and after weeks 2, 4 and 8 of the experiment. We analysed only females because of practical limitations, and cloacal swabbing was impossible for males. We evaluated time effects using four distinct sampling days, which we considered categorically in order to determine within-individual changes between these sampling moments.

Comparing treatment groups, IgY concentration was significantly elevated in the high diversity compared with the low diversity microbial environment (Fig. 5.1 b). This pattern remained when baseline values were excluded ($F_{1,44} = 4.35$, $P = 0.04$), which we tested separately as baseline values differed between treatment groups despite randomized allocation to treatments ($\chi^2 = 4.21$, df = 1, $P = 0.04$). Agglutination titer, lysis titer and haptoglobin concentration were unaffected (Fig. 5.1. a, c and d; Table 5.1). The effect on IgY was most strongly present after eight weeks of exposure to the different experimental microbial conditions (Fig. 5.1 b, Table 5.1). Using a multivariate distance-based redundancy analysis of the four immune indices combined we found no significant difference between treatment groups ($F_{1,\ 39\text{-}43} < 1.20$, $P > 0.26$). The elevated IgY levels in the high diversity microbial environment suggest that antigen-specific antibodies had increased with environmental microbial diversity, whereas agglutination, which is driven primarily by polymeric natural antibodies (e.g. IgM) with low specificity and low affinity, was not different between high and low diversity microbial environments.

We examined temporal shifts in the immune indices to determine if microbial environments altered host immune function. Absence of significant treatment by sampling moment-interactions indicated that changes in immune function between sampling moments were largely independent from experimental microbial conditions (Fig. 5.1; Table 5.1). Specifically, while agglutination titers showed no differences between sampling moments at all (Fig. 5.1 a; Table 5.1), total antigen-specific IgY concentrations increased by 0.19 absorbance units between sampling moments 2 and 4 ($\chi^2 = 12.16$, FDR $q = 0.003$; Fig. 5.1 b), and haptoglobin concentration increased by 0.16 mg ml^{-1} between

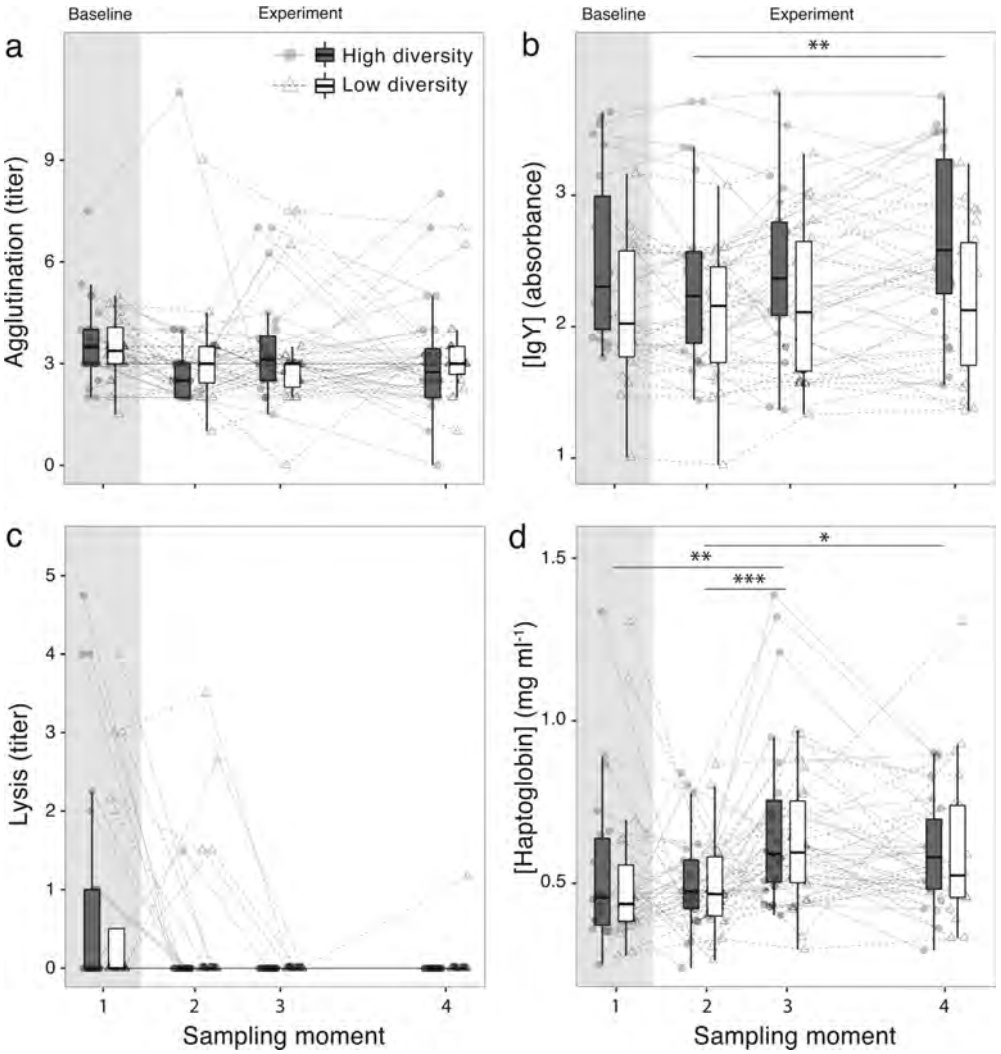

Figure 5.1: Experimental and temporal effects on host immune function. Relationships of population-level variation of (a) agglutination titer, (b) IgY concentration, (c) lysis titer and (d) haptoglobin concentration across sampling moments, stratified by experimental treatment. Faded circles (high diversity soil) and triangles (low diversity soil) represent repeated measurements connected by a line segment per individual female (solid = high diversity, dashed = low diversity). Boxplots show median and first and third quartile per group, with whiskers representing 1.5 · IQR. Experimental treatments are split along x-axis for visual clarity but sampled simultaneously. Grey areas highlight the baseline sampling moment. Experimental treatment and temporal effects on lysis titer were analysed as occurrence of lytic activity. Asterisks above plots denote pairwise contrasts among sampling moments; * FDR-corrected $q < 0.1$, ** $q < 0.01$. Statistics are detailed in Table 5.1. The experimental effect on IgY concentration is also significant after exclusion of baseline samples ($F = 4.35$, $P < 0.05$).

sampling moments 2 and 3 (Fig. 5.1 d). We observed complement-mediated lytic activity in only a few individuals at the baseline measurement, and the probability of lytic activity

further declined after exposure to experimental conditions (Fig. 5.1 c; Table 5.1). IgY concentrations tended to increase during the experiment only in birds exposed to the high diversity microbial environment (Fig. 5.1 b), but the interaction between treatment and sampling moment was not significant (Table 5.1), also when baseline measures were excluded ($F_{2, 87} = 1.53$, $P = 0.22$).

To examine the amount of variance in immune indices explained by differences among individuals, we examined the repeated measures on individuals over time, following (Stoffel & Nakagawa, 2017), and revealed that immune function differed consistently among individuals (Fig. 5.1; Table 5.1). The highest repeatability was highest for IgY concentration, and repeatabilities for agglutination titer and haptoglobin concentration were lower, but still significant (Table 5.2).

Table 5.1: Statistics of longitudinal analysis of experimental and temporal effects on innate immune function.

Response	**Predictor**	**df[a]**	***F***	***P***
Agglutination (titre)				
	Experimental treatment	1, 41	0.03	0.87
	Sampling moment	3, 125	0.79	0.50
	Interaction	3, 125	0.22	0.88
IgY concentration (absorbance)				
	Experimental treatment	1, 44	5.15	**0.028**
	Sampling moment	3, 129	4.12	**0.008**
	Interaction	3, 129	1.60	0.19
Lytic activity (probability) b			*z*	*P*
	Experimental treatment	1	-0.71	0.48
	Time (days)	1	-2.61	**0.009**
	Interaction	1	1.80	0.07
Haptoglobin (mg ml^{-1})			*F*	*P*
	Experimental treatment	1, 44	0.19	0.66
	Sampling moment	3, 127	6.20	**<0.001**
	Interaction	3, 127	0.40	0.76

[a]Denominator degrees of freedom based on Satterthwaite approximation
[b] No detected lysis titers at sampling moment 3 and 4 inhibited evaluation of differences among sampling moment categories; a logit link GLMM with continuous temporal predictor was fitted instead.

Table 5.2: Statistical analysis of host-associated microbiota alpha diversity.

Response OTU richness (log-scale)	**Predictor**	**df**[a]	***F***	***P***
	Experimental treatment	1, 43	4.56	**0.04**
	Sampling moment	3, 104	35.01	**<0.001**
	interaction	3, 104	1.42	0.24
Shannon diversity (log-scale)			*F*	*P*
	Experimental treatment	1, 42	0.00	0.99
	Sampling moment	3, 103	28.35	**<0.001**
	interaction	3, 103	2.43	0.07
Δ OTU richness (y_t - y_{t-1})				
	Experimental treatment	1, 76	0.75	0.39
	Sampling interval	2, 76	22.42	**< 0.001**
	interaction	2, 76	2.56	0.08
Δ Shannon diversity (y_t - y_{t-1})				
	Experimental treatment	1, 76	0.80	0.37
	Sampling interval	2, 76	17.37	**< 0.001**
	interaction	2, 76	1.98	0.14

[a]Denominator degrees of freedom based on Satterthwaite approximation

Microbial environment affects host-associated microbiota structure and composition

To investigate the diversity and resilience of host-associated microbiota traits in response to different microbial environments, we characterized the host-associated microbiota using cloacal swabs that were collected at the same four time points described above. We extracted DNA from these swabs and characterized the host-associated microbiota through 16S rRNA gene amplicon sequencing (V4/V5 region) using Illumina Miseq (see Supplementary information for more details on methods). Briefly, we assembled quality-filtered sequences into operational taxonomic units (OTUs; 97% ID) to analyse alpha and beta diversity. Rarefaction curves indicated that Shannon diversity but not OTU richness reached a plateau, which implied that our sequencing effort was insufficient to document rare OTUs (Fig. S5.2). Accordingly, we interpreted OTU richness as the richness of dominant OTUs. Our dataset contained 1084107 quality-filtered reads clustered in 1393 OTUs (each contributing > 0.001% of total abundance). Of these OTUs, 81% were shared between the treatments (Fig. S5.3), and 168 and 97 OTUs were detected only in birds on

high diversity and low diversity soils, respectively. To evaluate host-associated microbiota alpha diversity, we rarefied host-associated microbiota data to 1273 reads per sample (i.e. upper 80% of coverage distribution) for comparability: 173855 reads binned in 1310 OTUs. Beta diversity was calculated based on a non-rarefied and variance-stabilized community table (see Methods).

The experimental microbial conditions led to modest differences in alpha (Fig. 5.2 a, b) and beta diversity of host-associated microbiota (Fig. 5.2 c). Linear mixed model (LMM) analyses of alpha diversity (OTU richness and Shannon diversity) revealed significantly higher richness of dominant OTUs in the host-associated microbiota of birds living on high diversity soils compared with low diversity soils (Fig. 5.2 a, Table S5.3). We found no significant effect of microbial environment on Shannon diversity of host-associated microbiota (Fig. 5.2 b, Table 5.2). Principal coordinates analysis (PCoA) of weighted UniFrac distances revealed that the phylogenetic composition of host-associated microbiota differed significantly but modestly (1.9%) between experimental groups (PerMANOVA) (Fig. 5.2 c, Table 5.3). We observed that the composition of pre-experiment samples was more distinct from later sampling moments during exposure to experimental microbial environments (i.e. 2 to 4) (Fig. 5.2 c, Table 5.3). The relative abundance of major taxonomic groups in the cloacal microbiota of both experimental groups showed similar patterns, with Epsilonproteobacteria, Firmicutes and Actinobacteria representing the most abundant groups once under experimental conditions (Fig. S5.5). Transformed OTU counts were modelled with a *DESeq2* (Love *et al*., 2014) negative-binomial generalized linear model (GLM) with treatment and sampling moment as terms, which did not identify differentially abundant taxa between birds on high and low diversity microbial environments at OTU-level (FDR-corrected $q > 0.1$).

To address the resilience of host-associated microbiota in response to the novel environments, we evaluated the change in host-associated microbiota characteristics from outdoor aviary conditions to the indoor experimental treatments (at sampling moment 2). We found that alpha diversity declined (Fig. 5.2 a, b) and beta-diversity shifted in both treatment groups (Fig. 5.2 c; Table 5.3). Non-significant interactions between treatment and sampling moment indicated that these compositional changes were independent of the experimental microbial conditions (Table 5.2; Table 5.3). *DESeq2* analysis revealed that normalized OTU abundance changes were largely caused by a

(near) complete loss of some bacterial phyla after first exposure to experimental microbial conditions (e.g. loss of Bacteroidetes, Cyanobacteria and Fusobacteria). Subsequent analysis of changes of OTU abundances in the host-associated microbiota during the experiment (between sampling moments 2 and 4) revealed abundance changes that were inferior to those induced by outdoor-to-indoor translocation of birds (Fig. S5.5, Fig. S5.6). Shifts were most evident for Proteobacteria classes, where Epsilonproteobacteria, which were not dominant in soils (Fig. S5.1 e), became relatively more dominant in host-associated microbiota at the expense of Alpha- and Betaproteobacteria (Fig. S5.5). The detection of Chloroflexi, Chlamydiae and Firmicutes in host-associated microbiota was clearly associated with acclimation to experimental conditions irrespective of treatment group (Fig. S5.1 e). At the OTU level, nine taxa assigned to genus *Lactobacillus* (n = 5), genus *Campylobacter* (n = 2), family Enterobacteriaceae (n = 1), and family Micrococcaceae (n = 1) significantly changed in abundance with experimental duration (Table 5.5), but none of these responses were treatment-dependent (FDR-corrected q > 0.1).

To address the resilience of host-associated microbiota in different experimental microbial environments, we analysed within-individual changes in alpha and beta diversity between consecutive sampling moments, and then tested the experimental effect on these temporal shifts. The decline in OTU richness of host-associated microbiota stopped earlier in low than in high diversity experimental microbial conditions (Fig. 5.3 a). Shannon diversity showed a similar pattern but this was not significant ($\chi^2 = 2.61$, FDR $q = 0.32$) (Fig. 5.3 b). Moreover, after host-associated microbiota composition moved away from the baseline composition, temporal patterns indicated that compositions returned in the direction of the baseline (Fig. 5.3 c): the composition at sampling moment 4 was more similar to the baseline than to the composition at sampling moment 2 or 3 ($F_{1, 5034} > 6.47$, $P < 0.016$; Fig. S5.7). Furthermore, the shift away from the baseline was stronger in birds in the high diversity than in the low diversity microbial environment (Fig. 5.2 c; Fig. S5.7). Similar to OTU richness, a within-individual analysis of changes of phylogenetic composition between consecutive sampling moments revealed that host-associated microbiota indeed stabilized earlier in the low diversity microbial conditions (i.e. higher turnover; Fig. 5.3 c; Table 5.3; Fig. S5.8). In addition to the phenotypically plastic responses to environmental microbial conditions, analysis of within-individual repeatabilities of host-associated microbiota alpha and beta diversity indices demonstrated that OTU richness, Shannon diversity, and the second unweighted UniFrac PCoA axis were

significantly repeatable (Table 5.4), suggesting that host-related factors also shaped the host-associated microbiota.

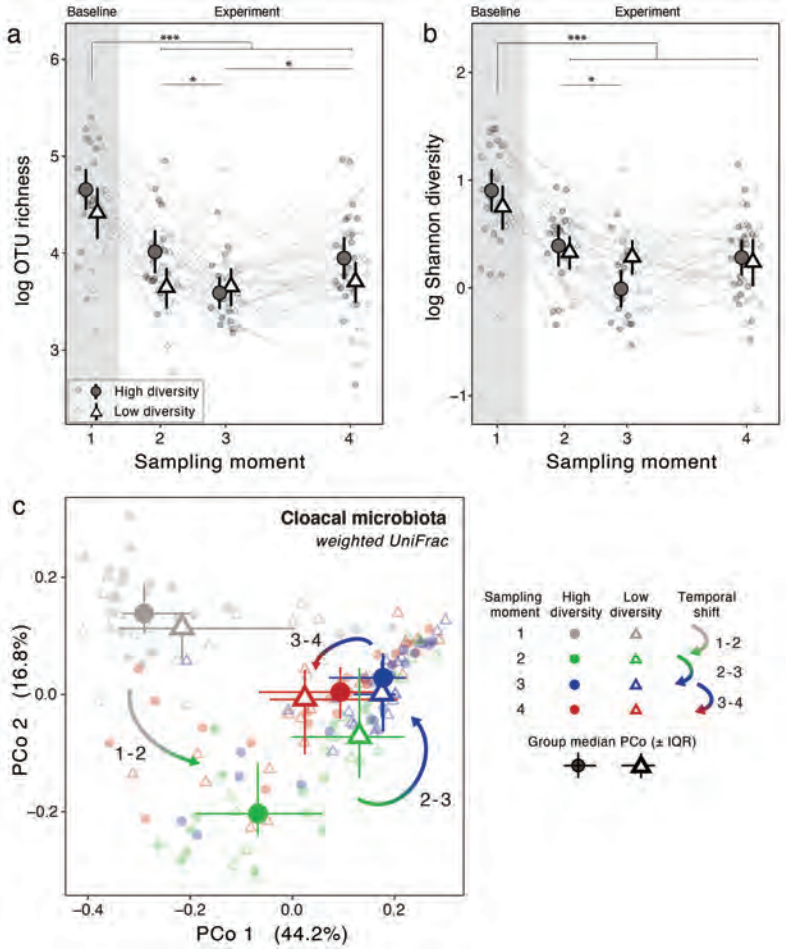

Figure 5.2: Experimental and temporal effects on cloacal microbiota alpha diversity and phylogenetic beta diversity. Relationships of population-level variation (mean ± 95% CI whiskers) of (a) dominant OTU richness and (b) Shannon diversity for each experimental treatment and across sampling moments. (c) PCoA of weighted UniFrac distances among cloacal microbiota samples; ordination of all samples including baseline samples shows differential clustering of experimental treatment and sampling moments, as well as a pattern of transitions that first diverges from, and later converges toward, the baseline state. Group medians and IQR are shown as large symbols and whiskers. (a, b) Faded circles (high diversity) and triangles (low diversity) represent individual measurements connected by line segments per individual. (a, b) Experimental treatments are taken simultaneously but split along x-axis for visual clarity. Grey area highlights the baseline sampling moment. Asterisks above plots denote pairwise contrasts among sampling moments; P or FDR-corrected $q < 0.1$ *, 0.01 **, 0.001 ***. Statistics are detailed in Table 5.2 and Table 5.3.

Table 5.3: Statistics of distance-based redundancy analysis and linear mixed model of experimental and temporal effects on phylogenetic beta diversity.

Phylogenetic beta diversity				
adonis(2)[a]	**R^2 (%)**	**df**	**Pseudo-*F***	***P***
Experimental treatment	1.86	1	3.87	0.01
Sampling moment	26.87	3	19.73	<0.001
interaction	2.21	3	1.64	0.08
within-individual weighted UniFrac distance (y_t - y_{t-1})				
ANOVA		**df**	***F***	***P***
Experimental treatment		1, 95	3.51	0.06
Sampling interval		2, 95	17.05	**<0.001**
interaction		2, 95	5.28	**<0.01**
Contrasts (sampling interval)	**effect**	**Df**	**χ^2**	**FDR *q***
High - Low diversity (1-2)	-0.02	1	0.14	0.87
High - Low diversity (2-3)	0.17	1	13.21	**<0.001**
High - Low diversity (3-4)	-0.01	1	0.03	0.87

[a]Group dispersions are shown in Fig. S5.4.

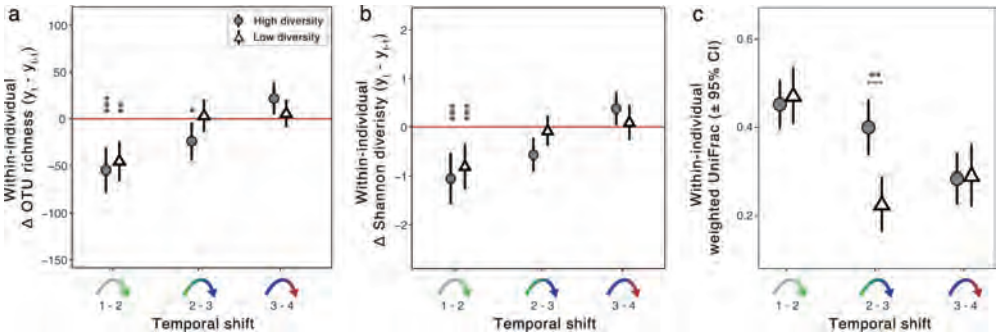

Figure 5.3: Temporal shifts in host-associated microbiota characteristics across experimental treatment and sampling moments. Average within-individual differences (± 95% CI whiskers) of (a) OTU richness, (b) Shannon's diversity and (c) weighted UniFrac distance between consecutive sampling moments, presented for each temporal shift and stratified by experimental treatment. Associated statistics are detailed in Table 5.3 and Table 5.4.

Table 5.4: Repeatability of innate immune indices and cloacal microbiota characteristics of female zebra finches.

Immune index	Treatment	Principal coordinates	*R*	SE	95% CI (lower, upper)[b]	*P*[c]
Agglutination (titre)			0.14	0.07	0.017, 0.301	**0.033**
Total IgY (absorbance)			0.80	0.05	0.65, 0.866	**0.001**
Lysis (presence /absence)[a]			0.06	0.20	0, 0.894	0.198
Haptoglobin (mg ml^{-1})			0.26	0.08	0.113, 0.423	**0.001**
Multivariate immune function	High diversity	axis 1	0.37	0.13	0.082, 0.584	**0.004**
		axis 2	0.56	0.13	0.265, 0.75	**0.001**
	Low diversity	axis 1	0.08	0.11	0, 0.333	0.242
		axis 2	0.74	0.11	0.437, 0.85	**0.001**
Cloacal microbiota						
OTU richness			0.18	0.10	0.005, 0.394	**0.019**
Shannon's diversity			0.23	0.09	0.064, 0.421	**0.005**
Cloacal taxon occurrence (unweighted UniFrac)	High diversity	axis 1	0.00	0.07	0, 0.223	1.000
		axis 2	0.46	0.13	0.168, 0.662	**0.001**
	Low diversity	axis 1	0.00	0.00	0, 0	0.980
		axis 2	0.28	0.14	0, 0.536	**0.008**

[a] No detected lysis titres at sampling moment 3 and 4 inhibited evaluation of differences among sampling moment categories; a logit link GLMM with continuous temporal predictor was fitted instead.
[b] confidence intervals based on 1000 parametric bootstraps
[c] *P*-values calculated based on 1000 permutations

Table 5.5: Log2 fold change and taxonomic affiliation of temporally changing OTUs in cloacal microbiota.

	Log2 fold change with experimental duration per day				
OTU ID	**Mean of normalized counts**	**log2FoldChange**	**SE**	**Wald statistic**	**FDR *q***
828667	1.26	0.07	0.012	5.71	0.000
221299	4.06	-0.02	0.006	-3.79	0.018
1107027	0.78	-0.05	0.013	-3.67	0.019
955052	2.40	-0.03	0.008	-3.57	0.021
333178	0.53	-0.04	0.015	-2.91	0.094
New.ReferenceOTU128	1.17	-0.04	0.012	-3.23	0.044
922761	0.44	0.06	0.019	3.22	0.044
New.ReferenceOTU261	1.95	0.02	0.006	3.33	0.041
New.ReferenceOTU434	1.17	0.03	0.009	3.03	0.072

	Taxonomic affiliation				
OTU ID	**Phylum**	**Class**	**Order**	**Family**	**Genus**
828667	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	unassigned
221299	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
1107027	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
955052	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
333178	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
New.ReferenceOTU128	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
922761	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unassigned
New.ReferenceOTU261	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*
New.ReferenceOTU434	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*

Immune function and host-associated microbiota correlate at the individual level

Given consistent individual differences of immune indices and host-associated microbiota traits (Table 5.4), we asked whether immune function and the host-associated microbiota covaried at the individual level. To examine these relationships, we performed Procrustes ordination analysis, which revealed that the dissimilarity matrix based on the immune indices (hereafter "multivariate immune index") correlated with the unweighted UniFrac distance matrix representing taxon occurrence in host-associated microbiota (Fig. 5.4 a and b), with (nearly) statistical support for both the high diversity ($M^2 = 0.26$, $P = 0.02$) and low diversity microbial environments ($M^2 = 0.24$, $P = 0.06$). In contrast, we found no significant correlations between immune function and host-associated microbiota structure based on weighted UniFrac (high diversity: Procrustes $M^2 = 0.18$, $P = 0.33$; low diversity: $M^2 = 0.18$, $P = 0.23$). Furthermore, for each experimental group, LMMs (that included individual identity and replicate room as random effects) resulted in significantly positive correlations between the PCo 1 scores for immune function and the PCo 1 scores for taxon occurrence in host-associated microbiota (unweighted UniFrac; Fig. 5.4 c and d). These models also revealed repeatability of the multivariate immune index and taxon occurrence in host-associated microbiota PCo scores along the first and second axes (unweighted UniFrac, Table 5.4). We also used LMMs to examine relationships between each separate immune index and OTU richness and Shannon diversity of the host-associated microbiota. Neither OTU richness nor Shannon's diversity accounted for significant variation in any of the individual immune indices (all LMM fixed effects: $P > 0.11$; Fig. S5.9). In contrast, PCo 1 scores of taxon occurrence in host-associated microbiota (unweighted UniFrac) were negatively associated with the probability of lytic activity (Fig. 5.5 e) and positively with haptoglobin concentration (Fig. 5.5 g). Microbiota PCo 2 scores positively associated with both IgY concentration (Fig. 5.5 d) and the probability of lytic activity (Fig. 5.5 f), but neither relationship was significant. Both PCo axes were unrelated to agglutination (Fig. 5.5 a and b).

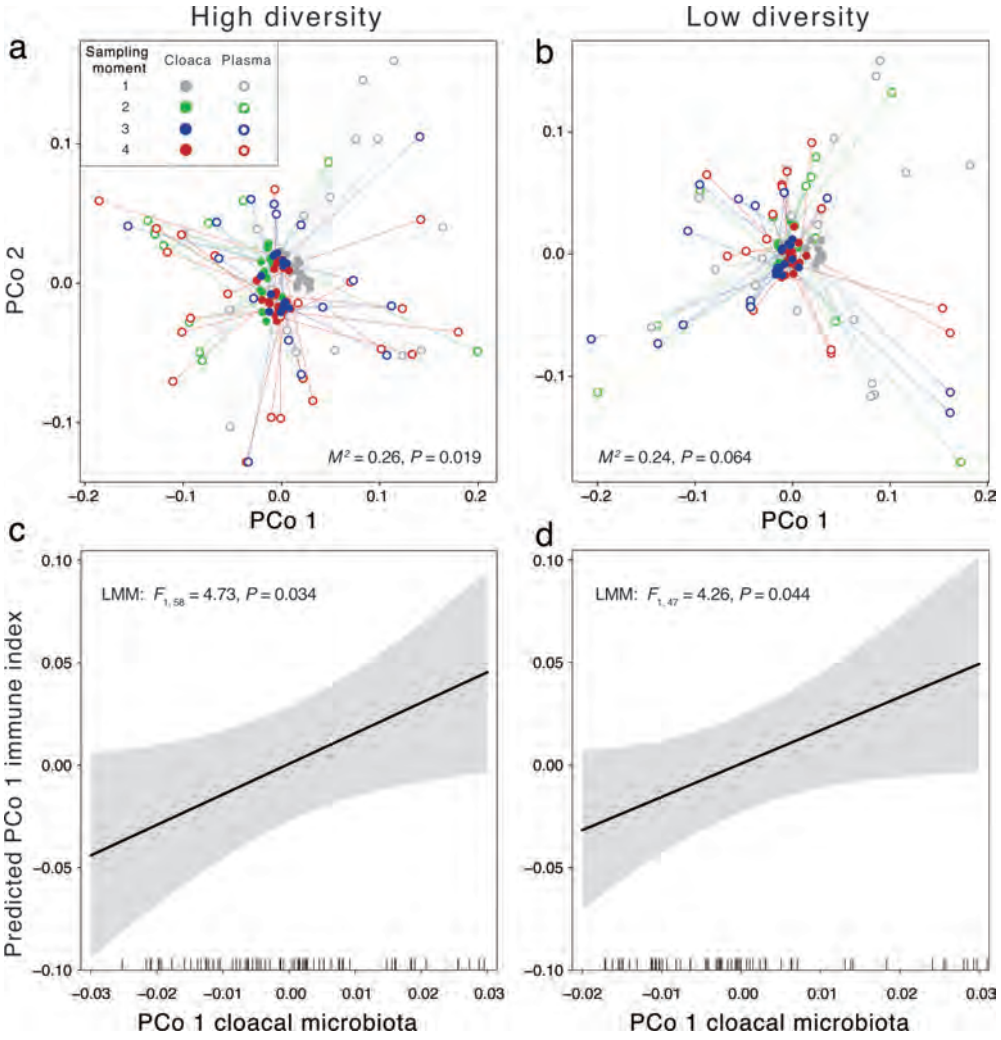

Figure 5.4: Procrustes analysis of immune function and cloacal microbiota states. (a, b) Procrustean superimposition of two multivariate data sets for birds exposed to (a) high diversity and (b) low diversity soils: multivariate immune index based on four immune indices (agglutination titer, IgY concentration, lysis titer, haptoglobin concentration) (open symbol) and taxon occurrence in cloacal microbiota based on unweighted UniFrac (closed symbol). Procrustes analysis scaled and rotated both ordinations to the best Procrustean fit (M^2) and protest statistics are shown in each plot. (c, d) PCoA scores of immune function of birds exposed to (c) high diversity and (d) low diversity soils predicted by PCoA scores for phylogenetic taxon occurrence of cloacal microbiota. The line depicts the predicted relationship and the shaded area depicts the 95% CI of the predictions. (c, d) Linear mixed-model inferences are controlled using subject identity and replicate room as random effects.

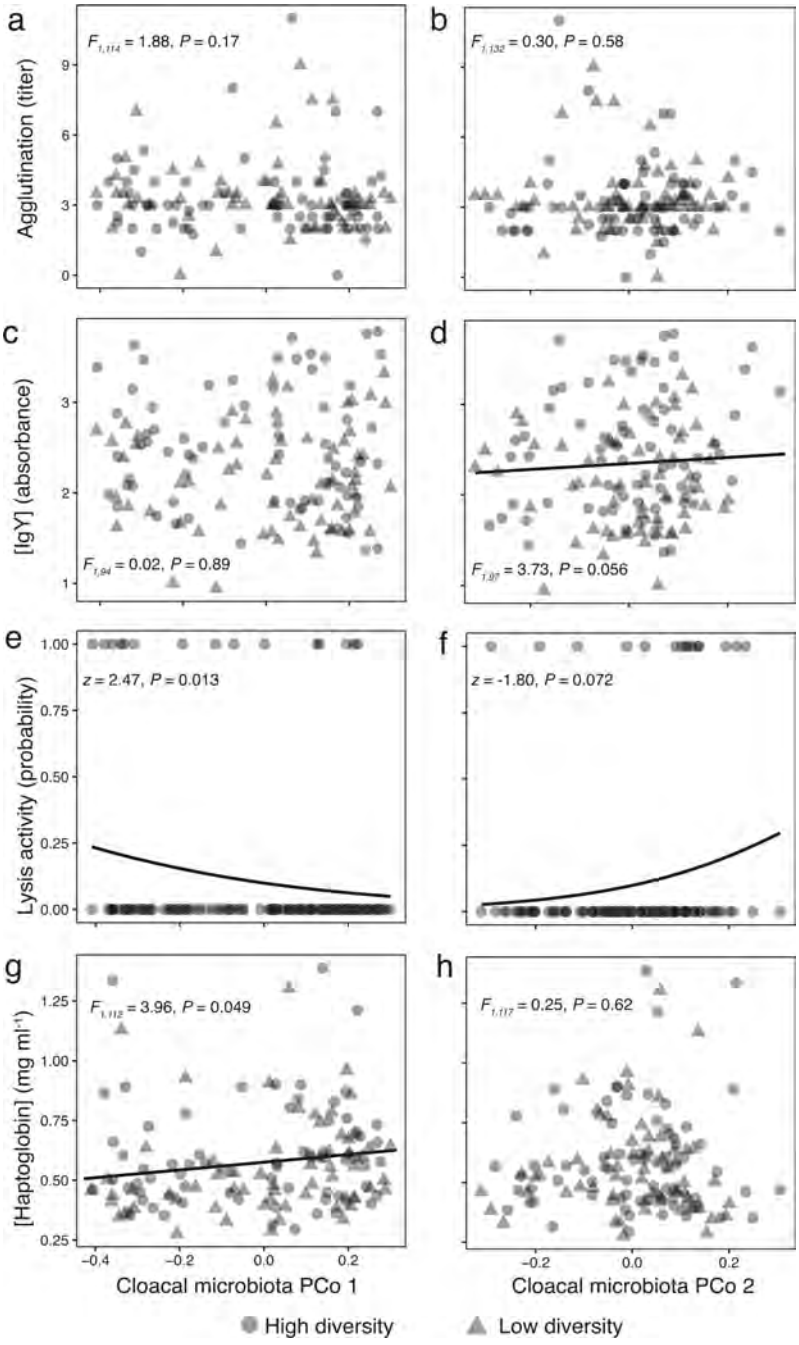

Figure 5.5: Relationships between individual immune indices and cloacal microbiota PCoA scores. Model predictions (black line) for (a, b) Agglutination titer, (c, d) IgY concentration, (e, f) Lytic activity and (g, h) haptoglobin along the first (PCo 1) and second (PCo 2) axis of unweighted UniFrac distances among cloacal microbiota, respectively. Black dots are individual plasma samples. (G)LMM statistics are shown in each plot.

Discussion

Exposure to distinct experimental microbial environments led to differences in adaptive immune function and in the composition, richness and dynamics of the cloacal microbiota in zebra finches. Importantly, at the individual level, immune function and the cloacal bacterial taxon occurrence covaried significantly, while individuals differed consistently for both immunological and microbiota variables. Indices of immune function changed over the time course of the experiment, but the temporal patterns were not different between experimental microbial environments. In contrast, the manipulated microbial environments did impact alpha and beta diversity, and cloacal microbiota resilience: the microbiota of zebra finches exposed to the low diversity microbial environment stabilized sooner, and microbiota returned in the direction of the baseline compositional state while maintaining individual differences. In the context of ecological immunology, our results suggest that adaptive immune function plastically responds to microbial communities in the surrounding environment, and that innate and adaptive immune function collectively correlate with host-associated microbiota variation at the level of the individual. With the inherent complexity of microbial communities in the wider environment, its impact on the physiological condition and evolutionary fitness of animals is likely more complex vis-à-vis classic ecological interactions like parasitism. A more thorough understanding of the impact of environmental microbes on animal immunity requires a better picture of within-individual flexibility of immune function and the host-associated microbiota.

The premise that environmental microbial communities may determine the immune defenses of animals underlies the increasing integration of microbial ecology research into ecological immunology (Horrocks *et al*., 2011; Kohl & Carey, 2016; McFall-Ngai *et al*., 2013; Tieleman, 2018). We hypothesized that animals may flexibly adjust immune defenses to the microbial environment at a given place and time. Our results suggest that different microbial environments can affect acquired antibody levels (IgY concentration) in captive zebra finches (Fig. 5.1). Caution is warranted for drawing firm conclusions, as IgY concentration slightly differed between the two experimental groups at baseline. Given the substantial differences among individuals, longer time series and larger sample sizes could help to affirm the observed pattern. The lack of distinction in agglutination titers in the face of different microbial environments is consistent with the unimportance of exogenous antibody stimulation to the production of natural antibodies (Lee, 2006). This highlights

that differences in the antigenic universe (*sensu* Horrocks *et al*., 2011), here as result of different environmental microbial communities, do not affect all immune defenses equally. Complement-like lysis was low in our zebra finches. This could be a feature of zebra finches (Matson *et al*., 2005). The observed lack of experimental treatment effect corresponds with earlier findings of lysis titers in zebra finches that did not change after manipulation of nest bacterial loads (Evans *et al*., 2016). The concentration of the acute phase protein haptoglobin signals inflammatory status (van de Crommenacker *et al*., 2010; Matson *et al*., 2012). Accordingly, the lack of any experimental effect on haptoglobin concentration suggests that the experimental microbial environments did not differentially induce inflammation in the birds. These patterns collectively suggest that, over a period of eight weeks, acquired immunity was more influenced by environmental microbial communities than innate immunity. Indeed, constitutive innate immunity is expected to fit evolutionary responses to different environments (Lochmiller & Deerenberg, 2000; Tieleman, 2018), but other studies have demonstrated that innate immunity can also be flexibly adjusted to environmental differences (not specifically related to microbes) (Buehler *et al*., 2008b; Hegemann *et al*., 2012; Matson, 2006). We did not find patterns implicating environmental microbial community features and innate immunity. This suggests certain rigor of the measured innate immune indices, at least at the time scale of this experimental study.

If the microbial environment affects animal immune function over short time scales, such as during several weeks, we expected to find changes in immune function to emerge over the course of eight weeks of experimental treatment. Life history theory predicts that nutritional and energetic reallocation between costly immune defenses and other efforts, such as reproduction, molting, migration and thermoregulation (Lee, 2006; Lochmiller & Deerenberg, 2000) invoke immunological variation between seasons or annual cycle stages (Buehler *et al*., 2008b; Eikenaar & Hegemann, 2016; Hegemann *et al*., 2012). Because such trade-offs were unlikely to be present here during eight weeks of non-breeding under controlled ambient conditions with unlimited access to sterilized food, this could explain why our zebra finches showed no adjustment of constitutive innate immunity. Yet, we documented adjusted adaptive (IgY concentration) and induced (haptoglobin concentration) immune responses within individuals independent of treatment (Fig. 5.1). While these temporal shifts coincided most prominently with the radical shift from outdoor aviaries to indoor cages, both indices also showed significant increments during the experimental phase. These patterns suggest that

adaptive and induced immune responses can adjust to novel microbial environments over relatively short time scales. We propose that the microbial environment may represent an important contributor to immunological variation, which should be considered in ecological immunology. Variation of immune function has been associated with variable environmental conditions in wild animals (e.g. variation imposed by long-distance migration or seasonality (Buehler *et al*., 2008a,b; Hegemann *et al*., 2012; Horrocks *et al*., 2012; Zimmerman *et al*., 2010)). Our results suggest that such effects could be (partially) due to variable environmental microbial conditions, in addition to well-documented factors driving nutritional and energetic tradeoffs.

In addition to these phenotypically plastic immune responses to changing microbial environments, our evidence for significant repeatability of immune indices, within the context of the imposed experimental conditions, indicates that immunity is a characteristic property of an individual (Table 5.4). If this individuality has a heritable component, it may be of great importance for microevolution to changing (microbial) environments (Tieleman, 2018; Tieleman *et al*., 2010). Devising host selection lines on different microbial conditions, and subsequent testing whether immune function upon exposure to high and low diversity microbial environments is different between animals of different lineages would strongly advance our understanding of the role of environmental microbes on evolution of animal immune systems.

Experimental microbial environment also impacted the richness, composition and stability of the cloacal microbiota of zebra finches (Fig. 5.2; Fig. 5.3). Our detection of more OTUs in the microbiota of birds on high diversity soil, and experimental effects on beta diversity suggest that environmental bacteria shaped the host-associated microbiota and highlight that animal microbiota to some extent may reflect the microbial environment that its host experiences. Furthermore, this suggests that invasion and recruitment of environmental microbes into the animal microbiota was not fully counteracted by the host's regulatory systems during eight weeks of exposure. We note that our sequence data were inadequate to capture the full cloacal microbiota diversity. This likely underestimated the true effect of environmental microbes on host microbiota since less dominant taxa were likely harder to detect. Despite that caveat, our data provides further support a role of environment on host-associated microbiota, which has become increasingly recognized (Eichmiller *et al*., 2016; Lucas & Heeb, 2005; Rothschild *et al*., 2018; van Veelen *et al*., 2017), and sheds new

light on the rarely addressed direct relationship between environmental microbes and microbiota of terrestrial vertebrates.

Nonetheless, several other studies suggested that animals also regulate their microbiota and implied importance of host genetic factors (e.g., Benson *et al*., 2010; Pearce *et al*., 2017). We previously reported finding no interspecific differences in cloacal, skin and feather microbiota of sympatric passerine species, and weak associations between cloacal and nest-environmental communities at the individual level (van Veelen *et al*., 2017). This suggested importance of a shared metacommunity but also some extent of host regulation. In the current study, the pattern that zebra finch microbiota seemed to return into the direction of their baseline state also suggests that environmental bacteria might be transient rather than establishing in the cloacal microbiota over a period of eight weeks, potentially due to host regulation. Moreover, the significance of host factors in shaping host-associated microbiota is also reflected by significant repeatability of host-associated microbiota characteristics. However, the compositional differences remained after eight weeks of experimental treatment and longer time series are thus required to determine if host-associated microbiota remain distinct over longer periods. Collectively, these results illuminate the presence and simultaneous influences of hosts intrinsic factors and environmental microbes on animal microbiota structure but leave open whether the microbial environment also influences the ability of hosts to regulate its microbiota. Recent work on healthy humans showed for the first time evidence for a mechanistic pathway linking microbiota and adaptive immunity (Fadlallah *et al*., 2019). Systemic IgG repertoires are produced in response to various symbiotic gut commensals. The authors further postulate a protective role for anticommensal IgGs, and IgG production appeared microbiota diversity dependent as well. This evidence suggests a potential underlying mechanism for microbiota-driven adaptive immune investment. Whether such connections between microbiota and IgG (and avian IgY) production are universal across vertebrates remains to be studied. Yet, whether such antibody responses to gut microbiota can be shaped by the microbial environment should remain a topic of investigation.

Effects of environmental microbial communities on animal gut microbiota dynamics, as shown here (Fig. 5.3), have to our knowledge not been documented before (Tasnim *et al*., 2017). Specifically, host-associated microbiota stabilized sooner in less diverse environments, indicating direct influence of the microbial environment on host-associated

microbiota dynamics. This could be due to the differences in the taxonomic breadth of environmental microbial communities between the treatments in which case, when assuming no dispersal limitations, more diverse communities (high diversity treatment) may lead to more diverse immigration and hence increased stochasticity and longer turnover rates in host-associated microbiota (i.e. reduced resilience) (Hubbell, 2006; Márquez & Kolasa, 2013). A fruitful avenue to test this could be to expose individual animals repetitively to a random sequence of high or low diversity microbial environments, with equal acclimation periods and simultaneous longitudinal monitoring to quantify microbiota resilience after each particular environmental transition.

Immune function significantly correlated with bacterial taxon occurrence in host-associated microbiota (Fig. 5.4; Fig. 5.5), suggesting that immune defenses respond to host-associated microbes, or vice versa, and most dependent on occurrence rather than abundance of taxa. While immune systems have evolved to cope with microbes and other antigenic compounds, our results suggest that individuals may flexibly respond immunologically to regulate their own microbiota (Fig. 5.4). Since birds were translocated from group living in outdoor aviaries to indoor cages in pairs, inevitably, changes toward a sterilized diet, a different temperature regime, and altered social and microbial environments all likely contributed to the observed shift between sampling moment 1 and 2. Because of the correlative nature of these findings, experimental manipulation of immunocompetence and host-associated microbiota are necessary to establish causal relations underlying the observed association. Yet, the correlation supports results from a field study that showed links between immune function and bird-associated culturable bacterial load, but not to airborne bacterial load (Horrocks *et al*., 2012). Although we did not explicitly consider bacterial load (total soil bacterial counts did not differ between experimental treatments, unpublished data), which has been shown previously to relate to fitness in birds (Peralta-Sánchez *et al*., 2018), this work documented an individual-level relationship between immune function and host-associated microbiota while simultaneously controlling for differences in diet and other environmental microbial factors.

Conclusions

We show that antibody-mediated immunity and the composition, richness, and dynamics of the cloacal microbiota in zebra finches varied in response to experimental microbial environments. The lack of associations between single immune indices and single host microbiota alpha-diversity measurements combined with the correlated multivariate summaries of the immune system and the microbiota underscore the complexity inherent in these systems and emphasize the challenge of interpreting immune function variation at different levels in eco-evolutionary contexts (reviewed in Tieleman, 2018). Yet, in a broader perspective, links between a host's immune system and microbiota highlight the importance of incorporating microbiota analyses into studies of ecological immunology. Doing so is expected, at least partially, to provide evidence about the immunogenic agents in an organism's environment with which an immune system must cope (Kohl & Carey, 2016; Lochmiller & Deerenberg, 2000; Tieleman, 2018). Consequently, we strongly encourage further experimental studies of the direct relationships between environmental and host-associated microbiota (e.g., Jacob *et al*., 2015; Risely *et al*., 2017a). Ecological immunology may benefit from future investigations covering a wide range of animals, particularly when accompanied by measures of fitness. Such efforts, though challenging, are expected to make major contributions to a more mechanistic understanding of host-associated microbiota community dynamics and the microbiota's influence on health of wild animals.

Ethics approval

This study was performed under animal welfare licence DEC6314 of the Institutional Animal Care and Use committee of the University of Groningen obeying the Dutch Law.

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Supplementary Information

Supplementary methods

Generation, maintenance and monitoring of experimental soil beddings

We commercially acquired (Loonbedrijf Ritzema, Zuidwolde, NL) 2.5 m^3 soil containing clay (∼40%), sand (∼40%) and organic matter (∼20%). We then divided the soil in two fractions and applied three cycles of 25 kGy gamma irradiation (Synergy Health Ede B.V, the Netherlands) to one soil fraction to generate a highly reduced microbial environment, referred to as 'low diversity' soil treatment in the main text (Fig. S5.1). We used the remaining fraction of non-irradiated soil as a high diversity microbial environment and referred to this fraction as 'high diversity' soil treatment. We applied either high or low diversity soil as a ∼2 cm deep bedding layer in the cages. Biweekly, throughout the experiment, we thoroughly cleaned bedding trays and then sterilised them with 70% ethanol, followed by replacement soils (mean ± S.E.M.: 15 ± 1 days, n = 4). Low diversity soil stocks were stored in a double layer of sealed polyethylene (PE) bags (∼10 kg per bag) under ambient conditions: (mean ± S.E.M.) 4.7 ± 0.41 °C (https://weerstatistieken.nl/eelde/). High diversity soils were stored in two layers of PE open-top bags, stored in a cooling cell at 4 °C enabling soil respiration while limiting bacterial activity to reduce temporal variations. We maintained soil moisture content of the cage beddings by daily spraying ∼30 ml autoclaved water per cage (i.e. average daily water loss determined as weight loss over 24 hours in the experimental rooms). To monitor the temporal stability of soil bacterial communities, we sampled soils at three sampling moments between soil replacements as follows: every 3 (n = 20), 10 (n = 20) and 14 (n = 18) days after soil (re)placement, we filled a sterile 15-ml tube with soil from three randomly selected cages in equal proportions using a sterilised spoon. Soil samples were then stored immediately at -20 °C. In addition, nine samples (natural n = 5, sterilised n = 4) were taken from storage bags to monitor changes during storage.

Housing and sample collection of zebra finches

To experimentally test gut microbiome responses to different microbial environments, we brought 53 adult female and 54 male zebra finches *Taeniopygia guttata* from single-sex outdoor aviaries to indoor cages (50 × 50× 40 cm) on 16 November 2014, housed as single-sex pairs to prevent inducing breeding behaviour. The group of females hatched between in May or September 2013 months prior to the experiment, except two birds of 3.5 years old. We exposed birds to high or low diversity soils, with two replicate rooms for high diversity soils and low diversity soils, respectively. In each room, we placed 12 cages in a block of 3 × 4, each containing a single-sex pair, each individual randomly assigned to a room and to a cage within the room. Male and female cages were spatially alternated in a block of 3 × 4 cages in each room. In addition, each room contained two separate cages to house single-sex groups of spare birds (n = 0-3). We kept birds for eight weeks under the following conditions: ambient temperature at 20 °C ± 1, relative humidity at 55% ± 15 and a 12:12 h light-dark (L:D) cycle.

We applied gamma-irradiation to the seed mixture fed *ad libitum* to all birds to reduce potential confounding effects of seed-associated communities on the cloacal microbiota.

In addition, we provided autoclave-sterilized water with 0.2 µm-pore filter-sterilized multivitamin / amino acid solution (final concentration 4 $g \cdot l^{-1}$; #181161; Omni-vit, Oropharma N.V., Deinz, Belgium) to compensate potential vitamin degradation from gamma-irradiation of seed. We thoroughly cleaned water and food dispensers twice a week followed by sterilisation with 70% ethanol.

To determine 'before experiment' levels of innate immune function and cloacal microbiome state, we collected a blood sample ($\sim$150 µl blood, 8 $ml \cdot kg^{-1}$) from the brachial vein and a cloacal swab from each female zebra finch (see Table S1) and we additionally recorded body weight, wing length and tarsus length ($\pm$ 0.1 mm) as a measure of general body condition. We then tracked temporal dynamics of innate immunity and the gut microbiome in the high and low diversity soil treatments by collecting cloacal swabs after two, four and eight weeks of acclimation to experimental microbial conditions. We refrained from sampling two females with congested cloacae (female ID 4208 and 4250) at the 'before experiment' sampling moment. We used a new pair of nitrile gloves per room when handling experimental equipment or soils and we used new gloves to handle birds, sterilised with 70% ethanol between birds. We centrifuged blood samples 10 min at 7000 rpm to separate plasma from blood cells. We then stored plasma and cloacal samples at -20 °C.

Laboratory analysis of innate immune function

To quantify non-specific antibody titres and complement-like lytic activity of female blood plasma, we used a hemolysis-hemagglutination assay with rabbit erythrocytes antigens (Envigo, Leicester, UK) (Matson *et al*., 2005). Plasma samples were randomized across plates and plate scans were scored using a double-blind procedure (by M. Havinga and M. A. Versteegh); average scores were analysed. We quantified total immunoglobulin Y (IgY) concentration in blood plasma in duplicate with an enzyme-linked immunosorbent assay (ELISA) using rabbit anti-chicken IgG (Sigma-Aldrich, St Louis, MO, USA) based on (Demas & Nelson, 1996; Grindstaff *et al*., 2005), optimised for zebra finch plasma. We used a chicken egg yolk (diluted 1:2000 in 0.1% (m/v) milk powder in 0.05% PBS-Tween 20) as standards to assess between-plate variability and as a reference to quantify total IgY concentrations in plasma samples. We examined the haptoglobin concentration in blood plasma as a measure of inflammatory status using a haem-binding assay (Tri-delta Diagnostics Inc., Morris Plains, NJ, USA) (Matson *et al*., 2012).

DNA extraction and 16S rRNA gene amplicon sequencing of soil and cloacal samples

We extracted DNA from 250 mg of homogenised composite soil samples and from cloacal swabs. We aseptically peeled the cotton from their cloacal swab stalks and placed them in extraction vials provided in the MoBio PowerSoil DNA isolation kit (MoBio laboratories, Carlsbad, CA, USA). We performed DNA extractions following the manufacturer's protocol, with addition of 0.25 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) to improve cell disruption during three cycles of 60 s bead beating (Mini-bead beater, BioSpec Products, Bartlesville, OK, USA). The V4/V5 region of the 16S rRNA gene was amplified in triplicate using primers 515F (Caporaso *et al*., 2011) and 926R (Parada *et al*., 2016) at Argonne National Laboratory, IL, USA, following the Earth Microbiome Project

protocol (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/) (Gilbert *et al*., 2010), followed by library preparation of pooled triplicates and 2 × 250 bp paired-end sequencing using V2 chemistry on an Illumina MiSeq. In total, we included seven technical negative controls in the sequencing run: three blank Powersoil DNA extractions with PCR-grade water instead of sample, as well as one with and one without zilconia beads, and two of the latter blank extractions with addition of a sterile swab. None of the samples detectably produced reads in the quality-filtered sequence data set.

Bioinformatic processing of 16S rRNA gene amplicon sequence data

We processed raw Illiumina sequence reads using the QIIME pipeline (v 1.9.1) (Caporaso *et al*., 2010a). Since samples were deliberately sequenced in random order, we did not use statistical correction for sequencing run (n = 2). We demultiplexed and quality filtered and paired reads, retained reads with fragment lengths ranging from 368 to 382 bp to discard non-specific reads ($\sim$ 267 bp) that mapped (99% identity) to the zebra finch's 12S rRNA gene using BLAST. We obtained 4.2 10^6 (51% of total read count) high quality bacterial 16S rRNA gene sequences. Using an open-reference strategy, we clustered sequences first into OTUs using the *uclust* algorithm (Edgar, 2010) at 97% identity against the Greengenes reference database (v. 13.8) (DeSantis *et al*., 2006), and *de novo* clustered (0.1%) reads that failed to match the reference set. We selected representative sequences per OTU, concatenated both OTU tables, removed singletons to reduce effects of sequencing error on richness estimation, and annotated the resulting OTU table with taxonomic information from the Greengenes reference set (97% identity). We then aligned representative sequences using PyNast (Caporaso *et al*., 2010b) and identified and removed chimeric sequences using the uchime algorithm in the *usearch81* toolkit (Edgar *et al*., 2011) followed by phylogenetic tree construction using FastTree (Price *et al*., 2009). We filtered OTUs assigned to Archaea, chloroplast and mitochondrial sequences from the data set, and offset the OTU table to retain OTUs at > 0.001% of the total abundance to reduce table sparsity.

Statistical analysis of innate immune function

We build linear mixed-effects models (LMMs) for each innate immune index to analyse effects of experimental soil treatment (fixed predictor) and temporal shift between sampling moment (fixed time predictor) (Pinheiro *et al*., 2017; Zuur *et al*., 2009) with individual female ID and experimental replicate room as random effects, as well as the interaction between fixed predictors. Because lytic capacity of plasma was mostly unobserved, we instead modelled the probability of lytic activity using a generalized linear mixed-effects model (GLMM) with a logit link function and the same set of predictors. After checking normality and homoscedasticity assumptions, we conducted ANOVAs using LmerTest (Kuznetsova *et al*., 2016) and calculated the repeatability *R* of each measure using the (G)LMM models using the *rptR* package (Stoffel & Nakagawa, 2017), controlling for fixed effects. Confidence intervals (95%) for *R* estimated by parametric bootstrapping and the significance inferred from permutation tests. Replicate room effects were not significant in all analyses. We noted that a significant experimental effect was observed in pre-acclimation samples (Fig. 5.1 b) even though birds had not yet been exposed to experiment soils. Nonetheless, the temporal increase of plasma IgY in the natural soil treatment group remained after exclusion of the five individuals designated

to the natural soil treatment that showed higher 'before experiment' values than the highest value of birds assigned to the sterilised soil treatment. This yielded significant support for the treatment:time interaction (t = -1.99, P < 0.05), suggesting that, when 'before experiment' plasma IgY levels were low or moderate, a more diverse microbial environment may induce an increase in circulating natural antibodies (IgY). In addition, we employed distance-based redundancy analysis (db-RDA) in *vegan* (Oksanen *et al*., 2017) as a multivariate approach to test immunological segregation between treatment groups. We analysed the treatment effect for each sampling moment separately to alleviate pseudo-replication complications and to remove the temporal effects.

Statistical analysis of diversity and composition of experimental soil communities

We used the *phyloseq* (McMurdie & Holmes, 2013), *vegan* (Oksanen *et al*., 2017) and *lme4* (Bates et al., 2015) packages in R (R Core Team, 2016) to analyse soil bacterial community characteristics. All R scripts and input tables are available online.

To evaluate the effect size and stability of gamma-irradiation on alpha and beta diversity of the bacterial communities in the soil treatments, we analysed 30 high diversity and 30 low diversity soil samples (i.e. comprising three cycles of soil replacement). We seemingly mislabelled two samples collected on the same day (Sample ID 8 and 67; 10 March 2015), which we conservatively removed from the data set for this analysis. Because the estimated total diversity of natural soils was consistently higher than sterilised soils (Fig. S5.1 a), we rarefied all samples to 1115 reads so all soil samples could be included for analysis alpha diversity in soils. Rarefaction curves for OTU richness (and estimated total diversity; Chao1) had not saturated at 1115 reads but for Shannon diversity curves reached a plateau at that coverage. We examined variation in OTU richness and Shannon diversity using linear mixed-models (LMMs) with the following predictors: experimental treatment ('high diversity' and 'low diversity'), time points among soil replacements (categorical; 3, 10 and 14 days), and replicate room as a random term in all models (Zuur *et al*., 2009). Interactions were not significant and were removed before estimation of fixed effects by REML. Both response variables fulfilled assumptions of normality and variance homogeneity. We estimated the marginal effect sizes using ANOVA with *lmerTest* (Kuznetsova *et al*., 2016). Where appropriate, we report adjusted P-values for multiple comparisons calculated using *multcomp* (Hothorn *et al*., 2016)

Because the library sizes of our experimental soil treatments did not differ (t = 0.47, df = 64.6, P = 0.64) and sample coverage was sufficient, we performed a variance-stabilising transformation of the full non-rarefied OTU table for soils (Love *et al*., 2014; Weiss *et al*., 2017). We used the transformed data table for ordination analysis of phylogenetic beta diversity using the weighted UniFrac metric, visualised by principal coordinate analysis (PCoA). We tested experimental treatment and temporal effects on unconstrained ordination using tests of marginal effects with the *adonis* and *adonis2* functions, with permutations stratified by replicate room. We tested the ordinations for multivariate group dispersions between all groups using *betadisper* and *permutest* with 999 permutations.

Statistical analysis of diversity, composition and taxon abundances of cloacal microbiomes

We performed analysis of cloacal microbiomes using a similar approach as described for soil communities. Based on visual evaluation of sample-wise rarefaction curves (Fig. S5.2), we estimated that a minimum of ~1200 reads sample^{-1} are sufficient to analyse within-sample diversity (OTU richness and Shannon diversity). Because some cloacal samples in our data set appeared to have a low coverage (median: 3398, range: 12-88999 reads per sample), we subset our OTU table to retain the upper 80% of the coverage distribution (min: 1273 reads per sample; n = 181), and rarefied the data set to the new minimum to examine variation of alpha diversity among experimental groups. We log-transformed OTU richness and Shannon's diversity index of cloacal samples to fulfil normality and homogeneity of variance assumptions. We used LMMs to estimate effects of soil treatment and sampling moment (1, 2, 3 and 4; representing 0, 2, 4 and 8 weeks) on alpha diversity with female identity and replicate room as random effects and an interaction of the fixed effects. To test effects of experimental soil treatment on cloacal microbiome alpha diversity at each sampling moment, we calculated pairwise contrasts of the experimental treatment factor at each sampling moment using the *phia* package (De Rosario-Martinez, 2015) and reported FDR-corrected q-values (critical q-value = 0.1). We calculated the repeatability R of each measure using (G)LMM using the *rptR* package (Stoffel & Nakagawa, 2017) while controlling for fixed effects, and we estimated confidence intervals for R by parametric bootstrapping with significance inferred from permutation tests.

We also examined the degree of change between the sampling moments to evaluate whether cloacal microbiomes stabilised in the novel microbial environments, and if so, how soon stabilisation was achieved. To characterise the degree of change for each female, we calculated the change in OTU richness and Shannon diversity by subtracting the alpha diversity at time t_i from t_{i-1}, referred to as within-individual change. We used LMMs with the interaction between experimental treatment and time, and including replicate room as random term, to test whether the within-individual change differed between soil treatments and varied across sampling moments.

To examine beta diversity, we applied the approach described for soil beta diversity to a subset of the female cloacal microbiomes comprising the upper 90% of the coverage distribution of cloacal samples (n = 204; minimum coverage: 469 reads per sample). Cloacal microbiome library sizes differed by a factor of 1.6 between treatments (t = 2.10, df = 129.2, P < 0.05), which is within acceptable range (Weiss *et al*., 2017). Accordingly, we continued to examine variation in beta diversity using variance-stabilised non-rarefied data and the weighted UniFrac as a measure for phylogenetic composition. We used *adonis* to calculate the explained variance portion for experimental treatment and sampling moment, and we used *adonis2* to determine the significance of the marginal effects. Permutations were stratified by replicate room in each model.

We used the weighted UniFrac distance matrix to examine within-individual shifts in the phylogenetic composition of cloacal communities: we extracted the within-individual distances of consecutive sampling moments, and we used an LMM with a random female identity term to test whether weighted UniFrac distances varied by soil treatment, differed

between sampling moments, and whether within-individual temporal shifts differed by soil treatment, i.e. an interaction term. We used Tukey-Kramer tests to evaluate *post hoc* group contrasts.

To examine differential abundances in cloacal microbiomes between soil treatments and with experimental duration, we used negative binomial generalised linear models as implemented in the DESeq function from the *DESeq2* package (Love *et al*., 2014) to appropriately deal with overdispersed taxon abundance data (McMurdie & Holmes, 2014; Weiss *et al*., 2017). We used the variance-stabilised non-rarefied data set (coverage: upper 90% of samples; n = 204) for this analysis. For this analysis, we selected only the sampling moments that took place during the experiment (i.e. no baseline samples). The models included experimental treatment, experimental duration (as continuous predictor) with their interaction to predict differential taxon abundances. We reported effect sizes as log2 fold (log2FC) changes and ascribed significance using the Wald statistic (ratio of log2FC and SE): with a critical FDR-corrected q of 0.1 (Love *et al*., 2014).

The relation between immune function and cloacal microbiome characteristics

We first generated dissimilarity-based PCoA ordinations (cmdscale function of *stats* package (R Core Team, 2016)) of the four immune indices and unweighted UniFrac distance matrices of the cloacal microbiome, for each experimental treatment group separately. We then applied Procrustes superimposition to test whether female innate immune function covaried with cloacal microbiome composition (Peres-Neto & Jackson, 2001). The employed symmetric Procrustes rotation minimizes the sum of squared distances between associated sample pairs (i.e. between simultaneously sampled blood plasma and cloacal swabs). To tests the significance of the Procrustean fit, we used the protest function (Peres-Neto & Jackson, 2001), which generates a *P*-value by comparing the observed goodness-of-fit statistic M^2 (i.e. sum of squared residual distances) of the Procrustes rotation with a simulated M^2 distribution generated by recalculating M^2 after each of 10 000 permutations of sample labels in our empirical data. We used LMMs to test the association between the first Procrustean axes of immune function and microbiome, with sampling moment, female identity and replicate room included as random terms. Predicted ordination scores were plotted using the *effects* package (Fox, 2003). Additionally, we used corresponding (G)LMMs to explore relationships between each distinct innate immune measure and OTU richness, Shannon diversity (alpha diversity), as well as PCoA axis 1 and 2 (beta diversity) based on an unweighted UniFrac distance matrix of the female cloacal microbiome. We calculated the repeatability R of the first PCo axes of immune function, and the cloacal bacterial beta diversity using the (G)LMM, while controlling for fixed effects, and confidence intervals for R estimated by parametric bootstrapping and the significance inferred from permutation tests.

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Supplementary tables

Table S5.1: Overview of collected cloacal gut samples of zebra finches on high diversity and low diversity soils.

	High diversity soil sampling time point					**Low diversity soil** sampling time point				
Female ID	**1**	**2**	**3**	**4**	**Total**	**1**	**2**	**3**	**4**	**Total**
2744						1	1	1	1	**4**
2784	1	1	1	1	**4**					
3666	1	1	1	1	**4**					
3759						1	1	1	1	**4**
3804	1	1	1	1	**4**					
3811						1	1	1	1	**4**
3824						1	1	1	1	**4**
3875	1	1	1	1	**4**					
4205						1	1	1	1	**4**
4207						1	1	1	1	**4**
4208							1	1	1	**3**
4211						1	1	1	1	**4**
4215						1	1	1	1	**4**
4218	1	1	1	1	**4**					
4227						1	1	1	1	**4**
4229						1	1	1	1	**4**
4231	1	1	1	1	**4**					
4232	1	1	1	1	**4**					
4237	1	1	1	1	**4**					
4238	1	1	1	1	**4**					
4244	1	1	1	1	**4**					
4250							1	1	1	3
4254	1	1	1	1	**4**					
4256	1	1	1	1	**4**					
4271	1	1	1	1	**4**					
4280						1	1	1	1	**4**
4298	1	1	1	1	**4**					
4406						1	1	1	1	**4**
4414	1	1	1	1	**4**					
4427						1	1	1	1	**4**
4428	1	1	1	1	**4**					
4432						1	1	1	1	**4**
4434						1	1	1	1	**4**
4438	1	1	1	1	**4**					
4445	1	1	1	1	**4**					
4453						1	1	1	1	**4**
4454						1	1	1	1	**4**
4457	1	1	1	1	**4**					
4459	1	1	1	1	**4**					
4460	1	1	1	1	**4**					
4464						1	1	1	1	**4**
4466	1	1	1	1	**4**					
4469						1	1	1	1	**4**
4471						1	1	1	1	**4**
4665	1	1	1	1	**4**					
4669	1	1	1	1	**4**					
Total	**24**	**24**	**24**	**24**	**96**	**20**	**22**	**22**	**22**	**86**

Supplementary figures

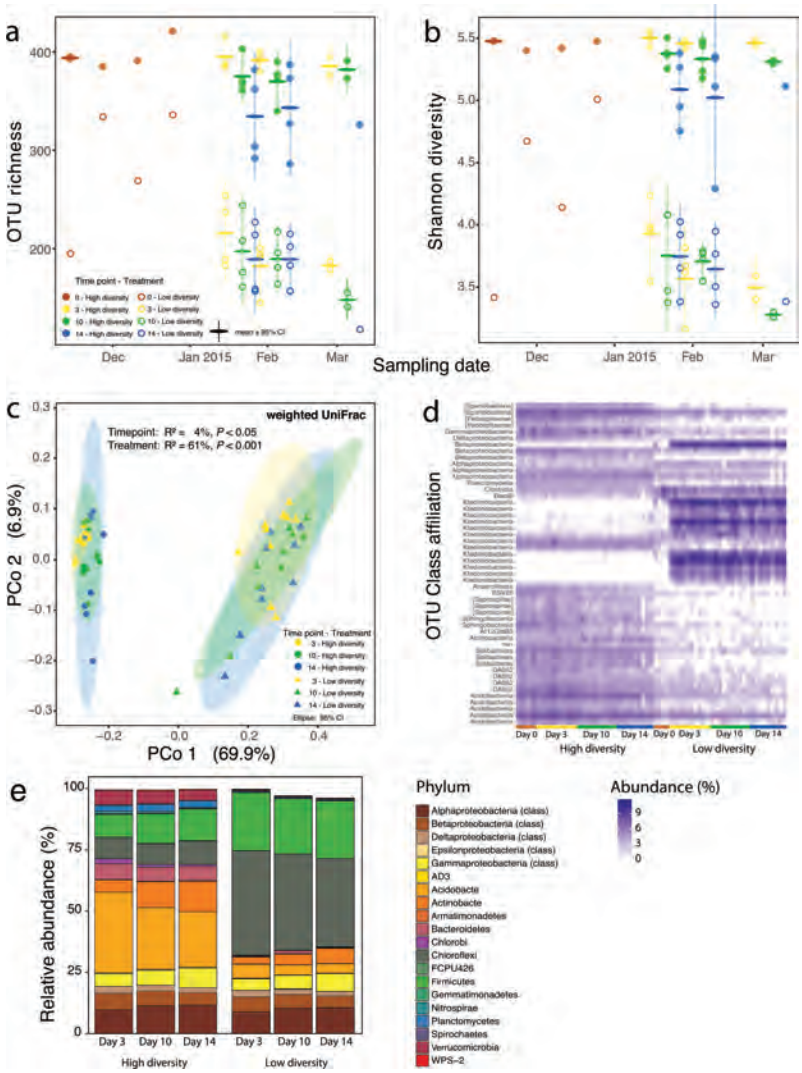

Figure S5.1: Experimental soil bacterial community characteristics. Relationship of a) OTU richness and b) Shannon diversity with sampling date during the experiment for each experimental soil treatment across time points between soil replacements. c) Principal coordinates analysis (PCoA) of weighted UniFrac distances of soil samples without 'day 0'. d) Heatmap of soil samples showing variance-stabilised abundances of 50 most differentially abundant OTUs. Day 0 samples have been taken immediately from the sterilised bags of soil. Plating Day 0 samples on TSA and LB media was negative. We concluded that Day 0 soil samples largely reflect relic DNA from dead bacterial cells after gamma irradiation. e) Bacterial community structure represented by mean relative abundances of major bacterial groups, stratified by time point for each of the two soil treatments. a) LMM OTU richness ANOVA; experimental treatment: $F_{1,54} = 551.2$, $P < 0.001$; time point: $F_{2,54} = 5.58$, $P < 0.01$; b) LMM Shannon diversity ANOVA; experimental treatment: $F_{1,54} = 600.7$, $P < 0.001$; time point: $F_{2,54} = 4.08$, $P < 0.05$. c) PerMANOVA: experimental treatment: Pseudo-$F_{1,54} = 96.7$, $P < 0.001$; time point: Pseudo-$F_{2,54} = 3.32$, $P < 0.05$.

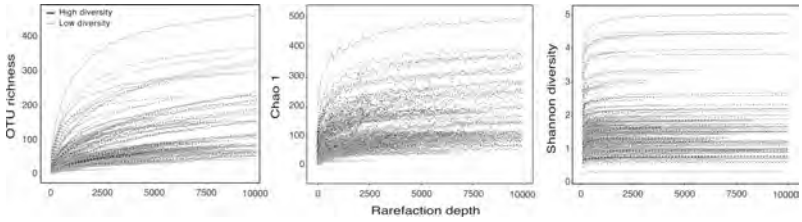

Figure S5.2: Rarefaction curves of cloacal microbiota samples. Rarefaction curves for four alpha diversity metrics. Alpha diversity was estimated as the average value of ten random sub-samplings at every depth. Shannon diversity saturated in all samples at ∼1200 reads per sample.

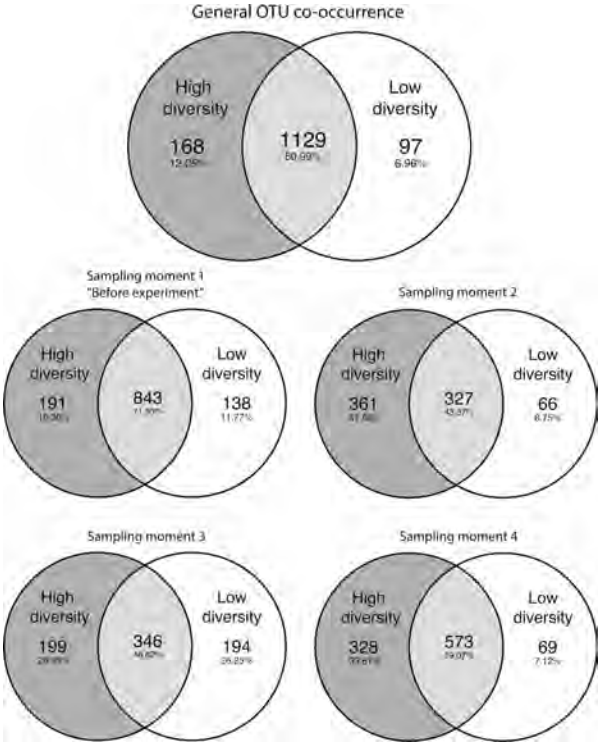

Figure S5.3: Uniqueness and co-occurrence between cloacal bacterial communities of birds on high and low diversity soils. Values (and percentages) in each compartment depict the (relative) number of unique or overlapping OTUs identified in the cloacal microbiomes of females acclimatising to either high diversity or low diversity soils. Small Venn diagrams depict separate analyses per sampling moment, showing that the average relative OTU co-occurrence between soil treatments decreased after birds were exposed to distinct microbial environments. Compartments are not scaled to the number of OTUs.

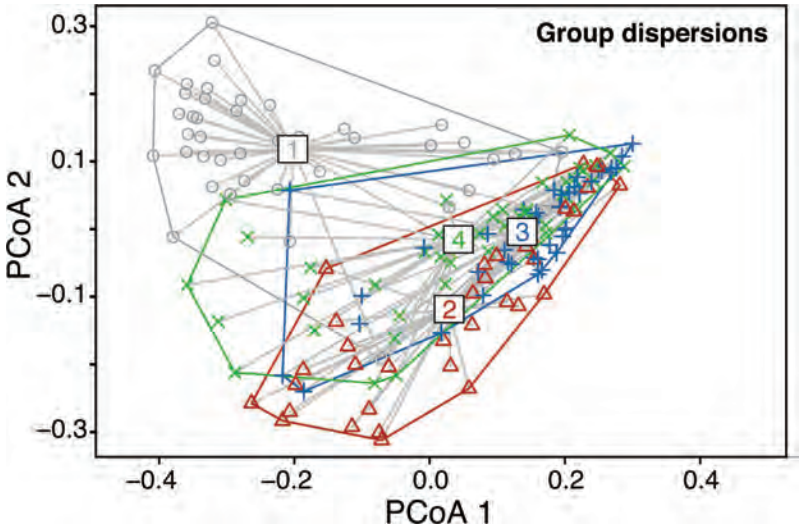

Figure S5.4: Group dispersions of PCoA clusters of cloacal microbiomes at different sampling moments during the experiment. Group dispersions are calculated as distances to the centroid for each sampling point. PerMANOVA statistics accompanying this Figure are reported in Table 3 in the main text.

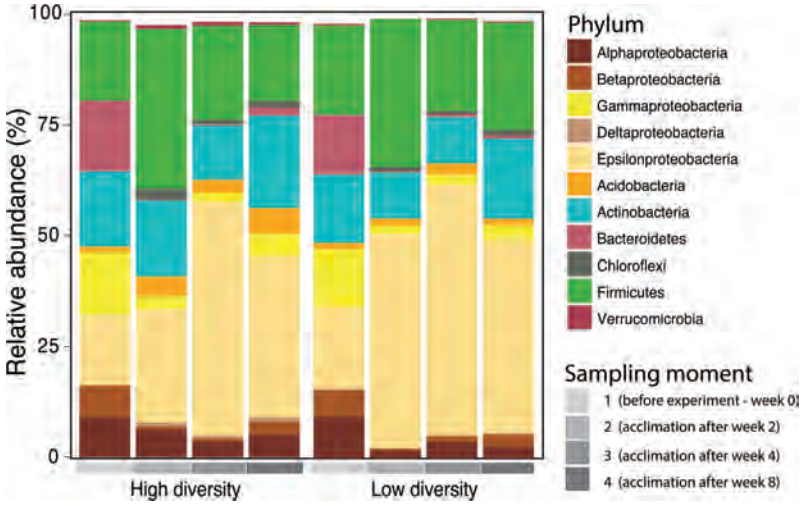

Figure S5.5: Relative taxon abundance depicting cloacal bacterial community structure. Relative read abundances as proxy for bacterial taxon abundances, for which the top seven dominant phyla have been shown. The dominant bacterial phylum Proteobacteria is divided into class-level taxa. Each stacked bar shows mean relative abundances for each sampling moment and for both experimental soil treatments separately.

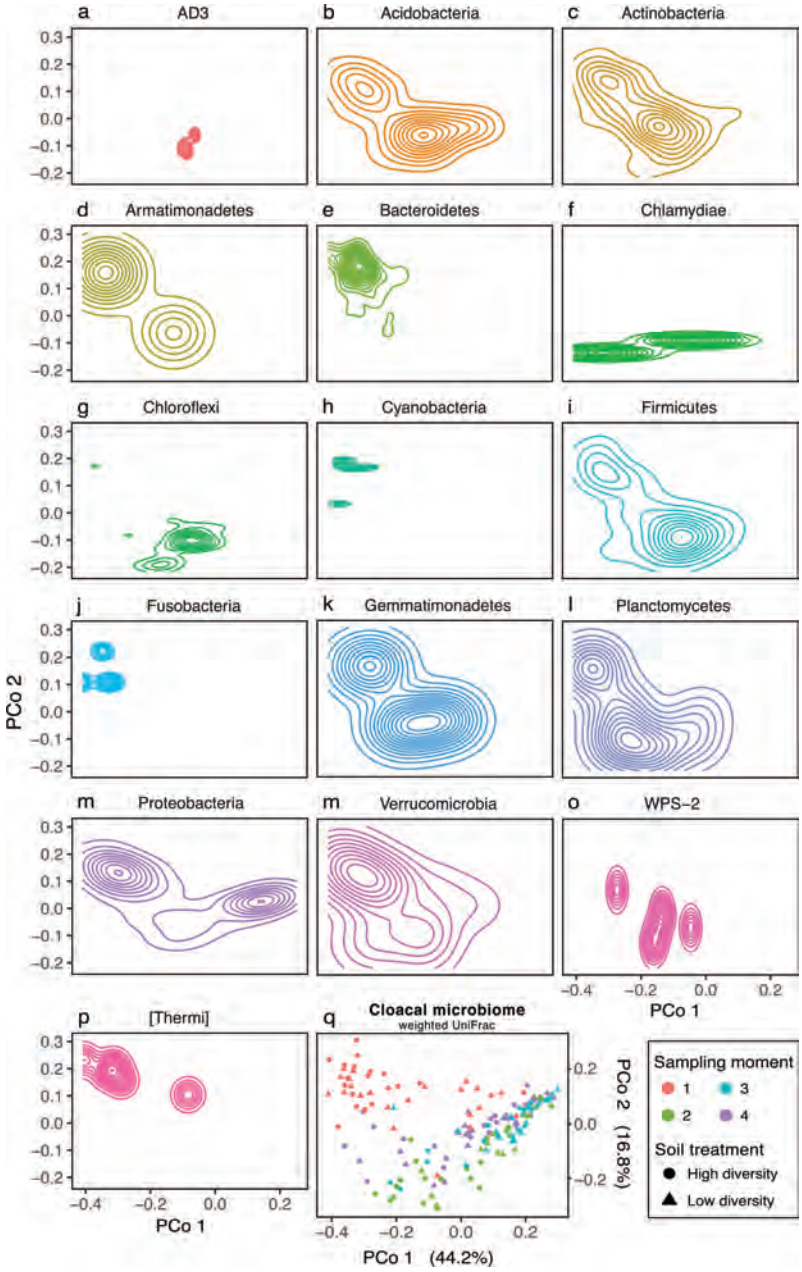

Figure S5.6: Ordination of bacterial phyla shows associations of bacterial phyla with sample ordination. Density plots of OTUs belonging to bacterial phyla (a-p) are plotted separate from the cloacal microbiome samples (Fig. 5.2 c in main text) in PCoA ordinations of weighted UniFrac distances. Phylum-specific patterns of bacterial OTU densities show associations with temporal shifts in bacterial community composition in the cloacae of female zebra finches.

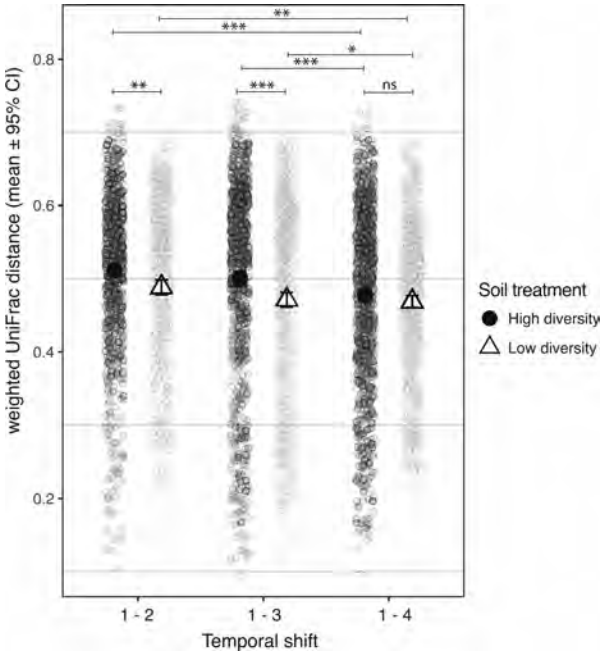

Figure S5.7: Pairwise weighted UniFrac distances between experimental and before-experiment states of cloacal bacterial communities. Cloacal samples of high diversity (circles) and low diversity (triangles) treatments during the experimental sampling moments (2, 3 and 4) are compared to the before-experiment samples (1). Cloacal samples in the low diversity soil treatment are significantly more similar (lower values) to the before-experiment samples during sampling moment 2 and 3, but not 4. However, all cloacal communities (on high and low diversity soils) tend to return back to the before-experiment state, as at sampling moment 4, cloacal communities are more similar to the before-experiment state compared with sampling moments 2 and 3. * FDR $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$, ns = not significant. Global test; treatment: $F_{1,5034} = 31.3$, $P < 0.001$; temporal shift: $F_{2,5034} = 19.3$, $P < 0.001$.

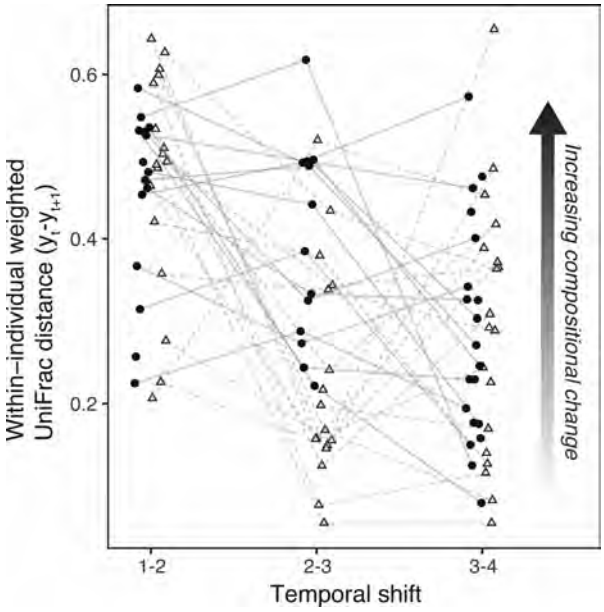

Figure S5.8: Longitudinal representation of within-individual shifts in phylogenetic community composition. Individual data points depict within-individual weighted UniFrac distances between consecutive sampling points (sampling interval). Data of females on high diversity soil are depicted with solid circles and females on low diversity soils by open triangles. See Table 5.5 in the main text for statistics.

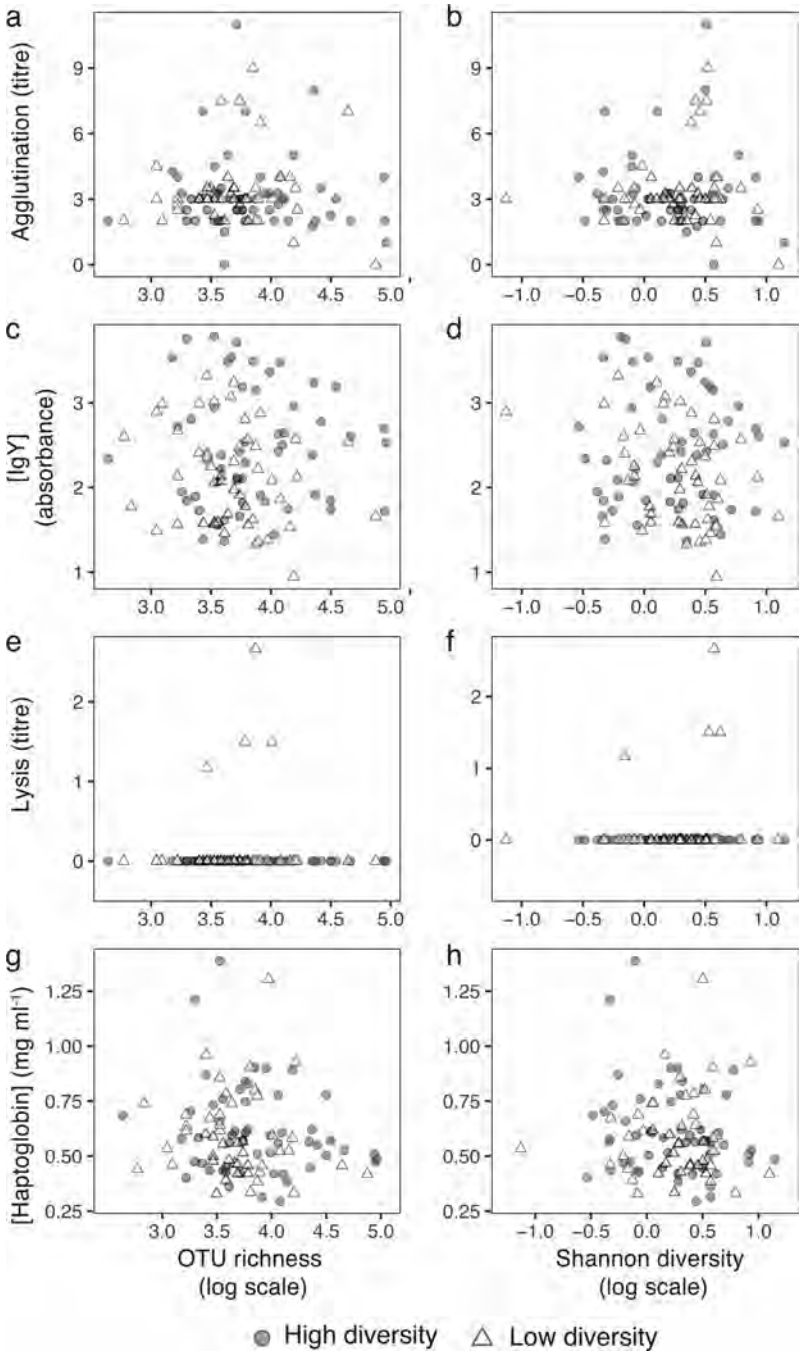

Figure S5.9: Associations between innate immune indices and alpha diversity of cloacal microbiomes. LMMs revealed no statistical support for experimental soil treatment, experimental duration or their interaction.

Chapter 6

The microbial environment modulates non-genetic maternal effects on egg immunity

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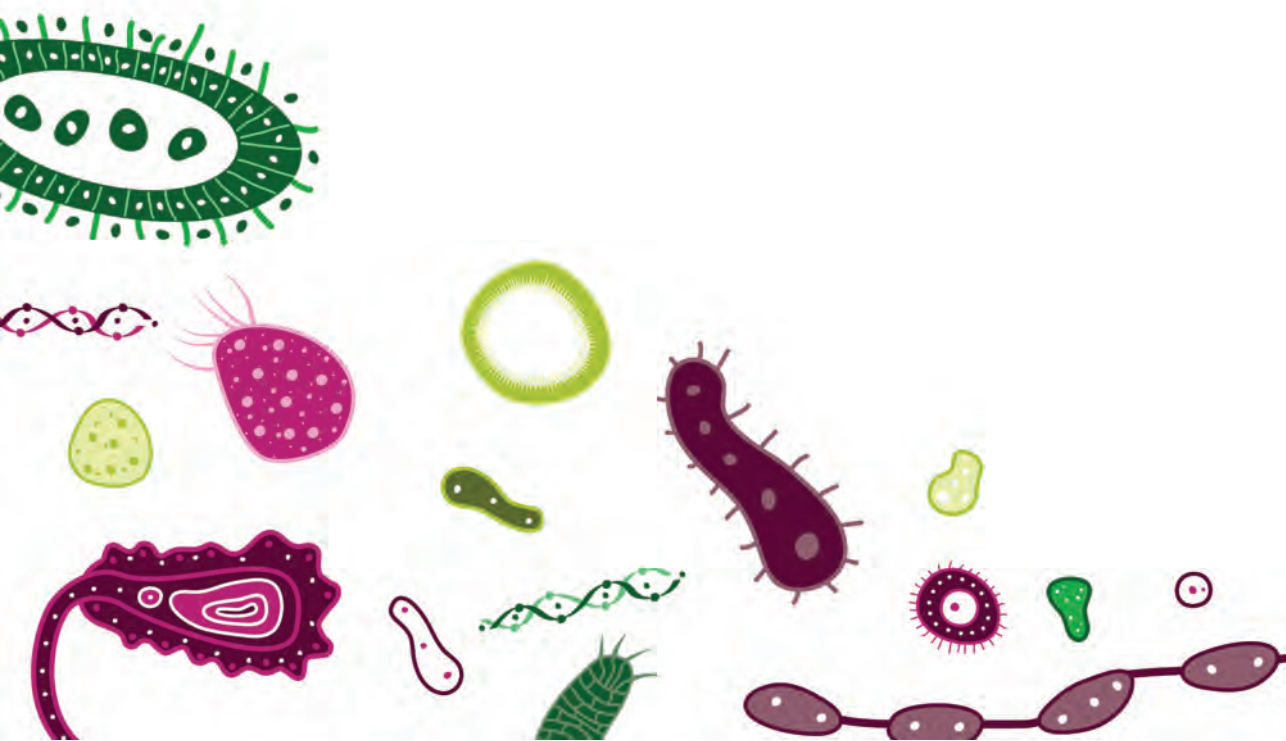
Submitted to *Journal of Animal Ecology*



Abstract

Immune function is essential to survival of animals in a world dominated by ubiquitous and diverse microorganisms. Immunological phenotypes of vertebrates vary widely among and within species and individuals, and encountering diverse microbial antigens may contribute to this variation. Maternal effects can improve offspring development and survival, but it remains unclear whether females use cues about their microbial environment to inform offspring immune function. To provide microbial environmental context to maternal effects, we asked if the bacterial diversity of the living environment of female zebra finches *Taeniopygia guttata* shapes maternal effects on egg immune function. By manipulating environmental bacterial diversity, we tested whether immunological investment in eggs is higher when environmental bacterial diversity is high (untreated soil) compared to low (gamma-sterilized soil). We quantified lysozyme and ovotransferrin in egg albumen and IgY levels in egg yolk and in female blood, and we used 16S rRNA gene sequencing to characterize maternal cloacal and eggshell microbiotas. We found a maternal effect on egg IgY concentration that reflected environmental microbial diversity: females who experienced high diversity deposited more IgY in their eggs, but only if maternal plasma IgY levels were relatively high. We found no effects on lysozyme and ovotransferrin concentrations in albumen. Moreover, we uncovered that variation in egg immune traits could be significantly attributed to differences among females: for IgY concentration in yolk repeatability $R = 0.80$; for lysozyme concentration in albumen $R = 0.27$. Furthermore, a partial least squares path model (PLS-PM) linking immune parameters of females and eggs, which included maternal and eggshell microbiota structures and female body condition, recapitulated the treatment-dependent yolk IgY response. The PLS-PM additionally suggested that the microbiota and physical condition of females contributed to shaping maternal effects on egg immune function, and that (non-specific) innate egg immunity was prioritized in the environment with low bacterial diversity. Since immunological priming of eggs benefits offspring, we highlight that non-genetic maternal effects on egg immune function based on microbial diversity cues from the parental environment may prove important for offspring to thrive in the microbial environment that they are expected to face.

Introduction

Immune function maturation depends on antigenic stimulation from the environment, which is a central process in shaping the immunological phenotype over the course of an individual's life (Klasing & Leshchinsky, 1999; Palacios *et al*., 2011; Stearns, 1989). The immunological phenotype of a female, accumulated during her life, can potentially drive a phenotypically plastic component of her investment into offspring. This maternal immunity investment provides direct protection and primes the development of early- and late-life immunological phenotypes of offspring (Grindstaff *et al*., 2006; Lemke & Lange, 1999; Moreno *et al*., 2008). Such environment-dependent maternal influences that causally affect development and survival of offspring are referred to as non-genetic maternal effects (Arnold, 1994; Boulinier & Staszewski, 2007; Mousseau & Fox, 1998; Wolf & Wade, 2009), and can be ecologically and evolutionarily significant (Bernardo, 1996; Moore *et al*., 2019; Mousseau & Fox, 1998). Microbial communities in an animal's surroundings are a ubiquitous and rich source of antigens, and could thus be environmental drivers of maternal priming of offspring immunity.

Consistent with the fact that antigens are stimulatory agents of an animals' immune system, we previously reported experimental evidence that bacterial diversity in the environment can shape immune function on short time scales (van Veelen *et al*., 2020). Whether these immunomodulatory effects of bacterial diversity cascade to immunological phenotypes of offspring through prenatal maternal effects has not been addressed. Thus far, immunological priming through maternal effects has been linked to other factors, such as resource limitation and postnatal parental care (Gasparini *et al*., 2007; reviewed in Grindstaff *et al*., 2003; Hasselquist & Nilsson, 2009), as well as epigenetic inheritance (Ho & Burggren, 2010). These factors have been identified by challenging females with one or more immunogens, followed by quantification of immune traits of offspring (Hasselquist & Nilsson, 2009), with particular focus on pathogens (e.g., Van Dijk *et al*., 2014). However, to investigate the influence of bacterial diversity more broadly requires a different approach, because animals typically encounter diverse bacterial communities that vary in composition through space and time.

Experiments that manipulate the microbial environment of animals are needed to fully understand causal mechanisms driving maternal immune investment. Such an approach would also incorporate numerous other (non-pathogenic) microorganisms that trigger

antibody production via a B-cell response (Evans *et al*., 2017; Fadlallah *et al*., 2019; Gensollen *et al*., 2016). Experimental evidence suggests that bacterial load (i.e. total bacterial abundance) has been linked to maternal immunological priming. For example, experimental reduction of bacterial load in nests lowered yolk carotenoid concentration in great tits Parus major and barn swallows Hirundo rustica (Jacob *et al*., 2015; Saino *et al*., 2003), and bacterial density on feathers predicted preen gland size and the composition of preen oil antimicrobials of great tits (Jacob *et al*., 2014). These findings suggested that environmental microbes can affect immunological priming, and alter immune function at short time scales, but did not implicate bacterial diversity. A basic understanding of whether environmental bacterial diversity affects immune investment requires explicit manipulation of bacterial diversity in an animal's environment, followed by quantification of (transgenerational) immune function (Evans *et al*., 2017; Horrocks *et al*., 2011a; Tieleman, 2018).

Prior work on adult zebra finches *Taeniopygia guttata* suggested that bacterial communities in the environment have immunomodulatory effects (van Veelen *et al*., 2020), but it remained unclear whether the degree of offspring priming associated with environmental bacterial diversity experienced by females. Here, we use the same study system to investigate if the diversity of the bacterial environment consequently shaped maternal immunological priming as non-genetic maternal effect. As the maternal microbiota can show signatures of the microbial environment (van Veelen *et al*., 2017, 2020), we hypothesized that the diversity and composition of environmental bacterial communities shape non-genetic maternal effects on offspring immune function. In this study, we experimentally created two levels of environmental bacterial diversity and investigated their effects on maternal immune investment. Our first objective was to test if biomarkers of innate and adaptive immunity of eggs (i.e., antimicrobial peptides in egg albumen and IgY in yolk) were affected. Our second objective was to investigate transmission of maternal IgY by linking IgY concentrations in blood plasma and egg yolk. Our third objective was to explore relationships among maternal and egg immune function and cloacal and eggshell microbiota, and whether such relationships differed between experimental microbial environments. We illustrated potential associations among the components in a conceptual model (Fig. 6.1). We used Partial Least Squares Path Modeling (PLS-PM) to explore the direct and indirect relationships among the immune biomarkers, cloacal microbiota, and body condition of the female, and immune

biomarkers and shell microbiota of eggs. We predicted that environmental bacterial diversity influences maternal immune investment in eggs, and we predicted positive relationships between maternal and egg immune function. Ultimately, we expected egg immunity to be contingent on the structure of the maternal microbiota (as a maternal effect) but not the eggshell microbiota (as a direct environmental microbial effect).

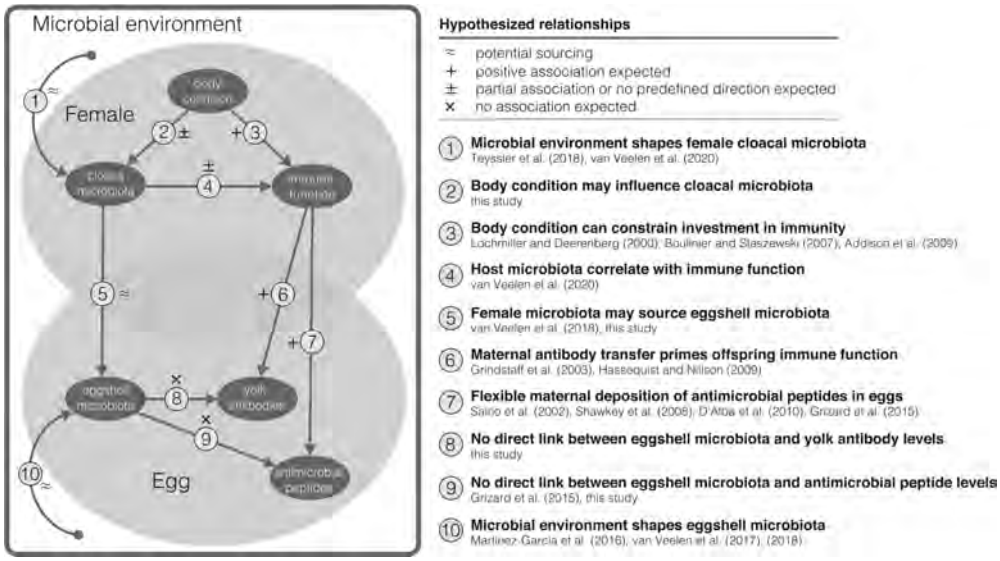


Figure 6.1: Conceptual model describing potential microbial environment effects on maternal immunological priming of avian eggs. (Addison *et al*., 2009; Boulinier & Staszewski, 2007; D'Alba *et al*., 2010; Grindstaff *et al*., 2003; Grizard *et al*., 2015; Hasselquist & Nilsson, 2009; Lochmiller & Deerenberg, 2000; Martínez-García *et al*., 2016; Saino *et al*., 2002; Shawkey *et al*., 2008; Teyssier *et al*., 2018; van Veelen *et al*., 2017, 2018, 2020)

Methods

Experimental design and sample collection

Experimental treatment

We divided commercially acquired soil in two batches and applied three cycles of 25 kGy gamma irradiation (Synergy Health Ede B.V, the Netherlands) to one batch, creating a highly reduced microbial environment, hereafter referred to as the 'low diversity' soil treatment (see Chapter 5, Fig. S5.1). We used the second soil batch as a high diversity microbial environment, hereafter referred to as the 'high diversity' soil treatment. We applied either high or low diversity soil as a ~2-cm deep bedding layer

in cages (50 × 50 × 40 cm) housing zebra finches *Taeniopygia guttata*. We maintained experimental microbial environments through biweekly cleaning of bedding trays, followed by sterilisation (70% ethanol) and replenishing with fresh low or high diversity soils (mean ± S.E.M.: 15 ± 1 days, n = 4). We maintained soil moisture content by spraying daily with ~30 ml autoclaved water per cage, which corresponded to the daily water loss (unpublished data). We analysed temporal patterns of soil bacterial community structure by sampling soil from cages three times between each replacement (at day 3, 10, 14). These analyses demonstrated that experimental soil diversity and composition remained stable over two-week periods (Chapter 5, Fig. S5.1) (van Veelen *et al*., 2020).

Species, housing and experimental time line

To experimentally test if females adjust investment in antimicrobial defenses of their eggs based on the microbial environment that they experience, we moved 53 adult female and 54 adult male zebra finches from single-sex outdoor aviaries to indoor cages. Birds were housed for eight weeks in single-sex pairs to prevent breeding but to allow physiological acclimation to experimental microbial environments (ambient temperature at 20 °C ± 1, relative humidity at 55% ± 15 and a 12:12 h light-dark (L:D) cycle). Birds were then randomly assigned to a treatment, to one of two replicate rooms, and to one of 12 single-sex cages (situated in a block of 3 × 4) per room. Up to three single-sex groups of surplus individuals were in the same rooms.

We fed birds with *ad libitum* gamma-irradiated (3 × 25 kGy) seed mixture and provided autoclave-sterilized water to limit potential dietary effects on the gut microbiota. The water was supplemented with multivitamin/amino acid solution (0.2 mm-pore filter-sterilized; final concentration 4 $g \cdot l^{-1}$, Omni-vit, Oropharma N.V., Belgium) to compensate potential vitamin degradation from seed irradiation. We thoroughly cleaned and sterilized (70% ethanol) water and food dispensers two times per week to reduce bacterial growth and its potential influence on the bird's microbiota.

After eight weeks of experimental conditions, we randomly paired males and females within each room, increased daytime (by 1 h per day to 16h:8h L:D), and supplied cages with sterilized (70% ethanol) plastic nest boxes (van Riel Distripet B.V., Waalwijk, the Netherlands) and autoclaved artificial nest material (Quiko GmbH, Bocholt, Germany) to stimulate breeding activity. Pairs with a single completed clutch were removed from the

experiment after 14 weeks, or shortly after a female completed two clutches within that period.

Sample collection

Birds

We measured body mass using a sterilized digital balance and collected a ~150 µl blood sample and a cloacal sample using a sterile cotton swab (Vacutest Kima, Arzegrande, Italy) from each female (n = 45) after the breeding period (i.e., after one or two complete clutches). We did not collect cloacal swabs during the egg laying phase in order to reduce disturbance and the risk of ceasing egg laying before a clutch was complete. We used new pairs of nitrile gloves upon entering every room when handling experimental equipment or soils, and we used new gloves to handle birds, which we sterilized with 70% ethanol between individuals. We collected cloacal swabs in sterile 2-ml screw-cap vials that were kept on ice. Samples were stored at -20 °C immediately after all birds had been sampled.

Eggs

We aseptically collected and stored eggs individually in sterile plastic bags (Whirl-Pak®, Nasco, Fort Atkinson, WI, USA), which were secured in sterile 50 ml tubes. We stored eggs immediately at -20 °C. All removed eggs were replaced with ethanol-sterilized plastic dummy eggs to encourage clutch completion and incubation. We marked the blunt end of first and second eggs of each clutch with a water-resistant marker to collect them when the first egg had been in the nest for seven days. The first two eggs of each clutch were left in the nest to be incubated and intended for a separate study, but we included 20 of these eggs without embryos (Table S6.1) to the analyses of egg immune defenses reported here. Subsequent eggs in the clutches (i.e., third to sixth egg of the laying sequence) were collected in the morning of the day they were laid. We collected a total of 262 eggs from first and second clutches (clutch size range: 3-6 eggs; see Table S6.1 for a detailed overview of the collected and analyzed eggs).

Laboratory analysis of immune function in egg albumen, yolk and female blood plasma

We dissected the eggs during the thawing process separating eggshells, albumen and yolk following Grizard *et al*. (2014). To remove residual albumen from the yolk sacks, we gently rolled thawing yolks on clean tissue before storing. We quantified lysozyme and ovotransferrin concentrations in egg albumen in duplicate following Horrocks *et al*. (2014) and Horrocks *et al*. (2011b), respectively, using 10 µl albumen per sample per analysis. We measured albumen pH using a digital pH meter (Jenco Instruments, San Diego, CA). We quantified IgY concentrations in egg yolk (25 mg yolk homogenized in 400 µl 0.1% milk solution) in duplicate using enzyme-linked immunosorbent assays (ELISAs) following Grindstaff *et al*. (2005) and Demas & Nelson (1996), using an adjusted protocol described in van Veelen *et al*. (2020). We quantified haemagglutination and haemolysis titers in blood plasma following Matson *et al*. (2005) and haptoglobin concentration in plasma following Matson *et al*. (2012). We reported averaged values of duplicate measurements for lysozyme, ovotransferrin, and IgY concentrations.

DNA extraction and 16S rRNA gene amplicon sequencing

We ground eggshells in liquid nitrogen using sterile mortar and pestle for DNA extraction following Grizard *et al*. (2014). We prepared cloacal swabs for DNA extraction by aseptically removing the stalk from the swab fibers and transferred the fibers per sample in extraction tubes. We then extracted DNA from $\sim$100 mg ground eggshell, 250 mg of homogenized composite soil samples, and from cloacal swab fibers using the MoBio PowerSoil DNA isolation kit (MoBio laboratories, Carlsbad, CA, USA) following the manufacturer's protocol with an additional step: 0.25 g of 0.1 mm zirconia beads was used in three 60 s cycles of bead beating (beads and Mini-bead beater, BioSpec Products, Bartlesville, OK, USA) to enhance mechanical cell disruption. The V4/V5 region of the 16S rRNA gene was amplified in triplicate using primers 515F and 926R at Argonne National Laboratory, IL, USA, according to the Earth Microbiome Project protocol (Gilbert *et al*., 2010). Amplification was followed by library preparation of pooled triplicates and 2×250bp paired-end sequencing using V2 chemistry on an Illumina MiSeq. The sequencing runs included 22 technical negative extraction controls to test for kit contamination (Salter *et al*., 2014). The negative controls covered every extraction kit

that was used and included blank extractions and extractions with sterile swabs with and without zirconia beads.

Sequence processing and assembly of amplicon sequence variants

In contrast to traditional 97% operational taxonomic unit (OTU) approaches, amplicon sequence variants (ASVs) lead to fewer false-positive taxon inferences while accurately illuminating cryptic diversity (Callahan *et al*., 2016). Hence, we quality-filtered and assembled sequences into error-corrected ASVs representing unique bacterial taxa using DADA2 (v 1.6.0) (Callahan *et al*., 2016). In total, we profiled 245 eggshell (excluding 20 eggshells with insufficient DNA content), 45 cloacal, and 69 soil bacterial communities, and assembled 9848 ASVs across these samples. We then assigned taxonomy to assembled ASVs using the Ribosomal Database Project (RDP) naïve Bayesian classifier implementation in DADA2 and the "RDP training set 16" and "RDP species assignment set 16" (Wang *et al*., 2007). As implementations in QIIME2 (Bolyen *et al*., 2019), we used MAFFT to align ASV sequences (Katoh & Standley, 2013) and FastTree2 to build a maximum-likelihood phylogenetic tree (Price *et al*., 2010). We then used *phyloseq* (McMurdie & Holmes, 2013) to remove ASVs assigned to Archaea, chloroplasts, or mitochondria and ASVs without a bacterial phylum assignment. Two out of 22 negative controls produced amplicons (NC1 and NC8) but with distinctly lower read numbers compared with samples after quality filtering. Because of the low read counts in only two negative controls, we did not remove any ASVs from the sample data set prior to subsequent analyses.

Data sets were filtered prior to data analysis. Based on substantial variation in the coverage distributions of each sample type, which included several low coverage samples, we selected the top 80% of the samples from eggshells (n = 198; new median coverage = 3101 reads per sample; range = 339-24815), the top 90% of the samples from cloacal swabs (n = 40; new median coverage = 4360; range = 726-78049) and 100% of samples from soil (n = 69; median = 7138; range = 717-21700). The remaining data comprised 7700 ASVs, which we used as input for beta-diversity analyses (McMurdie & Holmes, 2014; Weiss *et al*., 2017). Median sample coverage differed maximally 2.3 times between sample types (χ^2 = 39.9, df = 2, P < 0.001), which is acceptable (Weiss *et al*., 2017) for application of a variance-stabilizing transformation of the feature table using *DESeq2* (Love *et al*.,

2014; McMurdie & Holmes, 2014; Weiss *et al*., 2017) before calculating unweighted and weighted UniFrac as measures of phylogenetic beta-diversity (Lozupone *et al*., 2007).

Statistical analysis

All statistical analyses were performed in R statistical software (R Core Team, 2017). We used linear mixed models (LMMs) to test the effect of different microbial environments on egg immune indices (i.e., yolk IgY concentration (n = 154), albumen lysozyme (n = 139), and ovotransferrin concentrations (n = 119)). By including female identity as random effect, we statistically accounted for non-independence of eggs sampled from the same female when evaluating the effect of treatment. We tested treatment effect by modeling microbial environment as a fixed factor, clutch number as categorical confounding factor, egg sequence as ordinal covariate, and replicate room as additional random effect. Since albumen pH can influence lysozyme and ovotransferrin activity (Grizard *et al*., 2015), we included albumen pH as additional covariate in LMMs for these antimicrobial compounds. We performed a log-transformation of lysozyme concentration to meet the assumptions for residual normality and homoscedasticity. We performed ANOVAs using *lme4* and *lmerTest* (Bates *et al*., 2015; Kuznetsova *et al*., 2016), and then extracted model predictions using *effects* (Fox, 2003). To test if maternal investment consistently differed among females, we calculated within-female repeatabilities adjusted for fixed effects (R_{adj}) from the LMMs for each measure using *rptR* (Nakagawa & Schielzeth, 2013; Stoffel & Nakagawa, 2017). In addition, we summarized the variation of immune indices as a pairwise distance matrix among egg samples (referred to as 'immune index') using *vegan* (Oksanen *et al*., 2018) including those eggs that were fully analyzed for concentrations of lysozyme, ovotransferrin, and IgY (n = 115; Table S1). We then used distance-based redundancy analysis (db-RDA) to test the effect of microbial environment on multitrait egg immunity while constraining ordination by clutch size, egg number, and female identity using the capscale function.

Furthermore, since IgY levels could be compared directly between females and their eggs for each female-egg dyad, we analyzed this relationship to compare and interpret maternal immunological priming in the two experimental microbial environments. We first tested if female plasma IgY concentrations differed between environments. To test this we used a LMM with experimental treatment as fixed factor and modelled random intercepts for

female identity and replicate room. We then analysed the relationship between yolk IgY and plasma IgY concentrations using a similarly structured LMM with the additions of female plasma IgY concentration as fixed predictor of egg yolk IgY concentration and its interaction with treatment.

Partial Least Squares Path Modelling (PLS-PM)

We used Partial Least Squares Path Modelling (PLS-PM) to create a more holistic view of immune functions of females and eggs in the context of the microbial environment. PLS-PM is a statistical method that utilizes dimension reduction to allow analysis of a system of cause-effect relationships among blocks of (high dimensional) observational data (Sanchez, 2013). Our goal here was to refine existing hypotheses and potentially to generate new hypotheses about the complex system of interactions between microbial and immunological components of mothers and eggs in the nest environment. The unidirectional paths that we included in the path model reflect hypothesised causal relationships from the ecological immunology framework (Fig. 6.1). Because PLS-PM is primarily for generating hypotheses, not for testing them, the method does not impose formal restrictions on data distributions. The method is particularly suited to integrate data reduction with path modelling approaches to identify and quantify direct and indirect relationships among multivariate data sets (e.g. Barberán *et al*., 2014; Ossola *et al*., 2017). Hence, PLS-PM allowed for integration of maternal immune function and a body condition index (i.e., residual body mass after correcting for structural size using tarsus length), maternal cloacal microbiota (i.e., non-genetic maternal effects) and the eggshell microbiota (i.e., direct environmental effect) to explore if and how these components relate to egg immunity.

We selected empirical data as input for the path model (i.e., manifest variables) based on data completeness, pairwise collinearity among variables, and intrinsic structure of maternal and eggshell microbiota data. We simplified the microbiota data sets by selecting the largest clades identified by k-means clustering; maternal and eggshell microbiota each contained three distinct clusters. We used the clusters as formative indicators for latent variables representing the maternal and eggshell microbiotas. We excluded ovotransferrin concentration in albumen and lysis titer of blood plasma due to a lack of data and variance, respectively. We utilized data of 105 eggs (out of 198; 47 and 58 from high and low

diversity treatments, respectively) from 29 birds for which quantitative measures of all maternal and egg parameters were available. Maternal immune function was defined by two latent variables: one for natural antibody-induced agglutination titer as a measure of constitutive innate immunity, and one comprising both IgY concentration and haptoglobin level because of their collinearity, which we referred to as maternal 'immune index'. To ascertain that these indicators reflected the latent variable in the same direction, we inversed haptoglobin concentration. This adjustment enhanced the degree to which latent variables reflected the observed variables in the path model (Sanchez, 2013). Because haptoglobin concentration signals the degree of inflammation, inversed lower values indicated more inflammation, which were together with high IgY levels predicted to reflect bacterial diversity.

Under the assumption that the hypothesised causal relationships between variables (i.e., the 'structural model') are correct, it is possible to explore within the PLS-PM framework whether two experimental groups differ in the strength of particular associations between groups of variables. Hence, to assess whether maternal effects differed between experimental microbial environments, we compared the path coefficients (i.e., standardized partial regression coefficients) of the structural model between treatment groups using bootstrap resampling (n = 1000) and a t-test based on the bootstrap standard errors (Sanchez, 2013). Comparing between microbial environments, we interpreted significant differences (critical FDR-corrected $q < 0.1$) in the direction or strength of path coefficients between females and eggs as support for microbial environment-dependent maternal immune investment. Treatment-specific t-test results for the magnitude of path coefficients were extracted from the PLS-PM. Finally, we validated the robustness of path coefficients and coefficients of determination (R^2) for different variants of the structural model using 1000 bootstraps for estimating 95% confidence intervals. We used the R package *plspm* to construct the path models (Sanchez *et al*., 2017). Because of limited *a priori* understanding of causal links between microbiota and immune function, we remained cautious with inferring path coefficients as causal evidence and we avoided quantitative predictions. Instead, we limited the implications of PLS path model results to refine current hypotheses and for guiding new ideas about microbial environment-dependent maternal effects.

Results

No effect of environmental microbial diversity on egg immunity

Tests for overall experimental effects on the three egg immune parameters revealed no significant effect of experimental microbial environment on lysozyme and ovotransferrin concentrations in albumen and total IgY concentration in yolk of zebra finch eggs (Fig. 6.2 a-c; Table 6.1). Multivariate analysis (distance-based RDA) of egg immune defense traits, which simultaneously considered the variation of the three egg immunity measures, also did not reveal clustering of zebra finch eggs by experimental group (Fig. 6.2 d; Table 6.1). Log-transformed lysozyme concentration was 0.24 $mg \cdot ml^{-1}$ higher in second clutches compared to first clutches ($t = 2.34$, df = 125, $P = 0.03$; Table 6.1), and ovotransferrin decreased with 1.11 $mg \cdot ml^{-1}$ per egg along the laying sequence ($t = 4.46$, df = 116, $P < 0.001$). Absorbance of antigen-specific IgY in second clutches was 0.11 units (OD_{405}) lower than in first clutches ($t = 3.85$, df = 121, $P < 0.001$), but did not vary along the laying sequence ($t = 1.59$, df = 121, $P = 0.11$).

Consistent differences in egg immunity at the level of the female

In contrast to group-level experimental effects, among-female repeatability was significant for lysozyme concentration, IgY concentration and the multivariate immune index, but not for ovotransferrin concentration (Table 6.2; Fig. S6.1). These repeatabilities imply that immunological variation in eggs can be explained by consistently different transfer by females.

Maternal transfer of total antigen-specific antibodies to eggs is conditional on the microbial environment and maternal antibody levels

To discern environmental microbial effects and effects of females, we first assessed whether the maternal plasma IgY levels differed between experimental treatments after egg laying had been completed. Maternal IgY concentration was higher in the high diversity microbial environment compared with low diversity microbial environment ($F_{1,32} = 12.5$, $P = 0.001$; Fig. 6.3 a). We then analysed the relationship between maternal IgY concentration in blood plasma and in yolk of their eggs as a direct measure of maternal

immunological priming. Utilizing maternal IgY concentration to predict egg yolk IgY concentration showed a significant interaction between experimental treatment and maternal IgY concentration ($F_{1,31}$ = 4.96, P = 0.033; Fig. 6.3 b). Egg yolk IgY concentration was positively associated with maternal IgY concentration in birds that experienced the high diversity microbial environment, but not in birds that experienced the low diversity microbial environment.

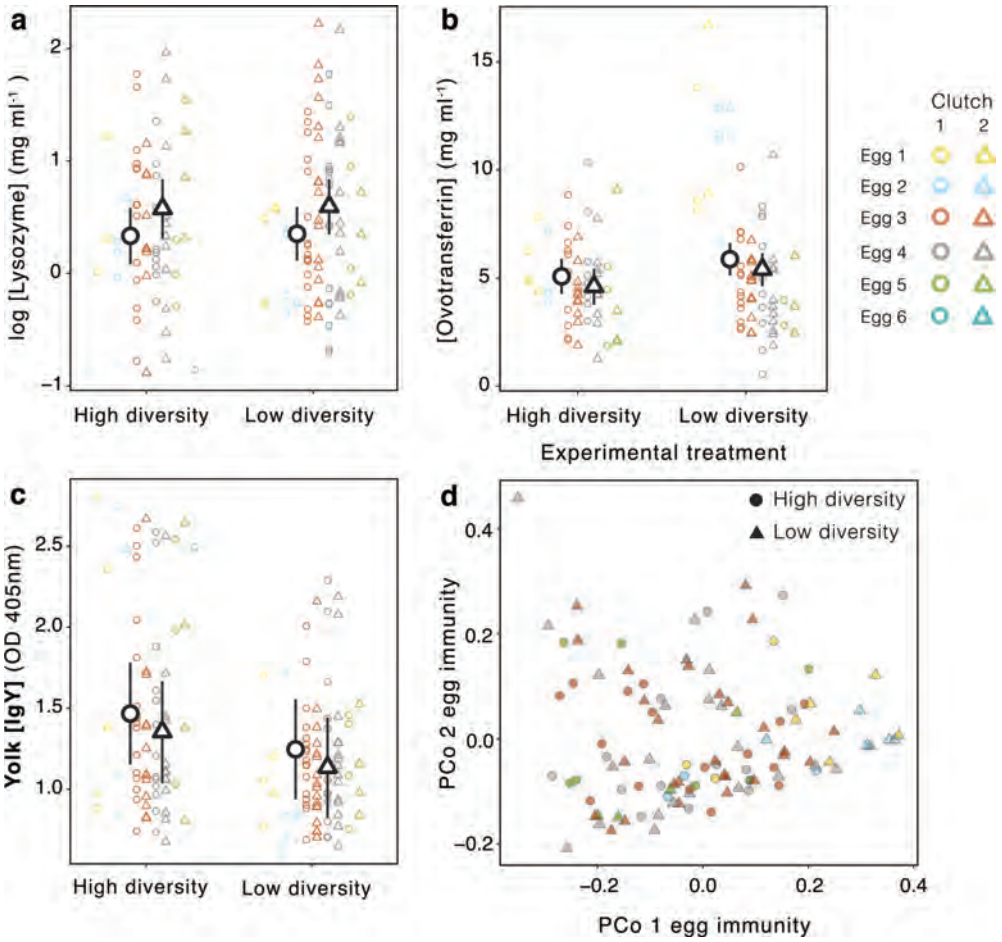


Figure 6.2: Experimental microbial environmental effects on egg immune function. a) Lysozyme concentration ($mg \cdot ml^{-1}$; log scale), b) Ovotransferrin concentration ($mg \cdot ml^{-1}$), c) IgY concentration in yolk (OD_{405nm}), and d) the first two principal coordinate axes of a multivariate immune index that represents the variation of the indices presented in a-c. Individual egg samples are presented by laying sequence (color) and stratified by clutch number (shape in a-c) or treatment (shape in d). None of the egg immune indices were significantly different between the two experimental microbial environments (Table 6.1).

Table 6.1: Analysis of variance of egg immune function indices.

Response	**Fixed**	**Df[a]**	***F***	***P***
Albumen lysozyme log-scale ($mg \cdot ml^{-1}$)	Experimental treatment	1, 33	0.01	0.908
	Clutch no.	1, 116	4.90	**0.029**
	Egg sequence	1, 128	0.14	0.709
	pH	1, 122	0.62	0.432
	Random	**Variance**		
	Female identity	0.130		
	Replicate room	0.000		
	Residual	0.357		
	Fixed	**Df[a]**	***F***	***P***
Albumen ovotransferrin ($mg \cdot ml^{-1}$)	Experimental treatment	1, 114	2.93	0.090
	Clutch no.	1, 114	1.08	0.301
	Egg sequence	1, 114	21.64	**< 0.001**
	pH	1, 122	0.24	0.627
	Random	**Variance**		
	Female identity	0.000		
	Replicate room	0.000		
	Residual	6.226		
	Fixed	**Df[a]**	***F***	***P***
Yolk IgY concentration (absorbance)	Experimental treatment	1, 1.88	0.98	0.433
	Clutch no.	1, 118	14.45	**< 0.001**
	Egg sequence	1, 119	2.62	0.108
	Random	**Variance**		
	Female identity	0.216		
	Replicate room	0.024		
	Residual	0.029		
Multivariate immune index (db-RDA)[b,c]		**Df[a]**	***F***	***P***
	Experimental treatment	1, 103	1.36	0.196
	Clutch no.	1, 103	1.37	0.195
	Egg sequence	1, 103	5.02	**< 0.001**

[a] Denominator degrees of freedom based on Satterthwaite approximation
[b] Distance-based Redundancy Analysis based on a Bray-Curtis dissimilarity matrix of three immune indices
[c] Marginal effects estimated with permutations stratified by female identity

Table 6.2: Adjusted repeatabilities of egg innate immune function for individual female zebra finches.

Immune index[a]		R_{adj}	SE	95% CI (lower, upper)[b]	P[c]
Albumen lysozyme		0.268	0.095	0.073, 0.442	**0.001**
log-scale (mg·ml^{-1})					
Albumen ovotransferrin (mg?ml^{-1})		0	0.052	0, 0.17	1.000
Yolk IgY concentration (absorbance)		0.804	0.113	0.503, 0.923	**0.001**
Multivariate immune index	High diversity PCo 1	0.214	0.2	0, 0.568	0.131
	High diversity PCo 2	0.406	0.18	0, 0.701	**0.011**
	Low diversity PCo 1	0	0.068	0, 0.236	1.000
	Low diversity PCo 2	0.277	0.141	0, 0.549	**0.010**

[a]Repeatabilities are estimated after adjustment for fixed effects: experimental treatment + clutch number + egg sequence (+ albumen pH in the cases of lysozyme and ovotransferrin) (Nakagawa & Schielzeth, 2013)
[b]confidence intervals are calculated from 1000 parametric bootstraps
[c]*P*-values calculated based on 1000 permutations

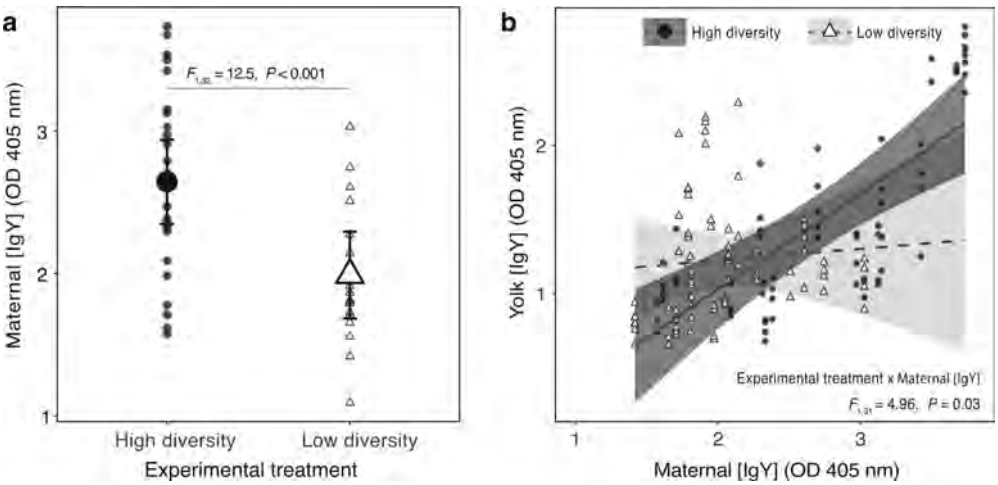


Figure 6.3: Effect of the microbial environment on the relationship between maternal and egg yolk IgY concentrations. a) Elevated maternal IgY concentration in a high diversity microbial environment. b) Egg IgY concentration increases with maternal IgY concentration only in the high diversity microbial environment. Lines depict linear mixed model predictions (± 95%CI).

The path model points out that maternal immunological priming of eggs may depend on the experienced microbial environment when females are in good body condition

We evaluated our conceptual ideas (see Fig. 6.1) on how maternal immunological priming may depend on the microbial diversity in the offspring's expected future environment using PLS-PM. The path model was indicative of strong and significant differences in maternal immunological priming of eggs between experimentally manipulated microbial environments that females (and their paired males) experienced in the eight weeks prior to nesting and egg laying (Fig. 6.4 c). We presented more detailed summaries of treatment-specific path coefficients and bootstrap t-test results for experimental differences in Table S6.2 and Table S6.3, respectively.

The maternal immune index which included maternal IgY and haptoglobin levels strongly and positively predicted the IgY concentration of egg yolk ($r = 0.79$, $P < 0.0001$; Fig. 6.4 a) of birds that experienced high diversity environmental microbial conditions, whereas it predicted an opposite association for birds that lived in the low diversity microbial environments ($r = -0.30$, $P = 0.032$; Fig. 6.4 b). Conversely, maternal innate immune function, measured as natural antibody-induced agglutination titer, negatively associated with the IgY conconcentraion in egg yolk in the high diversity environment ($r = -0.22$, $P = 0.015$; Fig. 6.4 a), whereas no association was predicted for the environment with low microbial diversity. The maternal immune index of females that experienced an environment with low microbial diversity positively predicted lysozyme concentration in egg albumen ($r = 0.30$, $P = 0.034$; Fig. 6.4 b). The lysozyme concentration was not different in eggs between experimental microbial environments. Hence, the associations between maternal immune index and egg yolk IgY concentration (Fig. 6.4 a), as well as the association between maternal agglutination titer and egg lysozyme concentration (Fig. 6.4 b), significantly differed between microbial environments (Fig. 6.4 c; Table S6.3). This suggests that females that experienced relatively high bacterial diversity in their environment invest in increasing adaptive immunity for their offspring rather than in non-specific innate defences, whereas under relatively low bacterial diversity the opposite is prioritized.

In our conceptual model, we included potential effects of general body condition (i.e. condition index defined as tarsus length-corrected mass) of females on maternal

effects. Our path model revealed that the maternal condition index negatively predicted agglutination titer in the low diversity (r = -0.50, P < 0.0001) but not in the high diversity microbial environment (r = 0.02, P = 0.92; Fig. 6.4 c). An opposite pattern was observed for the relationship of body condition with cloacal microbiome structure (Fig. 6.4 c), and no associations were found with the maternal immune index (Fig. 6.4 a, b). Because the phylogenetic composition of the maternal microbiota did not differ between experimental treatment groups (weighted UniFrac: pseudo-$F_{1, 39}$ = 0.03, P = 0.24; unweighted UniFrac: pseudo-$F_{1, 39}$ = 0.03, P = 0.39; Fig. S6.2) these results were based on the intrinsic structure in the cloacal microbiota (k-means clustering; k =3). The path coefficients did not differ between treatments (Fig. 6.4 c), likely because the variation among females within each treatment was considerable (Fig. S6.2).

Maternal cloacal microbiota structure additionally associated with the maternal immune index of females (high diversity: -0.30, P < 0.05; low diversity: -0.64, P < 0.0001; Fig. 6.4 a, b), but not differently between experimental microbial environments (Fig. 6.4 c), and it associated with maternal agglutination titer only in the environment with low microbial diversity (Fig. 6.4 b, c). These apparent associations between the maternal cloacal microbiota and the maternal immune index suggest within-individual processes linking the microbiota and immune function.

In addition, the structure of maternal and eggshell microbiotas were linked in both experimental microbial environments (Fig. 6.4 a, b), but statistical support for an effect of experimental treatment was lacking (bootstrap t = 0.52, df = 103, P = 0.302; Fig. 6.4 c). Eggshell microbiota predicted egg yolk IgY concentrations in both environments (Fig. 6.4 a, b). The maternal immune index and agglutination titer were not associated with pH of egg albumen (Fig. 6.4 a, b), whereas eggshell microbiota structure predicted albumen pH only in the high diversity environment (Fig. 6.4 a). Albumen pH did not predict lysozyme concentration, which was in contrast with our expectations.

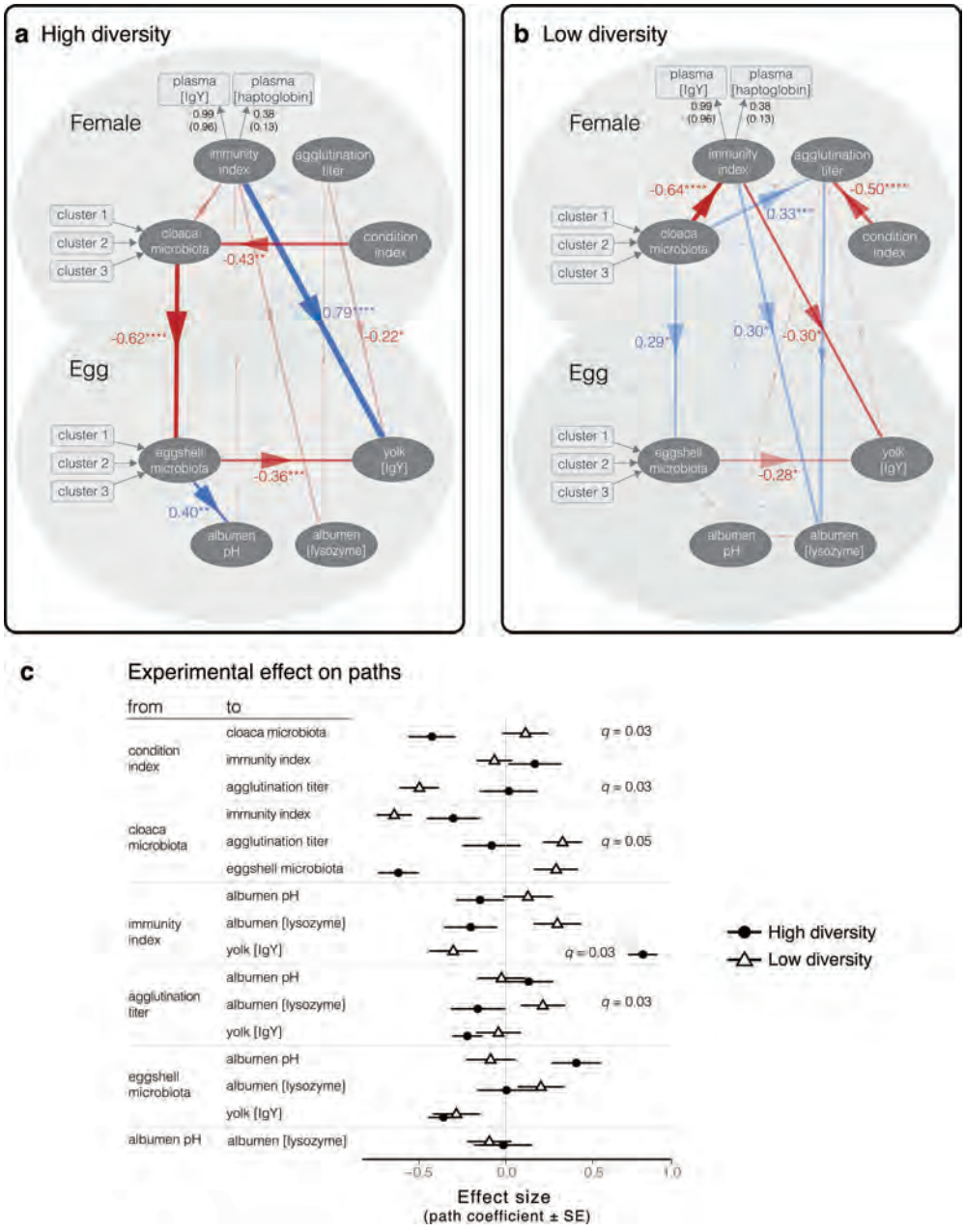


Figure 6.4: Partial Least Squares path model (PLS-PM) predictions link environmental and maternal microbiota to egg immune function. PLS-PM structural model representations (a, b) depict predicted path coefficients that were extracted from the global model for experimental microbial environments with a) High diversity and b) Low diversity. c) The experimental treatment effect on path coefficients was assessed with a bootstrap procedure and a t-test, where effects with FDR $q <$ 0.1 were considered significant. a, b) Dark grey ellipses depict (uni- or multivariate) latent variables and light grey rectangles represent manifest variables of either reflective or formative indicators of the latent variables. Colored arrows represent the path predictions (blue = positive; red = negative), line weight is proportional to the effect size (arrow labels); asterisks denote the probability that path coefficient is not zero: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** P <0.0001.

Discussion

We have previously shown that environmental microbiome can modulate immune responses in females (van Veelen *et al*., 2020). Immunological differences due to environmental bacterial diversity, and, independent of that, consistent differences in maternal immune traits and cloacal microbiota features brought up the possibility that maternal immunological priming of offspring may be similarly affected. Our results revealed that the microbial environment and female traits interactively determined maternal immunological priming of eggs. Variation in albumen lysozyme, albumen ovotransferrin and yolk IgY, biomarkers of egg immune function, could not be independently explained by the microbial diversity of experimental environments alone. Instead, consistent differences among females formed an important source of variation of these biomarkers. The relationships between levels of immunoglobulin Y (IgY) in maternal plasma and egg yolk depended on microbial environment: only in the high diversity microbial environment females transferred more IgY to eggs when their plasma IgY levels were relatively high. Path modeling subsequently provided a systems-level perspective that recapitulated this latter pattern, and suggested that maternal cloacal microbiota and body condition contribute to shaping maternal effects on egg immunity. It additionally suggested that the agglutination titre of female blood plasma and lysozyme in egg albumen, both non-specific innate defenses, were prioritized in the environment with low microbial diversity. Few associations between bacterial diversity and immunity have been studied so far. Hence, we anticipate that our results, and a more general perspective on linking pressure posed by microbes to immune function, encourage further investigation of the role of microbial diversity – and its different components – on vertebrate immunological development within and across generations.

Egg immune function

We found no independent effect of experimental microbial environment on levels of albumen lysozyme, albumen ovotransferrin and yolk IgY in eggs. Eggs varied markedly for all immune biomarkers and among-female repeatabilities for these biomarkers of egg immunity, up to 0.80 for egg yolk IgY, suggest that at least part of the immune variation among eggs could be attributed to differences among females. Since transfer of antibodies to egg yolk is associated to maternal plasma levels (Grindstaff *et al*., 2003),

and we previously found among-female repeatability of plasma IgY levels in these birds (van Veelen *et al*., 2020), our results comply with our expectation that among-female variation in IgY transfers to eggs. We found that a lesser degree of variation in lysozyme in albumen and IgY in yolk could be explained by clutch number, and of ovotransferrin in albumen by laying order. Effects of clutch number and laying order have been reported in other bird species, but their occurrence and directions can be species-specific and driven by other factors (e.g., D'Alba *et al*., 2010; Grizard *et al*., 2015; Hargitai *et al*., 2006; Svobodová *et al*., 2019). Differences in maternal transfer among females can arise due to both genetic and environmental factors (Boulinier & Staszewski, 2007; reviewed in Grindstaff *et al*., 2003; Okuliarova *et al*., 2014). We further discuss the environmental factors with a particular focus on the effects of the microbial component.

Maternal antibody transfer: interacting effects of microbial environment and female

Assessing maternal transmission of IgY, we found that eggs contained the highest IgY levels in the high microbial diversity environment, but only in eggs produced by females with relatively high plasma IgY levels. This result supports our hypothesis that maternal antibody transfer to yolk is microbial environment-dependent, which indicates that the microbial environment may reorder priorities for maternal resource trade-offs. That would also suggest that transfer of maternal antibodies is not simply passive, which contrasts with earlier ideas (Merrill & Grindstaff, 2014; Al-Natour *et al*., 2004). The consistent differences among females throughout the experiment raise the question which female traits influence maternal transfer. Body condition is a trait that in female King quails *Excalfactoria chinensis* has been shown to influence antibody transfer to eggs (Coakley *et al*., 2014). Factors implicated by other studies include energetic or nutritional budgets (Deerenberg *et al*., 1997; Gasparini *et al*., 2007; Hammouda *et al*., 2012; Ismail *et al*., 2015) and age (reviewed in Peters *et al*., 2019). We supplied *ad libitum* sterilized food in our experiment, which makes resource balance an unlikely explanation for our findings. Likewise, age is an unlikely explanation, because the zebra finches in this study constituted a single captive cohort aged between 1 and 2 years. Based on the role of the microbial environment reported here, we propose that pressure posed by environmental microbial communities may reframe priorities for maternal investment tradeoffs when transfer of immunity becomes more important for offspring fitness.

Path modeling: A systems-level perspective on maternal immune investment in eggs

We applied path modeling to explore maternal immune transfer in a systems-level perspective to identify unobserved relationships and indirect effects (see Fig. 1 for conceptual ideas), including associations among immune biomarkers, and data on cloacal microbiota and body condition as additional maternal traits. We caution that model results are based on the assumption that the structure of proposed relationships is correct. The model results suggested that relationships among maternal and egg immune parameters are microbial environment-specific. Particularly, the model fostered the hypothesis that adaptive immunity is prioritized by female zebra finches when they experience relatively high bacterial diversity, whereas innate defenses are prioritized under relatively low bacterial diversity. We propose that when microbial pressure is at least partly predictable, such as with annual or seasonal variations in environmental microbial communities (e.g. Cáliz *et al*., 2018; Shade *et al*., 2013) or with diet-associated microbial communities (e.g. Muegge *et al*., 2011; Youngblut *et al*., 2019), phenotypically plastic immune investment could be expected. Furthermore, we propose that this plastic response may act on overall investment in immunity, as well as on the balance between innate and adaptive defenses, both of which may subsequently translate into non-genetic maternal effects.

Moreover, the path model brought forward the hypothesis that the maternal cloacal microbiota and body condition may contribute to shaping maternal effects on immunity. Based on these outcomes, we suggest that balancing maternal investment in innate and adaptive immunity may depend on sequential effects of the experienced microbial environment through alteration of the maternal microbiota as a sensor for microbial pressure. Future challenges remain to discern relative contributions of these different factors on phenotypically plastic responses of females, and how they interact to shape maternal effects on immunity.

Conclusion and outlook: Non-genetic maternal effects on immune function in the context of microbial pressure

Our results constitute evidence of a direct link between bacterial diversity and female traits that interactively modulate egg immune function as maternal effects. These results

offer further prospects for manipulation of microbial pressure to unravel how microbial diversity shapes short term and life-long effects on health and survival through non-genetic maternal effects. Furthermore, microbial load likely also contributes to microbial pressure by influencing the probability with which antigenic stimulation is prompted (Leclaire *et al*., 2015; Soler *et al*., 2011). We postulate that microbial pressure effectively triggers immune systems as a function of microbial diversity and load, each of which may or may not independently influence investment in immunity and the tradeoffs between adaptive and innate defenses. We suggest that ecological immunology could greatly benefit from a framework to quantify relative influences of microbial diversity, load, and their predictability, and by integrating this knowledge to predict their relative importance for investment in immune defenses.

Ethics statement

This study was carried out obeying the Dutch Law on animal experimentation under licence DEC6314A of the Institutional Animal Care and Use Committee of the University of Groningen.

Acknowledgments

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Supplementary Information

Table S6.1: Overview of collected cloacal gut samples of zebra finches on high diversity and low diversity soils.

			High diversity soil treatment			
		n collected	**n analysed**			
			[lysozyme]	**[ovotransferrin]**	**total [IgY]**	**All measures**
Clutch 1	Egg 1	16	1	3	3	1
	Egg 2	17	4	3	3	3
	Egg 3	16	14	10	16	8
	Egg 4	13	10	8	13	6
	Egg 5	3	3	3	3	3
	Egg 6	1	1		1	
Clutch 2	Egg 1	13	2	2	2	2
	Egg 2	13	1	0	1	
	Egg 3	13	9	11	13	8
	Egg 4	13	11	9	13	8
	Egg 5	4	4	4	4	4
subtotal		122	60	53	72	43
			Low diversity soil treatment			
Clutch 1	Egg 1	18	3	3	3	3
	Egg 2	16	4	4	4	3
	Egg 3	17	17	14	17	16
	Egg 4	15	15	12	15	13
	Egg 5	4	4	2	4	3
	Egg 6					1
Clutch 2	Egg 1	16	2	2	2	2
	Egg 2	18	1	1	1	1
	Egg 3	17	17	14	17	13
	Egg 4	15	13	11	15	13
	Egg 5	4	3	3	4	4
subtotal		140	79	66	82	72
total		**262**	**139**	**119**	**154**	**115**

Table S6.2: Partial Least Squares path coefficients for associations between maternal and egg parameters per experimental microbial environment.

	High diversity				Low diversity			
cloacal microbiome								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.135	0.000	1.000	0.000	0.133	0.000	1.000
condition index	-0.425	0.135	-3.150	0.003	0.113	0.133	0.850	0.397
agglutination titer								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.150	0.000	1.000	0.000	0.111	0.000	1.000
condition index	0.017	0.166	0.099	0.921	-0.498	0.112	-4.440	0.000
cloacal microbiome	-0.082	0.166	-0.493	0.624	0.327	0.112	2.910	0.005
immunity index								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.138	0.000	1.000	0.000	0.102	0.000	1.000
condition index	0.167	0.152	1.100	0.278	-0.066	0.103	-0.641	0.524
cloacal microbiome	-0.302	0.152	-1.980	0.054	-0.643	0.103	-6.260	0.000
eggshell microbiome								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.117	0.000	1.000	0.000	0.128	0.000	1.000
cloacal microbiome	-0.619	0.117	-5.280	0.000	0.290	0.128	2.260	0.027
yolk [IgY]								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.083	0.000	1.000	0.000	0.128	0.000	1.000
agglutination titer	-0.222	0.087	-2.540	0.015	-0.043	0.130	-0.330	0.742
immunity index	0.789	0.085	9.260	0.000	-0.302	0.137	-2.200	0.032
eggshell microbiome	-0.359	0.086	-4.160	0.000	-0.285	0.136	-2.100	0.040
albumen pH								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.136	0.000	1.000	0.000	0.134	0.000	1.000
agglutination titer	0.131	0.142	0.920	0.361	-0.025	0.136	-0.180	0.855
immunity index	-0.148	0.139	-1.060	0.293	0.127	0.144	0.880	0.381
eggshell microbiome	0.405	0.141	2.880	0.006	-0.088	0.142	-0.620	0.539
albumen [lysozyme]								
	Estimate	**SE**	**t-value**	***P****	**Estimate**	**SE**	**t-value**	***P****
Intercept	0.000	0.149	0.000	1.000	0.000	0.126	0.000	1.000
agglutination titer	-0.161	0.157	-1.030	0.308	0.213	0.128	1.670	0.101
immunity index	-0.201	0.153	-1.310	0.196	0.297	0.136	2.180	0.034
eggshell microbiome	0.004	0.168	0.020	0.983	0.202	0.134	1.510	0.138
albumen pH	-0.014	0.166	-0.090	0.932	-0.095	0.128	-0.740	0.460

* bold values denote statistically significant associations (critical $P = 0.05$)

Table S6.3: Summary of bootstrap t-test for experimental effect on Partial Least Squares path coefficients.

Path from	To	Path coefficient global	Path coefficient high diversity	Path coefficient low diversity	Absolute difference high-low diversity	t	df	*P*	FDR *q*-value*
condition index	cloacal microbiome	0.091	-0.425	0.113	0.539	2.91	103	0.002	**0.030**
condition index	agglutination titer	-0.323	0.017	-0.498	0.514	2.47	103	0.008	**0.030**
condition index	immunity index	-0.006	0.167	-0.066	0.233	0.50	103	0.311	0.380
cloacal microbiome	agglutination titer	0.223	-0.082	0.327	0.408	2.13	103	0.018	0.057
cloacal microbiome	immunity index	0.283	-0.302	-0.643	0.340	0.65	103	0.257	0.374
cloacal microbiome	eggshell microbiome	0.121	-0.619	0.290	0.908	0.52	103	0.302	0.380
agglutination titer	yolk [IgY]	0.010	-0.222	-0.043	0.179	0.91	103	0.181	0.374
agglutination titer	albumen pH	0.054	0.131	-0.025	0.156	0.29	103	0.385	0.385
agglutination titer	albumen [lysozyme]	0.064	-0.161	0.213	0.374	2.54	103	0.006	**0.030**
immunity index	yolk [IgY]	0.702	0.789	-0.302	1.091	2.57	103	0.006	**0.030**
immunity index	albumen pH	-0.147	-0.148	0.127	0.275	0.81	103	0.211	0.374
immunity index	albumen [lysozyme]	-0.160	-0.202	0.297	0.498	1.04	103	0.149	0.374
eggshell microbiome	yolk [IgY]	0.015	-0.359	-0.285	0.074	0.88	103	0.191	0.374
eggshell microbiome	albumen pH	-0.254	0.405	-0.088	0.493	0.72	103	0.238	0.374
eggshell microbiome	albumen [lysozyme]	0.125	0.004	0.202	0.198	0.37	103	0.356	0.380
albumen pH	albumen [lysozyme]	-0.045	-0.014	-0.095	0.081	0.41	103	0.341	0.380

* bold values denote significant differences (critical FDR $q = 0.1$)

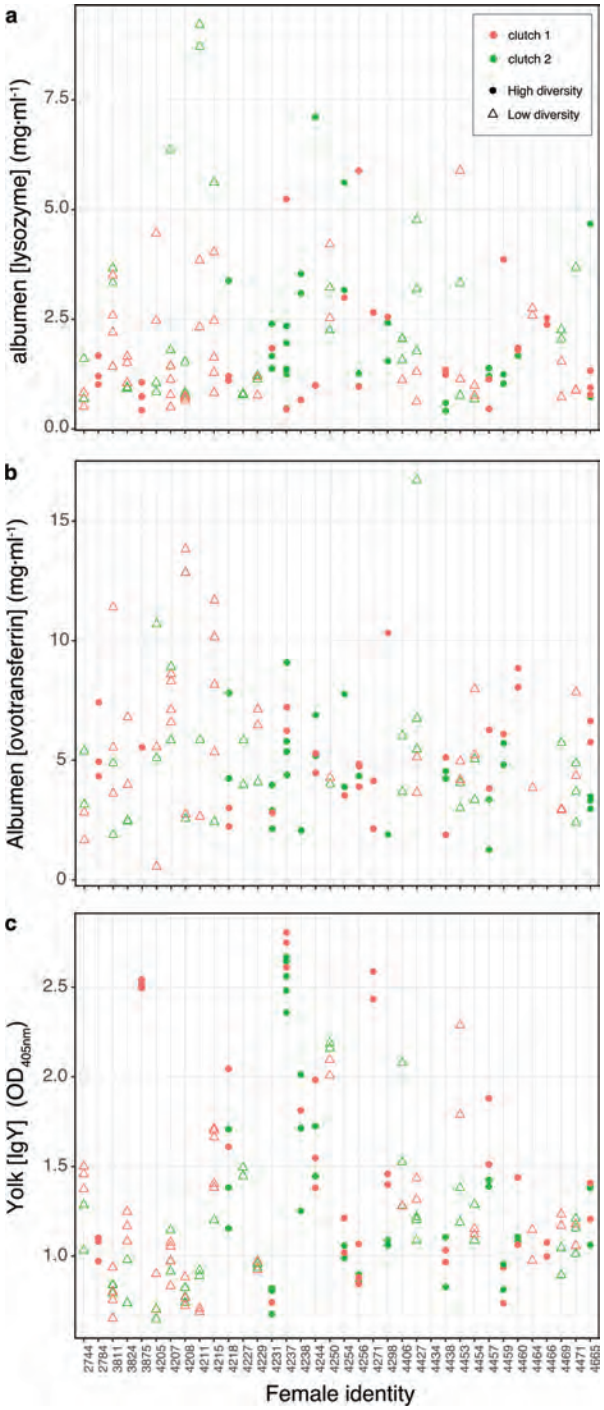


Figure S6.1: Repeatability of egg immune function parameters. See Table 6.2 in the main text for details.

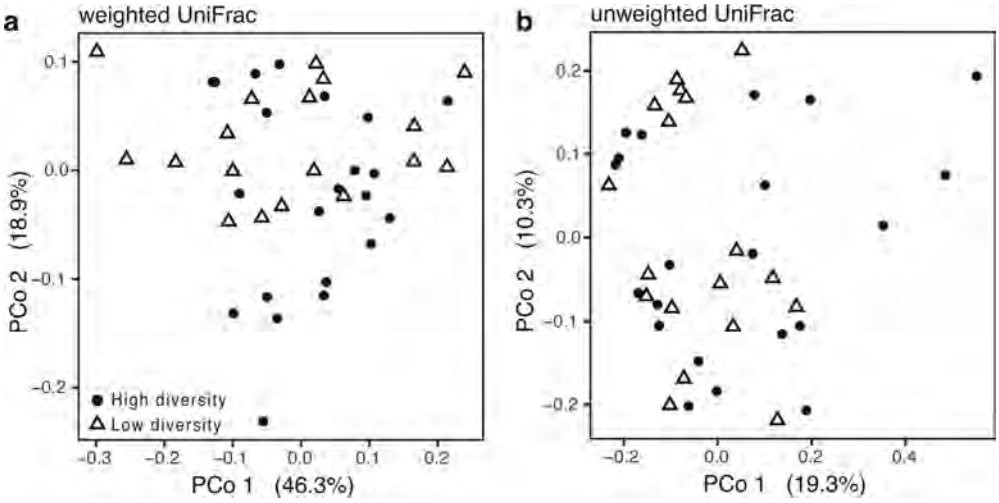
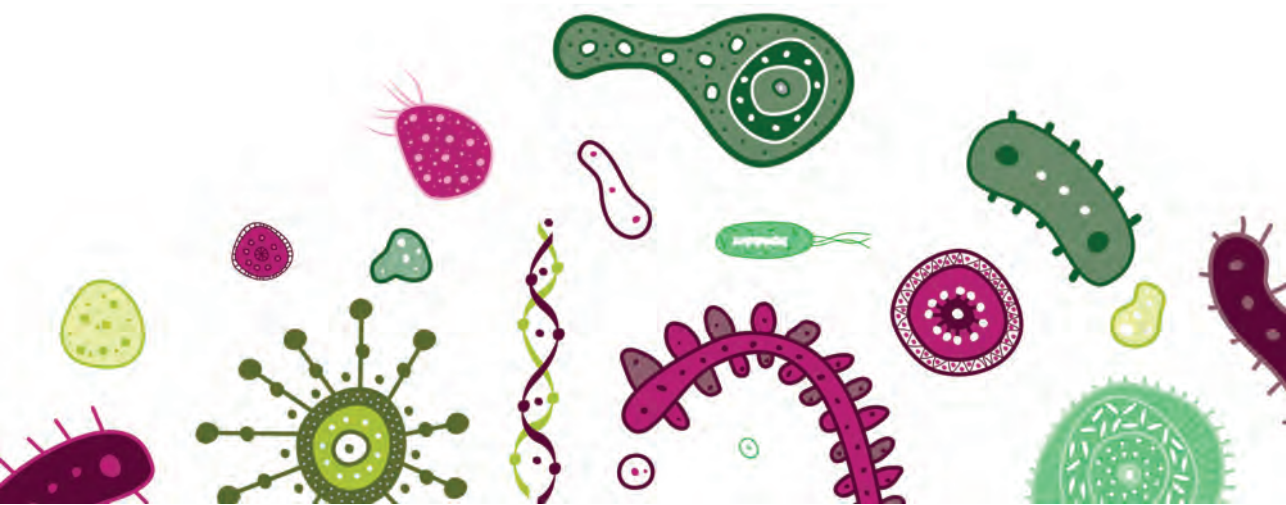


Figure S6.2: Phylogenetic composition of maternal microbiota in high and low diversity microbial environments. Post-laying cloacal microbiota compositions of female zebra finches as depicted by principal coordinates analysis. PerMANOVA analysis based on weighted (pseudo-$F_{1, 39} = 0.03$, $P = 0.24$) and unweighted UniFrac (pseudo-$F_{1, 39} = 0.03$, $P = 0.39$) indicated that cloacal microbiota compositions did not differ anymore between experimental microbial environments with high or low microbial diversities after egg laying, whereas the pre-laying period was characterized by different cloacal microbiota in high and low diversity environments treatments (van Veelen et al., 2020).

Chapter 7

The microbiota and immune function of birds in a microbial world: a synthesis

H. Pieter J. van Veelen



Overall conclusions and outline of this synthesis

The aim of this thesis was to explore ecological interactions between immune function of birds and microbial communities associated with birds and their environment. To address this topic that bridges the fields of animal ecology, ecological immunology and microbial ecology, together with my colleagues I have combined observational field studies with experimental research, and used immunological and molecular methodologies to answer the specific research questions.

This thesis demonstrates the substantial variation in the diversity and composition of bacterial communities that are associated with birds (hereafter 'bird microbiota') at different levels: within individuals, between sympatric individuals and species, and between species living in different climatic zones. These findings indicate that variation in bird microbiota is pervasive. This microbiota variation suggests that flexibility of immune systems may be particularly important to regulate animal-microbial interactions, maintaining a balance between protection against infection and facilitation of beneficial microbial functions. By experimentally manipulating microbial diversity in the environment of captive birds, my colleagues and I demonstrated that the microbial environment directly influences the composition of a bird's microbiota but also shows the bird microbiota's resistance to disturbance and its resilience thereafter. This dependency of the bird's microbiota on the microbial environment raises the question of whether the bird's microbiota is best considered a host trait or part of the host's environment. Because this experiment also affected a part of the bird's immune function, and the transmission of immunity to eggs by mothers, a host's microbial environment has possibly direct impact on life history trade-offs of animals.

In this synthesis chapter, I first present a summary of the preceding chapters. I then integrate the findings of Chapter 2, 3 and 4 to discuss the sources and processes that may govern the assembly of bird microbiota. Subsequently, I provide my perspective on how a bird's microbiota could be viewed from the host's perspective as a trait and from the microbial perspective as a bacterial community which is open to host and environmental influences, and I share my thoughts on how these views may be unified. I then synthesize the insights gained from the results in this thesis in the wider context of immunological variation in wild populations. The purpose is to provide my perspective on how influential environmental microbial pressure may be in shaping immune function

at different temporal and geographical scales, in which I speculate about the evolutionary and ecological processes that may be at play in shaping immune function.

Bird microbiota and immune function in the context of the microbial environment: a summary

In this microbial world, the vertebrate immune system has evolved to fight off threats posed by harmful microorganisms, and at the same time it enables the animal to take advantage of microbial communities that perform functions to its benefit (McFall-Ngai, 2007). In my view, a paradigm in ecological immunology that places not only emphasis on the costs of immunity, but places more weight on the host's benefits of animal-microbial interactions could improve our understanding of the eco-evolutionary dynamics of immune function. But before the evolutionary consequences of animal-microbial interactions to the host can be properly addressed, a thorough understanding should be gained of the microbial context in which animals function, as well as how animals deal with that microbial context. This entails understanding the influence of the microbial environment on animal-associated microbial communities.

In **Chapter 2** (van Veelen *et al*., 2017) my colleagues and I demonstrated how distinct bird-associated microbiotas relate to each other and to environmental microbiota. In a field setting, defining the environment of a bird is not straightforward. To enable useful comparisons of the microbial environment with a bird's microbiotas, we used a defined nest environment (i.e. nest material and soil close to the nest) and different microbiota (broodpatch skin, ventral body feathers and the cloaca) of female woodlarks *Lullula arborea* and skylarks *Alauda arvensis* that spent a substantial amount of time on the nest while incubating their eggs. We explored if the microbiota of the two larks differed, how different body sites compared to one another, and how each of these sites compared to the microbial environment of the nest. Our comparisons revealed that the two lark species did not differ, but that within individuals the microbiota of different body sites showed similarities and that individuals differed significantly. The birds' microbiotas showed similarities to the nest environment communities. Moreover, the phylogenetic composition of the microbiota indicated weak filtering of host microbiota at the body sites. While under less direct environmental influence, variation of cloacal microbiota composition was remarkably higher than skin and feather communities, and we suggest that individual

host traits influence microbiota assembly more strongly within the body than externally. Between-individual variation and direct influences of the microbial environment should be considered when causes of bird microbiota variation are addressed in future studies.

In **Chapter 3** we investigated how bird-associated microbiota of nine lark species (Alaudidae) differ among different habitats including temperate, desert and tropical bioregions. It remains unclear whether microbial diversity of host-associated microbes follows the biogeographical distributions of their hosts or the distribution of free-living microorganisms driven by environmental conditions (Delgado-Baquerizo *et al*., 2018; Groussin *et al*., 2020; Maestre *et al*., 2015). We showed strong geographic structure in cloacal microbiotas of nine lark species, where microbiota of desert larks had the lowest richness and were compositionally most distinct from larks of tropical and temperate grasslands. This pattern suggests that the biogeographical distribution of host-associated microbial communities co-varies with that of their hosts, implicating the potential functional association between host-associated microbes and host-adapted traits. While can covary, the mechanisms that shape this pattern of covariation between host traits and host-associated microbiota characteristics need further study. Horrocks *et al*. (2012) already noted that environmental microbes may affect host physiology, given correlated immune function and environmental bacterial density, feeding the debate on how animal microbiomes are assembled (i.e. horizontal or vertical acquisition). Results presented in **Chapter 2** and **Chapter 3** of this thesis highlight the potential impact of (microbial) environmental conditions on host-associated microbiomes, although data on the resistance and resilience of animal microbiota to extrinsic perturbation are scarce (see **Chapter 5**). However, host-associated microbiota may be more suitably considered part of a dynamic metacommunity that also comprises the environmental microbial communities surrounding them, which would place a host more firmly as a member of a community.

In **Chapter 4** (van Veelen *et al*., 2018) my colleagues and I explored the potential of eggshells as carriers of maternal microbial microbiota as a mechanism for vertical microbial transmission in birds. Eggshell microbial communities can directly impact host fitness by affecting embryo survival probability and egg hatchability (Cook *et al*., 2003, 2005a, 2005b; Martín-Vivaldi *et al*., 2014). Since eggshells harbour diverse microbial communities and parental incubation affects eggshell microbiome dynamics (Cook

et al., 2005a; Grizard *et al*., 2014, 2015; Peralta-Sánchez *et al*., 2012), we hypothesized that eggshells may have evolved as carriers assisting transgenerational transmission of maternal microbiomes in oviparous vertebrates. Using freshly-laid eggs of woodlarks and skylarks, we examined the similarities between maternal (cloacae, feathers and brood patch skin) and eggshell microbiota relative to similarities between egg and nest microbiota. Eggshell communities of freshly-laid eggs were mainly sourced by direct contact (horizontal uptake) with external communities (skin, feather and nest material) rather than maternal cloacal microbiota. Furthermore, some soil-derived bacteria thrived on freshly-laid eggs. These results indicate low potential for maternal (gut) microbiome transmission via eggshell communities. Future studies should address whether incubation may change this potential as the moment of hatching approaches.

In **Chapter 5** (van Veelen *et al*., 2020) my colleagues and I investigated whether immune function and cloacal microbiota are shaped by the microbial environment that birds experienced. We manipulated the diversity of the microbial environment of captive adult female zebra finches *Taeniopygia guttata* to test how the microbial environment affects aspects of innate and adaptive immunity. Simultaneously, we measured the altered cloacal microbiota characteristics and their temporal dynamics. Environmental microbial communities plastically shaped IgY levels, but not lytic activity, agglutination titer and haptoglobin concentration in blood plasma. Cloacal microbiota characteristics were more affected for birds exposed to an experimental microbial environment with high compared to low microbial diversity. Moreover, the cloacal microbiota of this group needed longer to recover after being introduced to the experimental conditions. These results indicate that metacommunity diversity affects host-microbiota dynamics. Among-individual differences in immune function and host-associated microbiota were consistent during eight weeks of experiment, and both aspects correlated at the individual level. We suggest that environmental microbial communities represent thus far poorly recognized drivers of variation in immune function in vertebrates. Microbial environment-dependent dynamics of cloacal microbiota further underscore the context-dependency of animal microbiota. Combined with established influences of diet (reviewed in Grond *et al*., 2017; Waite & Taylor, 2014, 2015) and sociality (Archie & Tung, 2015; Kreisinger *et al*., 2015; Raulo *et al*., 2018; Tung *et al*., 2015), we posit that horizontal acquisition of microbes from the environment is fundamental to microbiome assembly in birds, and that this idea likely extends to other (oviparous) vertebrates. The contribution of vertical transmission, either

strict heritability or non-genetic maternal effects, on microbiota assembly in vertebrates merits experimental investigation.

In **Chapter 6**, my colleagues and I utilized the experiment of **Chapter 5** to test if the microbial environment affects egg immune function by influencing maternal transfer of immunity to eggs. We contrasted patterns of lysozyme and ovotransferrin in egg albumen and IgY levels in egg yolk between microbial environments that differed in microbial diversity. Females in the environment with high microbial diversity deposited more IgY in their eggs, but only if the female's IgY levels were relatively high. Effects on lysozyme and ovotransferrin were not found. Furthermore, variation of egg immune traits could be partly explained by differences among females. Explorative path modelling suggested that the condition (residual mass), immune function and microbiota of females contributed to shaping maternal effects on egg immune function. Our results provide a systems-level perspective on how females may prime offspring for the microbial environment they will face later in life. We concluded that maternal immunological priming of eggs depends on both the female's microbial environment and the female, and that maternal immune transfer may be modulated by environmental microbial effects on the maternal microbiota.

Assembly of the bird's microbiota: environmental uptake of microorganisms may be the primary route for bird-associated microbial communities

The processes underlying assembly and maintenance of host-associated microbiota in and on a newly born or hatched non-human vertebrate body are scarcely studied in microbial community ecology (Burns *et al*., 2015; Costello *et al*., 2012; Stagaman *et al*., 2017). In a metacommunity framework (Leibold *et al*., 2004), four processes can describe how microbial community assembly takes place: dispersal, selection, speciation, and ecological drift (see Vellend, 2010 for a synthesis), and that framework can be applied to host-associated microbiota (Costello *et al*., 2012; Kohl, 2020). These processes probably occur simultaneously and in interaction with each other rather than in isolation. Based on this thesis, I propose that horizontal acquisition forms the basic mode of community assembly of bird microbiota through microbial dispersal from the environment, followed by deterministic processes such as ecological filtering by conditions of the niche (e.g., on

surfaces and in the cavities of an animal's body), selection by host filtering mechanisms (e.g. immune function), and microbial interactions (e.g. competition and facilitation).

Dispersal

In **Chapters 2**, **3**, and **5**, my colleagues and I report that the bird-associated microbiotas resemble the microbial communities that can be found in their environment. Comparisons in **Chapter 2** indicated that skin and feather microbiota of female larks resembled the microbiota of their nests. These patterns suggested that the external surfaces of birds horizontally acquire microorganisms from the microbial environment, implicating microbial dispersal as a process that contributes to their assembly. The cloacal microbiota, which inhabits a surface within the body, varied most strongly among individuals of these larks compared to external skin and feather microbiota. Differences in consumed food items may partly explain this variation, which could be interpreted as dispersal from the food-associated metacommunity, upon ingestion, to the cloacal microbiota. Another possible route is intake of soil-associated microbiota that may get ingested with food items during foraging on the ground. Similarly, horizontal acquisition from the environment is also suggested in **Chapter 3** to explain differences of cloacal microbiota compositions among nine lark species living in different bioregions. In **Chapter 3** we could not firmly conclude that environmental microbiota drove these differences, because a direct comparison of environmental and bird microbiota was not conducted. However, experimental evidence of the horizontal acquisition route is presented in **Chapter 5**, and indicated that the cloacal microbiota composition of zebra finches had changed solely as a result of a different experimental soil environment. In the absence of host selection, assembly of host microbiota solely through dispersal of microorganisms from the environment would cause the host microbiota composition to converge to that of the source environmental microbiota (Costello *et al*., 2012; Vellend, 2010). Across vertebrate groups, several lines of evidence support the contribution of environmental microbiota to skin microbiota in amphibians (Loudon *et al*., 2016), birds (**Chapter 2**), and mammals (Ross *et al*., 2018). However, in the case of the gut microbiota, filtering by host traits has been proposed to be a more important assembly process than dispersal alone (Mazel *et al*., 2018). Given the between-individual differences (**Chapter 2**) and within-individual consistency of cloacal microbiota composition (**Chapter 5**), this may also be true for cloacal microbiota.

Selection through host filtering

In two chapters of this thesis, evidence pointed to significant contributions of selection to the assembly of a bird's microbiotas. In **Chapter 2**, we show that the bird's different microbiotas remain distinct from their microbial environment despite their relative resemblance to it, and several bacteria were found uniquely on feathers and skin but not in cloacal samples, nest materials or soil. In **Chapter 5**, temporal changes of the cloacal microbiota composition of zebra finches in response to experimental microbial environments indicated that the cloacal microbiota was resilient to the biotic changes (environmental microorganisms) imposed by the experimental conditions. These patterns contrast with what would be expected if community assembly was neutral (Kembel, 2009), and thus could indicate that filtering by the host deterministically selects microorganisms to contribute to cloacal microbiota. It could also indicate that microbial interactions contribute to the cloacal microbiota's resilience. Across different body sites of the host, niches are expected to differ by how selective they are. Environmental filters (e.g., pH and salinity of skin; Bewick *et al*., 2019) could impose selective pressures that promote competition among microorganisms, and as a consequence determine the composition of the microbial community for that niche. More typical host-dependent filtering traits are described for several vertebrate groups, such as uropygial preen oil shaping feather microbiotas of different bird species (Javůrková *et al*., 2019; Martínez-García *et al*., 2015; Soler *et al*., 2016), and mucus composition shaping amphibian skin microbiotas (Walke *et al*., 2014). For other microbiota, such as differential skin microbiota among bats species (Avena *et al*., 2016) the mechanisms shaping the microbiota are not yet clear, whereas for vertebrate gut microbiota (Ross *et al*., 2018) the interplay of innate and adaptive immune function likely plays a significant role (Fadlallah *et al*., 2019; Magri *et al*., 2017; Thaiss *et al*., 2016). The variety of mechanisms by which assembly of host microbiotas occurs deterministically, and their relative contributions, may depend on host natural history (e.g. aquatic or terrestrial life style). Behavioural traits (e.g. diet, migratory tendency, sociality) could similarly determine the chance of encountering certain environmental microorganisms but not others. An illustrative example of ant-eating mammals highlights remarkable gut microbiota convergence (Delsuc *et al*., 2014) and underscores the importance of host ecology. I propose that host ecology and behaviour strongly govern the relative importance of different selective mechanisms in shaping host-associated microbiota. Hence, I suggest that to understand microbiota assembly of wild vertebrates

requires carefully selecting suitable hosts (species) as study subjects to reduce the impact of (a multitude of) confounding factors in explaining patterns of microbiome composition.

Speciation

The relevance of microbial speciation to the assembly of the microbiota within an individual bird is not clear and probably difficult to quantify. In particular, its impact relative to that of dispersal, selection and drift may be difficult to measure. In addition, it may be difficult to demonstrate microbial speciation within a bird, or other vertebrate, when horizontal acquisition from the environment prevails over vertical transmission. In **Chapter 3**, we demonstrate that closely-related lark species that inhabit vastly different bioregions (temperate and tropical grasslands and deserts) harbour cloacal microbiotas that cluster most strongly by the biogeographic location of the bird species, and their compositions are similar between species within habitats. A popular hypothesis to explain microbiota variation among species centers around the pattern of covariation between microbial community similarity and host phylogenetic relationships, and is referred to as "phylosymbiosis" (Brooks *et al*., 2016; Lim & Bordenstein, 2020). However, by which mechanisms phylosymbiosis emerges is an active topic of discussion (Kohl, 2020; Mazel *et al*., 2018) (discussed later). Reproductive isolation could shape a barrier for microbiota transfer (i.e., dispersal limitation), enabling co-speciation of host-associated microorganisms with hosts (Groussin *et al*., 2020), and could result in a phylosymbiosis pattern. As opposed to examples from insects and their endosymbionts, co-evolution between individual microbial species and hosts may be less likely to cause phylosymbiosis in vertebrates. While for co-evolution to occur, vertical transmission of microbial symbionts coupled with tight reciprocal obligatory association seems required, evidence from vertebrates is still lacking.

Ecological drift

The contribution of random fluctuations in microbial abundances (i.e., ecological drift) to the composition of host-associated microbiota may be limited. This may particularly hold true when deterministic processes such as selective pressures imposed by the host are involved in shaping a microbiota (Chase & Myers, 2011). The chance that ecological drift leads to differences in host-associated microbiota, may be most possible when

microbial member species follow independent evolutionary trajectories when living in allopatric hosts, leaving microbial members constrained of microbial exchange between hosts (Groussin *et al*., 2020). At the level of individual hosts, exposed external surfaces, such as feather plumage, probably experience most dispersal from the environment, as well as less selection by the host, possibly providing most room for ecological drift. A vast majority of bacteria shared between skin, feather, nest and soil communities in sympatric wild larks (**Chapter 2**) supported this hypothesis. However, unambiguous evidence for the influence of ecological drift on interspecific differences of microbiota composition may be particularly difficult to demonstrate when a metacommunity (e.g. the microbial environment) is shared by sympatric host species. Moreover, the outcome of stochastic species turnover that characterizes ecological drift may drive variation among hosts, but may not be easily distinguished from variation caused by biotic interactions with the host (e.g., through immune function). In addition, the outcome of microbial interactions within the host-associated microbiota, such as through competition, may similarly be challenging to discern.

As one of the most relevant and fundamental subjects in vertebrate microbiota ecology, the relative influences of dispersal, selection, speciation and drift to the process of host-associated microbiota assembly should to be better understood. I my view, expectations about the influence of each of these processes can only be made specifically for the ecological level (e.g. body site, individual, population, species) that these processes operate. Consider the influence of microbial dispersal from environmental microbial communities on assembly of feather microbiota, as an example. Dispersal could appear to strongly contribute to feather microbiota composition at the individual level when a bird's feather microbiota is compared with the microbial community of its nest. However, in a cross-sectional comparison of feather and nest microbiotas collected from a population of birds during a breeding season, weaker of no associations may be observed due to between-individual differences in feather microbiotas and microhabitat differences that affect nest microbial communities. Similarly, the influence of these processes to microbiota variation within individuals over time may fundamentally differ from how they influence average variation in a populations or species, posing a challenge to distinguish the processes at play. Common garden experiments involving wild birds from allopatric populations of a single host species can be a promising model to study the relative role of horizontal acquisition (i.e. dispersal from environmental microorganisms) and evolved

differential host filtering mechanisms that recapitulate intrinsic differences among hosts. Soil manipulation, comparable to **Chapter 5** and **Chapter 6**, for ground-dwelling birds (e.g., the horned lark complex Eremophila spp.) with a widespread distribution and disjunct populations could be an interesting experimental design. Moreover, the lark family (Alaudidae) could be of further interest to estimate the contribution of host phylogeny to interspecific microbiota variation, by contrasting the results of the proposed experiment with a second but similar experiment using multiple allopatric species. A third experiment using sympatric species could further provide insights into whether host local adaptation converges microbiota assembly outcomes, and by contrasting outcomes with patterns from allopatric species, it may experimentally shed light on effects of isolation by distance (dispersal limitation) on host microbiota assembly. Parallel investigation of the functional microbiome will then indicate how important (potential convergence of) microbiota composition is to the functional capacity of the microbiota to the host.

Should the bird's microbiota be viewed as component of a holobiont, as a host trait, or as an environmental variable?

The bird and its microbiota as a holobiont

One of the most fundamental discussions in animal microbiome research is whether the host-associated microbiota (and the functional microbiome) should be categorized as a host trait, as an extrinsic environmental variable that influences the host, or, jointly with the host animal's soma as a holobiont (e.g., Bordenstein & Theis, 2015; Zilber-Rosenberg & Rosenberg, 2008). The level at which natural selection acts seems of crucial importance to this discussion (Douglas & Werren, 2016; Foster *et al*., 2017; Moran & Sloan, 2015; Zapién-Campos *et al*., 2020). In fact, natural selection based on variation in other host traits can have different consequences for the host and for its microbiota. However, natural selection could act on community-level functional microbiome as well as on individual members of the microbiota, or simultaneously on all levels. Moving away from the common paradigm in evolutionary biology that natural selection acts primarily on individuals, and instead by considering multi-level selection, one could alleviate the desire of placing the host-associated microbiota in a particular category (Shuster *et al*., 2006; Whitham *et al*., 2020). In the following sections, I synthesize the observations that my colleagues and I made throughout this thesis in the context of the host-versus-environment discussion.

With this synthesis, I illustrate that the patterns can support predictions from both the host perspective and from the environmental perspective.

The microbiota as a host trait

Our understanding of how animals adapt to changing environments through genetic and epigenetic change continues to progress, but how adaptation is facilitated through the composition or function of host-associated microbiota is only starting to be unraveled (e.g., Suzuki & Ley, 2020). Classifying the host-associated microbiota as a host trait is complicated by the transient occurrence of many of its microbial members, which makes it particularly difficult to draw a conceptual line between the host and its environment. By altering the composition of host-associated microbiota in unpredictable ways, stochastic processes such as ecological drift and microbial dispersal demonstrate that host-associated microbiotas are part of a microbial environment as much as part of the host. Conversely, one could think of the degree by which hosts selectively filter interacting microorganisms as a factor that mediates that distinction. I advocate here against a universal categorization of host-associated microbiota as a host trait, because the degree of host filtering may vary widely between no filtering and very strong filtering within and among host taxa, and depending on the type of microbiota considered.

In birds, my colleagues and I observed among-individual differences of host-associated feather, skin and cloacal microbiotas in wild sympatric larks (**Chapter 2**; van Veelen *et al*., 2017) and in the cloacal microbiota of captive zebra finches (**Chapter 5**; van Veelen *et al*., 2020). Their general composition was consistent with other birds (Grond *et al*., 2018; Waite & Taylor, 2015) but individuality in microbiota composition was observed as well. Microbiota individuality in other vertebrate species is suggested to result from host genetic control (i.e. filtering), and this individuality in microbiota composition has been coined as a polygenic host trait (Benson *et al*., 2010; Org *et al*., 2015). The adaptive immune system was proposed to have evolved in order to regulate the commensal microbial community in the gut, especially to protect the microbiota's essential functions to the host (Lemke *et al*., 2004; McFall-Ngai, 2007). In support of this hypothesis, we observed that immunoglobulin Y concentration in female zebra finches, an aspect of adaptive immunity, was linked to cloacal microbiota composition (**Chapter 5**; van Veelen *et al*., 2020).

Further experimental studies and comparative analysis of paired immunological and microbiota data are required to identify how, and quantify the extent to which, adaptive immune function is shaped by microbial communities at ecological and evolutionary time scales, respectively. Yet, the composition of the microbiota can vary among individuals while providing similar community-level functions (Human Microbiome Project Consortium *et al*., 2012). Therefore, microbiota-manipulation studies are needed to assess whether hosts should select for particular microbiota compositions, or rather for maintaining particular community-level functional features independent of microbiota composition. In conclusion, redirecting future focus on microbiota functioning may become crucial to the field of ecological immunology, as compositional variation of host-associated microbiota may exist among individual hosts, populations, and species. The microbiota's functional composition (microbiome) may be particularly related to host ecology and physiology, and variation in microbiome rather than microbiota may have the largest fitness consequences for hosts.

The microbiota as an environmental variable

From the host perspective, viewing host-associated microbiota solely as an environmental variable that influences host functioning and fitness tends to neglect the extensive interactions between host and microbiota members. My colleagues and I advocate for embracing the versatile composition of host-associated microbiotas as the dynamic outcome of host-microbial interactions. Host-associated microbiotas thus should not be solely viewed as an extrinsic environmental variable, but rather as an ecosystem of which the host is one part (Christian *et al*., 2015). As outlined in the preceding section of this synthesis, the deterministic process of selection through host filtering can have a substantial influence on host-associated microbiota assembly, but depends on which microbiota is considered (e.g., skin, feather, cloacal). A role of the host's microbial environment in shaping host-associated microbiota is supported by similarities between different host-associated microbiotas and microbial communities of the nest environment of sympatric larks (**Chapter 2**; van Veelen *et al*., 2017). Differences of cloacal microbiotas among lark species that live far apart suggest that environments with vastly different climatic conditions may contribute to these interspecific microbiota differences (**Chapter 3**). Host-associated microbiome data paired with environmental microbial community data at large geographical scale are required to firmly test this hypothesis (Groussin *et al*.,

2020). In a single host species, we showed experimentally that the microbial environment influenced their cloacal microbiota composition of zebra finches, and changes in the cloacal microbiota composition during the experiment indicated that the microbiota was in part resilient to the initial change, which supports the impact of host control (**Chapter 5**), but how host control functions remains to be studied.

From a microbial ecology perspective, host filtering in vertebrates may best be considered as a distinct case of selection compared to how niche-based selection modulates free-living microbial communities (Costello *et al*., 2012; Leibold *et al*., 2004). The mechanisms underlying host selective filtering may consist of a complex interplay of specific and nonspecific immune components acting together to modulate host-associated microbiota (Suzuki & Ley, 2020). This complexity contrasts classical 'univariate' selective pressures (e.g. pH, salinity, temperature) of which the consequences on microbiota compositions are less multi-faceted. Changes in immune system configuration are expected to have far less predictable effects on microbiota composition or function (Foster *et al*., 2017). Adding to the complexity, the gut microbiota is further shaped by other indirect factors that depend on the host and on microbial interactions. For example, a host's dietary choices influence host-associated microbiota by determining the nutritional substrate niche for gut microbes, and by simultaneously harboring diet-specific microbial communities that become at least temporarily part of the host-associated microbiota. In our experimental studies (**Chapter 5**; van Veelen *et al*., 2020, and **Chapter 6**), we tried to eliminate confounding effects of diet by providing only sterilized water and food to all birds. Research on influences of environmental microbial communities on host-associated microbiota is still in its infancy. For animal ecologists, significant progress may depend on enhancing our understanding of the importance to microbiota functioning to host fitness. Therefore, I suggest that future studies continue to focus on unravelling how horizontal acquisition of microbes affects changes in microbiota composition, and whether compositional variations change microbiota functioning. The next avenue then is to shed light on how microbiota function affects host fitness.

Antigenic pressure to explain immunological variation: another axis in ecological immunology

Immune function evolved to protect an animal against disease, and imbalances of host-microbial interactions that cause disease receive considerable attention (Zheng *et al*., 2020). More interestingly from the perspective of life history evolution, Horrocks *et al*. (2011) refer to the goodness-of-fit between an animal's immune investment and the combined external threats imposed by antigenic agents as 'operative protection'. Operative protection thus entails sufficient immunological protection against disease while preventing unnecessary expenditure of resources to immune function, which could be vital for conserving long-term immunological homeostasis (Blaser, 2017). Within this context, in this section I aim to provide a more detailed perspective on how characteristics of antigenic pressure can be perceived as a function of (mostly microbial) antigenic diversity, antigen abundance, and their predictability. By integrating these aspects, I propose that investment in immune function should not only be optimized based on the diversity of antigens that an animal encounters, but also on the frequency and intensity of host-antigen interactions and their predictability. In a life history theory context, I emphasize the protective function rather than the cost of immune function, for which I propose that antigenic pressure acts as a constraint on resource allocation by determining a 'minimal immunity' for a given environment. In this way, I hypothesize that an axis of antigenic pressure dynamically sets an upper limit to the availability of resources for life history traits that are traded off against immunity. In this section I present my view on how microbial ecology (diversity, abundance and predictability) of microbial metacommunities (i.e. host-microbiota and environmental microbiota) may shape immunological variation, with a specific purpose to fuel the scientific debate on the causes of immunological variation with a microbe-focused perspective.

Antigenic diversity

A bird's immune system deals with a wide variety of antigens that originate from organisms interacting with the bird. Throughout this thesis, my colleagues and I used bacterial communities as a proxy to represent antigenic pressure on birds. A caveat of this approach is that contributions of viruses, archaea, protozoa and marcoparasites are necessary to capture a more complete and thus realistic image of selective pressures that shape the development and activation of a bird's immune system. Yet, the diverse

community of bacteria in a bird's environment is a fruitful system to study effects of antigenic pressure on immune function. Using eight weeks of experimental manipulation of environmental bacterial communities that vary in diversity, we showed in **Chapter 5** that the total concentration of all antigen-specific antibodies (IgY concentration) was higher for birds in the experimental environment with highest bacterial diversity. This suggests that the antigen diversity aspect of antigenic pressure shapes a bird's adaptive immune function as a phenotypically plastic response. Emphasizing the potential evolutionary consequences of this immunological response, Blaser (2017) fittingly reiterated the context of vertebrate immune function evolution: "The remarkable aspect of vertebrate life is not that we respond to pathogens, but that we so easily tolerate the overwhelming numbers of commensal microorganisms that we host". Our experimental results in **Chapter 5** did not indicate that indices of innate immune function were affected, but predictions from a path model based on variation in all quantified innate and adaptive immune indices and other female traits such as condition and cloacal microbiota, suggested that innate immunity was prioritized in the experimental environment with lower microbial diversity. These insights represent pioneering steps toward unravelling how the diversity of environmental microorganisms shape a bird's investment in immune function and how resources are divided between innate and adaptive defenses. Effects of antigenic diversity on host functioning and fitness remain to be uncovered, and I believe that future correlative studies on associations between host-microbiota diversity and immunological variations in wild animals can provide the necessary context for further experimental studies in this exciting research topic.

Antigen abundance and the probability of host-microbial interactions

Antigen abundance generally determines the probability that antigenic stimulation of the host's immune system is prompted (Leclaire *et al*., 2015; Soler *et al*., 2011). Antigen abundance is an aspect of antigenic pressure that my colleagues and I have not explicitly investigated in this thesis. However, variations in the number of microorganisms, and thus the number of antigens, in a bird's environment may be expected to determine the probability of (or rate at which) a bird and its immune system encounter a specific microbe. For a bird in its microbial environment, this would mean that the number of individual microbes a bird and its immune system encounter in a given amount of time shapes antigenic pressure as 'the magnitude' of host-microbial interactions. In environments

with high antigen abundances, I hypothesize that because of increasing host-microbial encounters hosts should invest relatively more in innate immunity and especially in constitutive defenses, especially when host behaviours further promote host-microbial encounters, such as feeding on carrion (Roggenbuck *et al*., 2014) or having many social interactions (Raulo *et al*., 2021; Tung *et al*., 2015). To discriminate between effects of antigen abundance and diversity, exposing animals to experimentally manipulated environments that differ in microbial abundance while antigen diversity remained constant could be fruitful. Quantification of immune function indices after exposure could then provide insights into antigen abundance effects on immune investment, and the relative investments in innate and adaptive defenses.

Predictability of antigenic pressure

The predictability of antigenic pressure has not been studied in birds. However, that predictability may be particularly relevant for the evolution of immune function. I hypothesize that adaptive change in immune function may follow when local antigenic conditions are (in part) predictable to a bird. However, the relevant spatial scale for which antigenic pressure may be predictable likely influences the adaptive potential of particular strategies of immune investment, and thus warrants further investigation. I hypothesize that host responses to unpredictable fluctuations of antigenic pressure should favour phenotypically plastic immune responses, such as by increasing antibody levels following seasonal surges of antigenic pressure. Conversely, in case of predictable patterns of antigenic pressure, an evolutionary response could boost average constitutive levels of immune defenses, which may correspond to larger geographical variation in antigenic pressure. Our direct comparisons of wild bird microbiota with environmental microbial communities represent pioneering work in this field (**Chapter 2**), after which we uncovered associations between immune function and antigenic pressure in a study system of captive birds (**Chapter 5** and **6**). Because research of antigenic pressure in wild birds is in its infancy, research opportunities are endless but necessary to unravel how antigenic pressure contributes to evolution of immunity. An important start should be made with mapping antigenic pressure at different spatial scales and across the bird phylogeny. In parallel, direct comparison between antigenic pressure and immunological variation holds promise to identify key features for further study (both microbial taxa and immunological components).

Host traits may be determining the impact of antigenic pressure as well. For example, the dietary niche of highly specialized ant-eating animals (e.g., Delsuc *et al*., 2014) represents a host trait that may be more predictable for gut microbiota variation among host species, than may be expected in dietary generalists that opportunistically consume diverse diets. With respect to host features more generally, I hypothesize that behavioural and life history trait variation between vertebrate species, populations, and individuals (e.g. trophic niche specialization, migratory behaviour, longevity, sociality) determine how predictable antigenic pressure is to a host. On ecological time scales (e.g. within an individual's life), I hypothesize that antigenic pressure should increases with the bird's mobility, where the frequency and extent of movement (e.g. migration or a large home range) cumulatively builds up antigenic pressure by raising chances of encountering new microorganisms. Thus, antigenic pressure of migratory birds likely varies throughout the annual cycle as they visit various geographically distant locations. Therefore, I hypothesize that among bird species, migratory propensity suppresses the predictability of antigenic pressure compared with non-migratory species with possibly repercussions for immune function. Furthermore, temporal variations of environmental microbial communities could add to antigenic pressure, but which are challenging to quantify and are poorly understood. How host fitness may depend on investments in immune function shaped by antigenic pressure are questions yet to be explored in birds. I propose that ecological immunology could benefit from a framework to estimate the relative influences of antigenic pressure components (i.e. antigenic diversity, abundance and predictability) on immune function, which may additionally elucidate causes underlying inconsistencies between predicted and observed patterns of immune function in life history and pace-of-life contexts (Tieleman, 2018).

Although host traits partly determine the antigenic pressure experienced by a host, antigenic pressure is principally exerted by microbial (and parasitic macro-) organisms (sensu Horrocks *et al*., 2011). Thus, predictability of antigenic pressure should depend on inherent properties of antigenic agents and their communities as well. Future studies are necessary to uncover the mechanisms by which antigenic pressure varies and potentially affects immune function of birds. For example, average virulence potential or pathogenicity of microbial communities may differ between environments and can thereby functionally increase antigenic pressure even when diversity and abundance are equal. When it comes down to how the immune system deals with antigenic pressure, I hypothesize that

antigenic diversity has additive effects by only growing the immune system's experience, whereas antigen abundance can decline over time and thus lower antigenic pressure. A potential approach to test this is to characterize the antibody repertoire (e.g. through antibody sequencing; Meyer *et al*., 2019). An experiment that contrasts the antibody repertoire of migrants and non-migratory birds in a population of partially migratory birds after a winter season could provide these insights.

Non-genetic maternal effects on the development of microbiota and immune function in birds

The importance of vertical microbiota transmission as a non-genetic maternal effect

The routes by which microorganisms become part of host-associated microbiota is a fundamental but unresolved topic in animal microbiome research. Our analysis of eggshell microbiota of wild larks showed more correspondence with nest-associated and soil microbial communities than with maternal cloacal microbiota (**Chapter 4**; van Veelen *et al*., 2018), suggesting that eggshells do not function as important vehicles for vertical transmission of maternal microbiota in these birds. In other birds, stronger evidence of vertical transmission of microbiota was demonstrated by comparing microbiota of maternal faeces and the first faeces of hatched homing pigeons Columba livia forma domestica (Dietz *et al*., 2020) and in chicken (Ding *et al*., 2017), suggesting prenatal vertical transmission of a subset of the maternal microbiota prior to oviposition. If incorporation of maternal microbiota in the pre-oviposition stage is a universal mechanism of vertical microbial transmission in birds, and possibly other oviparous vertebrates, many new questions arise. For example, are transferred microorganisms a specific subset of the maternal microbiota? Are transferred microorganisms of particular functional relevance to offspring (immunological) development? Contrasting results challenge the universality of this transmission mode: the gut microbiota of fully-grown shorebird embryos showed no evidence of gut microbiota presence in several bird species, and suggested that shorebirds acquire their microbiota horizontally from their diet and their surroundings (Grond *et al*., 2017). As earlier discussed in the context of microbial community assembly processes, in this thesis my colleagues and I presented evidence of the influence of the microbial environment on host-associated microbiota assembly of adult birds (**Chapter 2** and

Chapter 5). Our observational and experimental findings underscore that horizontal acquisition contributes significantly to host-associated microbiota assembly in songbirds. Therefore, with mounting evidence for both vertical and horizontal transmission in birds and other vertebrates (Douglas & Werren, 2016; Funkhouser & Bordenstein, 2013; Kohl *et al*., 2017; Tung *et al*., 2015), the time has come to study the relative influences of these acquisition routes on microbiota-dependent fitness to animal hosts. Particularly the development of robust community heritability estimators would be helpful for quantifying the relative fraction of variation in microbial (or functional) composition that can be attributed to vertical transmission (van Opstal *et al*., 2015; Shuster *et al*., 2006; Whitham *et al*., 2020).

Importance of non-genetic maternal effects on offspring immune function

In the previous section I proposed that spatial and temporal variation of environmental antigenic pressure may modulate life history tradeoffs in birds. Building on this idea, in this section I hypothesize that non-genetic maternal effects of immune function become more important to offspring development and fitness as antigenic pressure increases. This means that providing that the maternal phenotype is vertically transferrable, the phenotypically flexible response of females to changes in antigenic pressure may have beneficial consequences for the offspring phenotype, in addition to optimal immune protection and resource use for themselves. In the more specific context of immune function in birds, my colleagues and I postulated that antigenic pressure shapes the physiological mechanism for prenatal maternal care in birds: transgenerational immunological priming of eggs (Grindstaff *et al*., 2003, 2006).

By manipulating the microbial environments of zebra finches, my colleagues and I showed to our knowledge the first support for this hypothesis (**Chapter 6**), because transfer of antigen-specific antibodies (IgY) to egg yolk depended on the microbial diversity of the manipulated microbial environment. We then tried to zoom out to have a broader look at immune function of birds and their eggs in the context of manipulated environments that differed in microbial diversity. The use of a systems biology approach allowed us to simultaneously evaluate the microbiota and several adaptive and innate immune components of mothers and their eggs. Most notably, the resulting network of associations suggested that tradeoffs between innate and adaptive immune function possibly depend on antigenic pressure experienced by the female. Future studies are needed to test that,

and other fundamental questions remain about the mechanisms that underlie antigenic pressure-induced maternal effects on offspring immune function. For example, which of the characteristics of antigenic pressure (i.e. antigenic diversity, abundance, predictability) or which specific microbial traits most strongly affect the strength of maternal effects? Whether antigenic pressure induces maternal effects on offspring immune function that improve offspring early-life survival probabilities remains to be investigated. Similarly, it is unknown whether maternal priming may enhance protection later in life through priming of the developing offspring's immune system (Addison *et al*., 2009). I hypothesize that adaptive benefit of maternal immunological priming should increase with the magnitude of antigenic pressure, but should diminish with predictability on an evolutionary scale, in which case evolved responses should be favoured over phenotypically plastic maternal responses. However, adaptive potential only lasts if maternal immunological priming leads to improved survival and recruitment, either through enhancing direct protection to the developing offspring, or indirectly by improving ontogenetic development by allocating resources to growth rather than immune function (Lemke *et al*., 2009). Based on our results in **Chapter 6**, I propose that high antigenic pressure enhances maternal transfer of (antigen-specific) adaptive immunity, and that low or unpredictable antigenic pressure should promote transmission of non-specific innate immune defenses. Yet, how antigenic pressure affects the balance between maternal investment in adaptive and innate immune components of eggs, and what the consequences are for offspring development remains to be unraveled.

Concluding remarks

Animal ecologists nowadays are challenged by the complexity of host-microbiota interactions and the intimate and intricate ways animal ecology and evolution may be dependent on these interactions. This thesis builds a case for animal ecology to embrace a community ecology perspective, in which a bird represents an ecosystem as well as a community member in its microbial world. At the same time, to better comprehend host-associated microbiota assembly, microbial community ecology may need to find ways to incorporate the multifaceted selection imposed by host ecology and physiology, including immune function.

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Samenvatting

Een microbieel perspectief op het leven van vogels

In dit proefschrift onderzoek ik de vraag hoe de diversiteit aan micro-organismen in de buitenwereld de vorming en verdere ontwikkeling van microbiota van vogels beïnvloedt, en hoe het immuunsysteem van de vogel - als gastheer - in die ecologische interacties met micro-organismen een rol speelt.

Onderliggend aan dit proefschrift is de realisatie dat dierenleven zich voltrekt in een wereld vol micro-organismen. Vanuit het perspectief van bacteriën en andere micro-organismen kunnen (mensen en) dieren worden beschouwd als veelzijdige ecosystemen. Ieder individu dient als gastheer voor gemeenschappen van micro-organismen ('microbiota') die leven in en op het lichaam. Om te begrijpen wat de onderliggende principes zijn die de totstandkoming van microbiota bepalen is uitgebreid onderzoek nodig. Wetenschappelijke inzichten die de laatste decennia zijn verkregen benadrukken niet alleen de complexiteit van deze microbiota, maar ook de positieve effecten van microbiota op de fysiologie en het functioneren van de gastheer. Ook buiten het lichaam is de aarde bezaaid met microbiële gemeenschappen. Over de invloed van die microbiële gemeenschappen in de buitenwereld op de vorming en verdere ontwikkeling van de microbiota van vertebraten is nog maar weinig bekend. Terwijl het immuunsysteem de gastheer beschermt tegen ziekmakende effecten van microbiële infecties, zou het tevens een regulerende functie kunnen vervullen in de continue interacties met diverse microbiële gemeenschappen.

Samen met mijn collega's heb ik onderzoek verricht naar de bacteriële microbiota van leeuweriken in hun natuurlijke omgeving en naar de bacteriële microbiota en immuunfunctie van zebravinken in gevangenschap. De volgende vragen staan in dit proefschrift centraal:

- Hoe verhouden microbiota van verschillende onderdelen van het lichaam van een vogel zich tot de microbiële gemeenschappen in de omgeving van de vogel? (Hoofdstuk 2)
- Reflecteert variatie in de samenstelling van vogelmicrobiota van verschillende soorten leeuweriken de biogeografie van de vogels? (Hoofdstuk 3)
- Wat zijn de bijdragen van maternale microbiota en nest microbiota aan de vorming van de microbiota van eieren? (Hoofdstuk 4)
- Hebben microbiële gemeenschappen in de omgeving een invloed op immuunfunctie en microbiota van vogels? (Hoofdstuk 5)
- Is maternale overdracht van immuunfunctie in eieren afhankelijk van de microbiële omgeving? (Hoofdstuk 6)

Het definiëren van 'de microbiële omgeving' van een vogel is geen gemakkelijke taak. In hoofdstuk 2 hebben we ons gericht op broedende vrouwelijke boomleeuweriken *Lullula arborea* en veldleeuweriken *Alauda arvensis* en hun nesten op het Aekingerzand in Drenthe. We hebben de bacteriële microbiota van leeuweriken vergeleken met de bacteriële gemeenschappen in hun nestomgeving, waarbij we gebruik hebben gemaakt van monsters van de huid, van de broedvlek, lichaamsveren rondom de broedvlek, de cloaca, nestmateriaal en de zandgrond aan het oppervlak voor het nest. De microbiota van beide leeuweriksoorten verschilden niet, maar binnen het individu vertoonden de microbiota van verschillende plekken op het lichaam duidelijke overeenkomsten.

Verschillen in de samenstelling van de microbiota van individuele vogels was ook duidelijk. Deze verschillen bleken deels terug te voeren op de nestomgeving, omdat met name huid- en veermicrobiota de bacteriële gemeenschappen van de nestomgeving reflecteerden. De relatie met de nestomgeving was minder duidelijk voor de microbiota van de cloaca, terwijl deze cloaca microbiota juist het meest verschilden tussen individuele vogels. Deze gegevens suggereren dat de invloed van microbiële gemeenschappen in de omgeving sterker is op externe microbiota (zoals van huid en veren) dan op microbiota in het lichaam, waar regulerende fysiologische processen zoals immuunfunctie mogelijk een sterkere filterende werking hebben.

In hoofdstuk 3 hebben we op grotere geografische schaal en met negen leeuweriksoorten de relatie tussen samenstellingen van vogelmicrobiota en de microbiële omgeving van vogels onderzocht. Het is bij vertebraten nog onduidelijk of de biogeografie van de microbiota de evolutionaire verwantschappen tussen de gastheersoorten volgt, of dat variatie van microbiota samenstelling vooral een reflectie is van de micro-organismen die vrij in de omgeving van de gastheer leven. Door de patronen van diversiteit en samenstelling van de microbiota van leeuweriken te analyseren, lieten wij zien dat leeuweriken die leven in de woestijn microbiota hebben die minder divers en van een andere samenstelling zijn dan leeuweriken in de gematigde en tropische graslanden. Ondanks de grotere tussenliggende geografische afstand lijkt de samenstelling van microbiota van leeuweriken in tropische (Kenia) en gematigde graslanden (Nederland) meer op elkaar dan op leeuweriken die leven in de woestijn (Saudi-Arabië). Deze gegevens suggereren dat de variatie in microbiota een reflectie is van de microbiële omgeving van de vogels, of dat vogels aan de hand van de selectiedrukken in hun omgeving bepaalde bacteriën selecteren voor functionele baten.

De overdracht van microbiota van moeder op jong is een fenomeen waar nog relatief weinig over bekend is bij vertebraten, vooral voor vertebraten die eieren leggen zoals vogels. Dergelijke kennis is cruciaal om te kunnen onderzoeken in welke mate microbiota overerfbaar kunnen zijn, wat inzicht kan verschaffen in het evolutionaire pad dat heeft geleid tot functionele relaties tussen micro-organismen hun gastheren. In hoofdstuk 4 hebben we onderzocht of vrouwelijke vogels via de microbiota van de eischaal micro-organismen zouden kunnen overdragen naar de volgende generatie. We hebben de eischaal microbiota van boomleeuweriken en veldleeuweriken vergeleken met de microbiota van de cloaca, huid en veren van de moeder, het nestmateriaal en de bodem. We vonden dat de samenstelling van microbiota van vers gelegde eieren meer overeenkomsten had met microbiota van huid, veren en nestmateriaal dan met de moeders cloaca microbiota. Verder vonden we in eischalen ook bodembacteriën die goed gedijden op vers gelegde eieren. Deze bevindingen suggereren dat eischalen geen prominente rol spelen in de overdracht van darmbacteriën van moeder naar jong, maar dat vooral direct contact met andere bacteriële gemeenschappen de eischaal microbiota vormgeeft.

Het wordt steeds duidelijker in welke mate en op welke manieren microbiota van belang zijn voor de gezondheid en functioneren van vogels en andere vertebraten. Ondanks dat, is de invloed van microbiële gemeenschappen in de omgeving van dieren op hun immuunfunctie en hun microbiota nog weinig in kaart gebracht. In hoofdstuk 5 hebben we zebravinken *Taeniopygia guttata* acht weken laten leven in een van twee experimenteel

gecreëerde omgevingen die verschilden in bacteriële diversiteit. We hebben onderzocht of de bacteriën in de omgeving veranderingen teweegbrengen in onderdelen van de aangeboren en verworven immuunfunctie. Tegelijkertijd hebben we veranderingen in de microbiota van de cloaca van deze vogels in kaart gebracht. We vonden dat IgY antilichaam concentraties in het bloedplasma, een onderdeel van verworven immuniteit, hoger was bij vogels in de omgeving met een grotere diversiteit aan bacteriën. Ook in de microbiota van de cloaca van deze vogels vonden grotere veranderingen plaats, vergeleken met de omgeving met een minder diverse bacteriële gemeenschap. Ook duurde het bij deze vogels langer voordat die veranderingen waren hersteld. Deze bevindingen betekenen dat de bacteriële omgeving op een directe manier effect heeft op de immuunfunctie en op de dynamiek van de microbiota van vogels. De microbiële gemeenschap in de omgeving van de gastheer blijkt een factor waar onvoldoende rekening mee wordt gehouden als bron voor het opbouwen van microbiota en verdient het een prominentere plaats in het conceptuele raamwerk voor studies naar microbiota ecologie.

In tegenstelling tot microbiota overdracht, begrijpen we veel beter hoe vrouwelijke vogels het immuunsysteem van hun nageslacht via het ei voorbereiden op wat er zich buiten het ei staat te wachten, op basis van de opgebouwde ervaring van het eigen immuunsysteem. Echter, wat de invloed is van de microbiële omgeving van de moeder op die immunologische voorbereiding is weinig bekend. Dit vraagstuk staat centraal in hoofdstuk 6. We onderzochten of de depositie van antimicrobiële stoffen en antilichamen in eieren was verhoogd in de omgeving waar vrouwelijke vogels een hoge bacteriële diversiteit in plaats van een lage diversiteit ervaren. We ontdekten dat alleen vogels die zelf een hoge concentratie IgY antilichamen in het bloed hadden de depositie van antilichamen in het eigeel verhoogden, maar dat eieren van vogels met relatief lage concentraties en vogels in de omgeving met een lage microbiële diversiteit niet een dergelijke respons lieten zien. Ook vonden we geen effect van de bacteriële omgeving op de andere antimicrobiële stoffen in het ei. Daarnaast hebben we door gebruik te maken van padanalyse de samenhang van immuunfunctie en microbiota van vrouwelijke vogels en hun eieren verkend. Die analyse suggereerde dat de conditie en microbiota van de vrouwelijke vogels een rol zouden kunnen spelen in hoe vrouwelijke vogels investeren in immuniteit van het ei. Het lijkt er dus op dat de mate waarin vrouwelijke vogels investeren in de immunologische bescherming van eieren en jongen afhangt van de microbiële omgeving waarin zij leven, alsmede de ervaring van hun immuunsysteem en de conditie waarin zij verkeren.

Concluderend laat dit proefschrift zien dat de omgeving sterk bijdraagt aan de grote variatie in diversiteit en samenstelling van vogelmicrobiota op verschillende niveaus: binnen het individu, tussen individuen en soorten die leven in dezelfde omgeving, en tussen gerelateerde soorten die in klimatologisch verschillende omgevingen leven. Hoe die variatie tot stand komt, en welke functionele consequenties die variatie heeft voor overleving of reproductie zijn belangrijke vervolgvraagstukken. Middels experimentele manipulatie is in dit proefschrift laten zien dat de microbiële omgeving niet alleen direct van invloed is op de samenstelling en dynamiek van vogelmicrobiota, maar dat ook de immuunfunctie afhangt van de microbiële omgeving. Informatie van de microbiële omgeving wordt door de vogel ook gebruikt om via het ei de volgende generatie immunologisch voor te bereiden. Een dergelijk maternaal effect, zoals hier

door de microbiële omgeving gedreven, heeft de potentie om de overlevingskans van het nageslacht te verhogen. De invloed van de microbiële omgeving op de microbiota van een vogel suggereert dat een flexibel immuunsysteem van belang is voor het reguleren van de vele interacties tussen een vogel en micro-organismen. Flexibiliteit in immuunfunctie zal zorg moeten dragen voor een balans tussen het faciliteren en reguleren van een functioneel microbiota en het beschermen van de vogel tegen infecties. Vanuit evolutionair ecologisch perspectief bestaat discussie over of microbiota gecategoriseerd kunnen worden als onderdeel van de vogel, of dat de vogel en micro-organismen beter worden beschouwd als onderdelen van een metagemeenschap. Dit proefschrift ondersteunt het laatste, door te laten zien dat horizontale opname van bacteriën van invloed is op vogelmicrobiota en immuunfunctie. Op basis van deze bevindingen bepleit ik dat de microbiële omgeving een prominentere rol toebedeeld krijgt in microbiota ecologie en ecologische immunologie van vogels en andere vertebraten.

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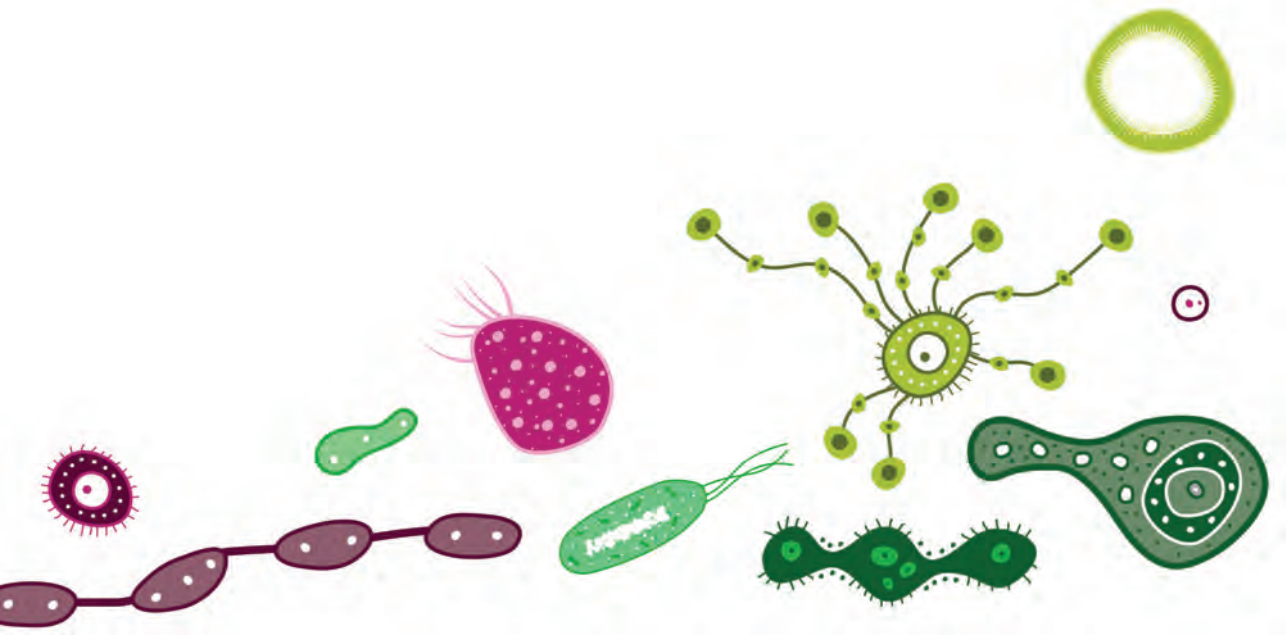

In een prachtige tuin die ons basiskamp was aan de oevers van het Naivasha Meer in Kenia lag er in pannenkoekvorm 'PhD' geschreven op mijn bord (bedankt Maaike). Op die dag en op die plek begon mijn onderzoekstraject dat hier eindigt. Terwijl ik rustig kon wennen aan het zoeken naar leeuweriknesten, stroomden de nieuwe soorten in Kenia binnen. Een wel heel fijne start van een promotietraject voor een vogelaar. Nesten zoeken was trouwens een hele andere manier van vogelen dan ik gewend was. Achteraf was dat het eerste, maar zeker niet het laatste dat ik gaandeweg dit project heb geleerd. Die kans om in Kenia aan dit onderzoek te kunnen starten was een prachtige kans, Irene, waar ik je nog erg dankbaar voor ben.

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Pieter van Veelen
Haren, September 2021

Curriculum Vitae

About the author

Pieter van Veelen was born on 5 September 1986 in Melegama, Sri Lanka. Pieter first started in 2004 at the University of Applied Sciences Rotterdam to study Biology and Medical Laboratory Research and graduated in 2008. Pieter then continued his studies in Biology at Wageningen University, from which he graduated in 2012. His Master's theses focused on stopover behavior of dunlins *Calidris alpina* at Boundary Bay, Vancouver, and on the genetic signatures of the versatile mating systems of Sanderling *Calidris alba* of Zackenberg, Greenland, respectively. This second project was based at the University of Groningen. There, Pieter started his PhD project on bird-microbe interactions in 2013. Pieter continued his career at Wetsus Centre of Excellence for Sustainable Water Technology as Microbial Analyst and Bioinformatician since 2018, and as Manager of the Laboratory for Microbiology since 2020.

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