Protozoal and bacterial infections, including the gut microbiota, in wild rodents and the effects of environmental and host variables on their composition

Doctorate of Philosophy Thesis

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General introduction

Purpose of study

The present thesis will aim to explore the composition and variability of wild rodent gut microbiota and how environmental as well as host variables impact on its composition. Specifically, a wild house mouse (*Mus musculus domesticus*) population, living on the Isle of May, was investigated as well as a wild population of field voles (*Microtus agrestis*) living in Kielder Forest.

The gut microbiota composition was determined for each rodent population separately. The microbiota of house mice from the Isle of May will be discussed first. The thesis investigated whether there are any spatial differences in microbiota composition on the Isle of May as well as the effect age, sex and weight of the host. Furthermore, effects of parasite infections on the gut microbiota composition were investigated to determine any variability in the microbiota in response to parasites. To add further to this investigation, blood parasites (haemoparasites) were indentified for the first time within the mouse population living on the Isle of May and will be discussed separately. Finally, the associations between the host's immune response and the gut microbiota composition were investigated, focusing on the expression of immune markers in the mesenteric lymph nodes.

Similar investigations were carried out on the microbiota of the voles from Kielder Forest. Additionally, temporal changes of the microbiota were investigated, focusing on the differences between individuals kept in a captive environment for 24 hours compared to individuals in captivity for up to 48 hours. Two different sample types were collected from the voles, faecal pellets and proximal colons; differences between these sample types were also investigated to establish

similarities and dissimilarities between the sampling methods. Finally, relatedness was investigated with regards to the gut microbiota composition to establish whether individuals which are more closely related to each other showed greater similarity in their gut microbiota composition or not.

The Immune System

Throughout their lives mammals are exposed to a multitude of pathogenic and non-pathogenic microbes which contain potentially toxic or allergenic substances impacting on the host's homeostasis (Chaplin 2009). Most microbes can be classed as either obligate pathogens, causing potential harm, or commensals which may be beneficial to the host (Mueller and Macpherson 2006). The host must respond to these microbes by either tolerating them or by expelling or killing them in order to ensure survival (Chaplin 2009).

The immune system responds in different ways to either remove microbes from the host or tolerate them by detecting and responding to specific structural features of the pathogens or the toxin/allergen that the microbe produces and by being able to differentiate between host cells and foreign cells (Chaplin 2009).

Innate and adaptive immunity

The immune system can be divided into two general categories: the innate, hard wired responses and the adaptive, learned responses. Innate responses are more generic and will recognise molecular patterns shared by many microbes or allergens which are not present within the host (Chaplin 2009; Holmskov, Thiel, and Jensenius 2003). The innate response will act rapidly after pathogens are encountered in the host's system and are the first line of the immune system to respond (Chaplin 2009).

The adaptive immune system is made up of cells which have specificity for any individual pathogen, toxin or allergen (Dempsey et al., 2003; Reiner 2007). After encountering the antigen adaptive immune cells must proliferate to increase cell numbers and mount an effective response against the microbe (Flajnik and Kasahara, 2010). The adaptive immune response produces long-lived cells which persist in small numbers in a dormant state until the antigen is encountered again at a later stage (Chaplin 2009; Reiner 2007). This allows the adaptive response to manifest immune memory, contributing to a more effective host response against specific pathogens when they are encountered a second time, even decades after the initial encounter (Dempsey et al., 2003).

Dendritic cells recognize differences between different classes of pathogens using microbial pattern-recognition receptors and then relay this information into signals to the microbe-specific T cells. Among the most important signals that direct the induced patterns of gene expression in maturing helper T cells are cytokines (Murphy and Reiner 2002; Weaver et al. 2006). Once antigens stimulate the immune cells naive CD4+ T cells differentiate into two subsets, Type 1 Helper (Th1) and Type 2 Helper (Th2) cells. Th1 and Th2 cells represent two polarized forms of the CD4 T cell-mediated immune responses with Th2 being associated with parasite infections, such as nematodes and helminths, and Th1 associated with bacteria and viruses (Romagnani 2000). These responses are characterised by different cytokine productions and effector functions (Chaplin 2009; Iwakura and Ishigame 2006). The majority of cytokines are produced in small numbers by T cells which will either release them into the microenvironment or transfer the cytokines from cell to cell. This does create a methodological problem as it is difficult to detect all of the produced cytokines in biologic fluids (Romagnani 2000). By identifying and measuring cytokines and transcription factors associated

with either a Th1 or Th2 response it is possible to try and assess the immune expression of the host in association with any pathogens or parasites infecting the host.

Cells belonging to the Th1 response produce interferon (IFN)-g, interleukin (IL)-2 and tumour necrosis factor (TNF)-a, which have effect on the activation of macrophages, cell cytotoxicity, and induction of cell-mediated immunity (Romagnani 2000). IL-12, a very powerful Th1-inducing agent, is produced by dendritic cells (DC) and up-regulated by the presence of IFN-g. (Romagnani 2000; Armant et al. 1996). High exposure to bacteria and their products, such as lipopolysaccharides (LPS), is linked to the stimulation of IL-12 production (Romagnani 2000; Ulevitch and Tobias 1995). IL-12, secreted by dendrritc cells and macrophages, is an inducer of Th1 responses as it represses the expression of transcription factor GATA-3. IL-12 also promotes survival, growth, a gene expression of Th1 cells (Reiner 2007).

Cells associated with a Th2 response produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL 13, which evoke strong antibody responses including Immunoglobulin (Ig) E (Romagnani 2000). Nematode resistance in the gastrointestinal tract is mostly mediated by Th2 cytokine responses, especially the expression of IL-13 (Wynn 2003). The transcription factor GATA-3 is also widely involved in the induction and maintenance of Th2 cytokine secretion and it is expressed by both mature and immature T cells (Romagnani 2000; Zheng and Flavell 1997).

Another important immune response mechanism is the T helper (Th) 17 response which is mainly driven by the IL-23 and IL-17 axis (Iwakura and Ishigame 2006). Th17 plays an important role in chronic inflammation and host defences against bacteria. Dendritic cells which are stimulated by antigens during chronic infection

will produce IL-23 which in turn promotes the development of IL-17 (Kolls et al., 2003; Langrish et al., 2005). IL-23 also stimulates the generation of pro-inflammatory cytokines IL-1, IL-6, and TNF-a (Iwakura and Ishigame 2006). IL-17 enhances T cell priming and triggers further inflammatory responses (Kolls et al., 2003; Park et al., 2005). Th17 immune responses and the associated Th17 cells promote the integrity of epithelial surfaces and are therefore important in regulating gastrointestinal immunity (Weaver et al. 2006). IL 17 and IL-22, for example, have been found to be involved in barrier function by inducing growth and differentiation of the epithelial barrier (Reiner 2007).

T cells are crucial for immune response mechanisms and will differentiate into discrete subpopulations, each with defined effector functions. The major subsets are defined by their selective surface expression of either CD4+ or CD8+ (Chaplin 2009). A portion of the circulating CD4+ T cells, specifically the so called T regulatory cells (Tregs), play an important role in down modulating immune responses (Langrish et al., 2005). Natural Tregs will develop in the thymus and are characterized by surface expression of CD4 and nuclear expression of the transcription factor FoxP3 (Chaplin 2009).

CD8+ T cells defend by direct killing of infected target cells. In contrast to the other lymphocytes, CD4+ T cells mobilize a variety of other cell types to eliminate the infection (Germain 2002). CD4+ T cells, for example, recruit other lymphocytes, innate immune cells, and even host cells, such as the epithelia, to achieve clearance of the pathogen (Reiner 2007).

The response of the immune system is most commonly studied utilising laboratory settings often focusing on a single pathogen or infection at a time. In the wild, however, individuals will commonly be exposed to a multitude of pathogens and

parasites simultaneously which may lead to differences in the mechanisms and responses of the host and its immune system (Pedersen and Babayan, 2011). It is therefore of great importance to investigate immune functioning in wild living mammals. Utilising a wild rodent models allows us to better understand associations between ecological variables, the host immune system and the microbiota in natural populations which includes humans (Turner and Paterson 2013).

Wild Immunology

Studies investigating the functioning and role of the immune system are commonly carried out in controlled environments. The effect of co-infections on the immune response often goes undetected as the focus is on single infections and the effects of host genotype as well as pathogen genotype variation is not taken into account as animals will often be very inbred and have little variation (Pedersen and Babayan 2011). This is not very representative of natural interactions and immune development as multiple infections may occur at once all of which will influence the host and its immune response. Lab reared mice will often lack differentiated T cells in their immune response and are therefore comparable to newborns in the wild but not mature adults and will not be representative when testing immune response and variation over time to multiple infections (Beura et al. 2016). By introducing pet store mice to lab mouse populations it was noted that the microbial diversity in the lab mouse increased to match the introduced “wild-type” mice (Beura et al., 2016). This suggests that the environment and contact with peers plays a significant part in immune cell response which can even impact on the cell composition and immune response of the animal over time (Beura et al. 2016).

Using wild animal systems and observing their immune responses along with their infection status is a new emerging field in immunology - referred to as “wild immunology” - and is important to further our understanding of the complex multi-way interactions within the host to combat infection and increase fitness as well as the evolutionary development of the immune system and its associations to the outside world. Arguments have been made to support the idea that multiple pathogens within the host should be viewed and assessed as a community rather than individually as they will not only interact with the host but also impact on one another (Friberg et al. 2011). Using wild populations of study organisms and studying the influence of external factors on immune response could be a link between lab based studies in immunology and wildlife or even human health which will be greatly shaped by environmental influences (Pedersen and Babayan 2011). In the wild, populations will display a wide genetic and environmental diversity which will impact their fitness and ability to survive illness depending on their innate and adaptive immune response (Pedersen and Babayan 2011). Studying how genetic and environmental variation can influence immunity and hence survival of the host could help understand key processes needed for improved fitness and survival (Pedersen and Babayan 2011). By measuring antibody and cytokine levels in the animal and any changes in these levels over time a “immune phenotype” can be established from the average. Comparisons over time can show any deviations from this average in response to differences in multiple infections, which can be caused by geographical differences as well as temporal changes (Friberg et al., 2011), to understand the immune system’s role and changes in host fitness according to outside pressures (Pedersen and Babayan, 2011).

Host-parasite interactions can take many forms. The host might develop a resistance to the pathogen or the pathology of the disease it causes. It might also completely clear the infection of the pathogen or some immune phenotype may even give the host the ability to avoid infection in the first place (Boots et al. 2009). The way the host responds to the pathogen is an important driver in evolutionary dynamics and population dynamics as defence against harmful infection is critical to survival and requires a large energetic investment as part of life history. Resistance or elimination of infection, such as macroparasites, depends also on the relative energy investment into other host biological variables such as body condition and reproduction (Jackson et al. 2011b). It was found that more mature male voles, for example, will invest more into their overall body condition when accumulating macroparasite infections rather than trying to eradicate the pathogen; this did lead to a lowered reproductive output especially when comparing to younger male voles (Jackson et al. 2014). Greater investment into body condition could be traced by looking at temporal changes and increase in the expression of the immune regulatory biomarker GATA3 - which is associated with a Th2 immune response which is an anti-inflammatory response (Jackson et al. 2014). With increased age it seems to be of benefit to the survival of the vole to switch from a resistance or elimination and Th1 inflammatory response to macroparasites to a non-inflammatory Th2 driven tolerance to increase survival (Jackson et al. 2014).

Animals suitable for this type of study need to be well understood in their own biology and immunology, such as the house mouse *Mus musculus* which has been extensively studied in the past and is still often used in lab studies (Dea-Ayuela et al. 2007; Matsumoto et al. 2013, Langille et al., 2014). Another suitable study system for wild immunology is the field vole, *Microtus agrestis*, from Kielder

forest as it is a well studied ecological system in the wild. Its diet, habitat use and population dynamics are well recorded and being a close relative to the mouse it will respond in similar ways in its immunology.

Mus musculus domesticus on the Isle of May

One of the sampling sites of the present thesis, the Isle of May, lies approximately 8km off the south-east coast of Scotland in the Firth of Forth. The island is approximately 1.6km long and 0.5km wide (Berry 1964; Triggs 1991). The only terrestrial mammal species known to be living on the island are rabbits (*Oryctolagus cuniculus*) and a population of house mice (Berry 1964). The mice live in a range of different habitats throughout the island from shrubs and low grasses, to cracks in rocks and stone walls, to the boulders at the shallow northern end of the island and can even be found inside of puffin (*Fratercula arctica*) and rabbit burrows (Berry 1964). The home ranges of the mice have been studied and found to be largely overlapping, forming an island-wide panmictic unit with random mating occurring (Triggs 1991). Mice have preferred but not exclusive ranges to one another and those living in open or unprotected habitat exhibit a greater tendency to roam compared to individuals living in more covered areas (Triggs 1991). Males and females are not known to differ in their home ranges and movement patterns (Triggs 1991). Earliest records of mice populations on the Isle of May date back to the late 19th century (Berry 1964).

In the 1980s seventy-seven mice from the island of Eday (Orkneys) were introduced to the existing population on the Isle of May (Berry et al. 1991). Six months after the introduction, alleles from the Eday mice had introgressed into the resident population and were found in individuals from all trapping locations on the island, suggesting that successful hybridisation and free gene flow had

occurred across the entire island (Berry et al. 1991). This is supported by observations from re-trapping studies showing that mice move across wide ranging territories which overlap across the island (Triggs 1991) and suggests that the mouse population on the Isle of May is able to freely interbreed (Berry et al. 1991).

Microtus agrestis in the Kielder Forest

The present thesis investigates the associations of host and environmental variables with the composition and diversity of the gut and faecal microbiota of wild caught field voles (*Microtus agrestis*) taken from Kielder forest, Northumberland. Kielder is the largest and one of the oldest state-owned forest in Britain and the ecology of the field vole population found there has been extensively studied (Telfer et al. 2010; Bown et al. 2002; Turner et al. 2014; Majazki et al. 2013). This allows current studies to take their parasite dynamics and ecology, such as the cyclical nature of vole populations (Korpimäki et al. 2005), into consideration when investigating their health and immune response. The population in Kielder forest differs in its population cycles from other Northern European vole population as it has a 3-year population cycle with lowest densities dropping to approximately 25-50 individuals per hectare (Korpimäki et al. 2005). The lowest observed densities of voles in Kielder Forest correspond to the peak densities in North European taiga and tundra habitats (Oksanen and Henttonen 1996) making Kielder a suitable study sites for voles as densities remain high enough throughout the cycles to allow for plenty of captures. The cycles of Kielder Forest are spatially synchronous at small scales only, between 8 and 20 km (Lambin et al.2000; Korpimäki et al. 2005) and will only vary in their peaks and lows by a 10-fold differences unlike Northern European vole

populations which will show 50-500 fold changes between peak and low cycles (Korpimäki et al. 2005). The small-scale synchrony in field vole cycles is most likely caused by the patterns of clear-cutting happening in Kielder, which will impact vole dispersal (Lambin et al. 1998; Lambin et al. 2000). Field voles prefer clear-cut habitats, which will develop into grassy vegetation approximately 2 to 3 years after tree felling occurred. The area remains suitable as vole habitat for up to 12 years until the trees regenerate and outcompete ground vegetation (Lambin et al. 1998; Lambin et al. 2000).

Voles are non-selective graminivores (Wheeler 2005) and will eat most grasses with little preference or selection (Wheeler 2005). Any preference between grass species observed has been linked to palpability and nutrient content of the plants as voles often live in low nutrient conditions and need to maximise their intake (Wheeler 2005). Vole diet has not been found to change significantly between seasons and dormant shoots as well as dead material of grass species have been found in faeces throughout the year, meaning vole diet does not depend on availability of fresh plant material (Wheeler 2005).

Infections and Parasites

Co-infection

Infection with multiple parasites at the same time is common in wild animals (Clerc et al., 2018) and will even occur in humans living in less sanitised housing or living in close proximity to animals as certain parasites have a zoonotic potential.

Multiple pathogens may infect an individual simultaneously, where one infection may interfere with the host's immunity and render them more susceptible to other infections. On the other hand, infection with certain pathogens can also lead to a decreased susceptibility to other disease, either by outcompeting the

other pathogens for resources or by up-regulating parts of the immune response which may be detrimental to other infections (Telfer et al. 2010). Studies which identify more than one parasite infection at a time and consider them in relation to one another will often be limited by being cross sectional only, not allowing for changes over time within the same individual to be considered (Telfer et al. 2010). Long-term studies of interactions between hosts and their parasites are not often done but can give great insight into evolutionary processes (Bajer et al. 2014).

A study in wild voles showed that being infected with one pathogen had a greater influence on susceptibility to other infection than the host's own biological variables such as age or environmental factors such as season (Telfer et al. 2010). In rodent populations it was also found that the most significant environmental impact on host susceptibility to infection is seasonality (Telfer et al. 2010).

Common rodent parasites and their life cycles

Common parasite infections in European rodents include gastrointestinal helminths such as *Trichuris* species, as well as *Syphacia* species (Reynolds et al., 2015). Rodents are also often secondary hosts to microparasites in their blood, such as *Babesia*, *Bartonella*, *Anaplasma* and *Trypanosome* species (Bown et al., 2003; Telfer et al., 2005).

The bacterium *Anaplasma phagocytophilum* is transmitted by tick bites but unlike *B. microti* it is a limited infection, usually detectable for four to eight weeks (Telfer et al. 2010). It will predominantly infect the neutrophils of its host. *Bartonella* species that are common in western European rodents are *B. grahamii* and *B. taylorii* (Taylor et al. 2018). Typically, *Bartonella* is transferred by fleas, usually via faecal inoculation, and then entering the blood stream. Infection with *Bartonella*

is detectable for approximately four to eight weeks and will predominantly invade the erythrocytes in the blood of the infected host (Schülein et al. 2001).

Trypanosoma species are typically insect vector borne and can infect humans (Vickerman 1985). Three species occur most commonly, namely *Trypanosoma brucei, T. cruzi* and *T. rangeli* and their transmission is cyclical (Vickerman 1985). The parasite species have slightly varying methods of infecting their hosts as *Trypanosoma brucei* multiplies in the blood and other body fluids of a variety of mammals, whereas *Trypanosoma cruzi,* multiplies inside host cells, such as mononuclear phagocytes and muscle cells (Vickerman 1985). The genus *Trypanosoma* belongs to the flagellated parasitic protozoa and is found worldwide. Ticks are potential transmission vectors of the parasite (Noyes et al. 1999).

Sarcocystis is the largest genus within the family Sarcocystidae and consists of species which infect a range of animals including mammals, birds and reptiles (Mackenstedt et al., 1990). *Frenkelia*, another genus within this family, consists of parasites that use rodents as intermediate hosts and birds of prey as definitive hosts (Mackenstedt et al., 1990). The parasite reproduces sexually in the gut of the definitive host, is passed with the faeces and ingested by the intermediate host. Once the intermediate host is consumed by the definitive host, the cycle is completed (Markus, Killick-Kendrick, and Garnham 1974).

Parasite competition

Microparasites will often compete with each other and have different strategies infecting their host usually relying on abundance in available red blood cells (Graham, 2007). They are very common in wild populations of voles. 90% of bank voles sampled over a 3 year period tested positive for a microparasite (Bajer et al.

2014). Infection with microparasites can have benefits for the host as the presence of macroparasites leads to equilibrium in the host by decreasing occurrence of microparasite epidemics and instead display stable levels of endemic micro- and macroparasites (Fenton 2008). Typically intracellular microparasites will be causing a higher expression of Th1 related cytokines (IL12, INFg) whereas macroparasites are known to increase a Th2 response in their host (IL10, IL5) (Fenton et al., 2008). Coexistence of these parasites depends on a "compromise" in the expression of these counteracting immune responses (Graham, 2007). Ecological mechanisms are at the heart of co-infection of parasites: the presence of macroparasites that may induce anaemia, for example, will limit the number of microparasites relying on blood cells as resource. This is an example of bottom-up control which is resource competition (Graham 2008). A top-down example of control between parasites would be the presence of many helminths which will lead to an increasing expression of anti-inflammatory cytokines which will allow for a greater density of microparasites to infect the host (Graham 2008). Immune mechanisms involved with nematode infection are usually opposing mechanisms to host infections with microparasites. Th1 immune responses lead to an increased death of infected cells by macrophages, which are detrimental to microparasites, which infect the host's cells (Fenton 2008). Th2 responses, on the other hand, can lead to an increased production of mucus which can help remove helminths from the gut (Fenton 2008). This highlights the difficulty of responding to infections in the wild as the host may have to regulate its response to eliminate one type of pathogen which will, however, allow for another type of parasite or pathogen to infect (Mosmann and Sad, 1996; Romagnani, 1996; Pritchard et al. 1997; Stewart et al. 1999). The host's innate and adaptive immune responses play an important role in the co-infections of

pathogens – these responses in turn are made dependent by the host's overall health such as their diet, reproductive status and age (Fenton 2008).

The effects of co-infections on the host are usually studied specifically linked to one host only (Lello et al. 2018). It would be of great value to be able to predict the effects co-infection – such as increased susceptibility to other pathogens, greater disease severity and changes in immune expression – in other host species, testing whether these interactions act the same or differently depending on their host (Lello et al. 2018). A recent study showed that when comparing the effects of co-infecting nematodes in wild rabbits and using these ecological predictions to hypothesise similar patterns in sheep, co-infecting nematodes had very similar effects on the sheep host as they did on the rabbits (Lello et al. 2018).

The Role of the Microbiota

What is the microbiota and how is it established

Humans, as well as most other animals, are colonised by thousands of microbial organisms, such as bacteria and fungi, which can be found on the skin as well as the mucosal layers of the body. This group of micro organisms is referred to as the "microbiota". The microbiota is first established during birth when the infant gets in direct contact with the bacteria found in the birthing canal of the mother (Palmer et al. 2007). Studies in human babies have found that the dominant phyla of bacteria in the gut microbiota are Bifidobacteria and Lactobacillus, also commonly associated with breast feeding infants (Bode et al., 2012). The exposure to non-harmful pathogens is vital in the early stages of development of the innate immune system. The bacteria that make up the microbiota in the gut interact with the host's own immune system – which is trained to detect any foreign bodies in the host – and has been suggested to play a major role in early

development and “teaching” of the immune system by training it to recognise beneficial bacteria which do not require a full inflammatory response from the immune system (Ursell et al. 2012): Studies have suggested that a the lack of exposure to non-harmful pathogens in the early stages of life and the resulting interactions with the immune system can lead to overreactions of the immune system – in the shape of allergies and autoimmune disease for example – later in life when exposed to harmless antigens (Okada et al. 2010).

Toll-like receptors of the host are the primary way the innate immune system recognises commensal bacteria in the gut and the bacteria will display antigens which are easily recognisable by these proteins – the interaction between commensal bacteria and the host’s TLRs is what leads to a lowered inflammatory response and greater immunological tolerance to allow the presence of the beneficial bacteria (Clemente et al. 2012).

During early development the microbiota is prone to significant shifts and changes in composition (Palmer, 2007; Dominguez-Bello et al., 2011), whereas the adult microbiota remains relatively stable with only minimal fluctuation due to dietary, environmental, or disease related changes (Palmer et al. 2007). The exact interactions and association between the microbiota and it’s host’s biology and environment have started being studied very recently as technological advances in sequencing have enabled scientists to increase both the quality and the quantity of samples (Bálint et al. 2016). Bacterial 16S sequencing is commonly used to investigate the microbiota community of the gut and will be supplemented either by studying the host’s environment and biology or by controlling for any external variables as is the case in studies utilising laboratory animals (Linnenbrink et al. 2013). Understanding how the microbiota reacts and adapt depending on external factors could be useful in the treatment of disease

as deviations of the microbiota composition to an “average” norm value has been observed in many different types of disease such as Celiacdisease, gastric cancer as well as conditions such as obesity, allergies, Type 2 diabetes – all of which will display an either below or above average amount of certain bacterial groups in their faecal microbiota compared to healthy adult individuals (Clemente et al. 2012).

The role of the gut microbiota

Bacteria living in the gut often play an important role in the processing of nutrients, for example helping with the breaking down of fats, carbohydrates and proteins (Sergeant et al., 2014). An example of this is the phylum Bacteroidetes which primarily derives its energy from carbohydrates and protein fermentation. Bacteria of this phylum have a broad saccharolytic potential (Arumugam et al. 2011). The most dominant or common bacterial phyla found in the gut microbiota are often linked to the host’s diet due to requiring specific resources (Arumugam et al. 2011).

Commensal bacteria also colonise and take up the available space in the gut mucosal layers which prevents other, harmful bacteria from spreading and multiplying, an example being the relative abundance of *Helicobacter pylori* which, if found in very high quantities in the human gut, can be a leading cause of stomach ulcers and other disease (Abadi et al. 2012).

The gut microbiota has been shown to have direct links to many other parts of the human body – most interestingly a potential link to the brain and its functions by directly influencing levels of serotonin (Ridaura and Belkaid 2015; Yano et al. 2015). Spore forming bacteria found in the gut microbiota are involved in the synthesis and overall production of most of the body’s serotonin which in turn

influences a multitude of bodily functions including platelet aggregation, gastrointestinal motility and immune function (Yano et al. 2015). In addition, studies have found a link between the microbiota and the so-called "hypothalamic-pituitary-adrenal" axis which is known to contribute to anxiety and depression periods if in dysregulation (Foster and McVey Neufeld 2013).

The microbiota, in turn, is also affected by the host's biology. An example is the notable changes in microbiota composition observed after exposing newborn animals to stress in the form of removing them from their mothers for prolonged periods of time (O'Mahony et al. 2009). Increased stress could be measured by an increase in plasma corticosterone and a shift in the gut microbiota was observed in the faeces compared to control groups (O'Mahony et al. 2009).

Dysbiosis in the gut is associated with disease in the host overall – not just localised to the gut but impacting on the overall body , examples including Crohn's disease and IBS in the gut itself, as well as asthma, allergies and mental health. It is clearly a very vital system in the body but little is known about what drives any changes and variation of the gut microbiota, especially in wild systems which are more comparable to humans than studies carried out in sterile lab conditions (Nguyen et al. 2015).

We do know that the microbiota is highly variable between individuals. Some of this variation can be linked to the host's genetic makeup and predisposition (McKnite et al. 2012). The majority of variation, however, is suggested to be influenced by the environment in which the individual grows up and lives (Suzuki and Nachman 2016; Snijders et al. 2016).

Composition of the gut microbiota

The microbiota can be grouped into so called operational taxonomic units (henceforth referred to as OTUs); the microbiota is made up of hundreds of bacterial species but often dominated by a select few phyla, the most common ones in rodent studies being Bacteroidetes and Firmicutes (Arumugan et al., 2011). Species composition of the gut microbiota is still an emerging field of science but studies have found that at a phylum level the microbiota in humans can usually be classed into one of three broadly applicable "enterotypes" of microbiota – the ones predominantly composed of Bacteroidetes, those made up of Firmicutes and the less common third enterotype usually dominated by Prevotella or Clostridium depending on host species studied (Eckburg et al., 2005; Arumugam et al. 2011; Wu et al., 2011).

The species found in the gut microbiota undergo selection pressure from the host but also have to compete for space and resources among each other. This will lead to an overall homeostasis of the microbiota with some species occurring at higher abundances and some very low abundances. The overarching composition of the microbiota at phylum level seems to be relatively stable within an individual as it can even be completely restored to previous levels after significant perturbation such as the prolonged taking of antibiotics or a drastic change in diet (Arumugam et al. 2011).

Diet has a very significant effect on the microbiota. However, changing from one type of diet to another does not necessarily lead to permanent changes in the microbiota composition but only for as long as the new diet is being consumed (Wu et al. 2011). A diet high in animal fats and sugars is associated with a

Bacteroides dominated enterotype, whereas a diet high in fibre and carbohydrates is linked to Prevotella and Firmicutes (Wu et al. 2011).

A link has been suggested between the ratio of Bacteroidetes and Firmicutes and obesity in the host, specifically a decrease in relative Bacteroidetes proportion compared to Firmicutes in obese individuals compared to lean individuals (Armougom and Raoult 2008; Mariat et al. 2009). The ratio between Firmicutes and Bacteroidetes also changes with age and is significantly different in older individuals compared to young adults and has been suggested to be one possible indicator of total microbiota diversity and health (Mariat et al. 2009).

The gut microbiota in wild rodents

Most microbiota studies carried out in the wild have utilised house mice (*Mus musculus domesticus*) as well as wood mice (*Apodemus sylvaticus*) and sometimes rat species (Kohl and Dearing 2014; Maurice et al. 2015a; Kreisinger et al. 2015). Predominantly, studies consider a single time point in the rodent's life – the time of cull - with repeat sampling occurring within the same sampling site across time scales ranging from a few months to a couple of years (Kohl and Dearing 2014; Maurice et al. 2015a). The geographical distances across which wild rodents are caught varies across the different studies, some having a few miles between all of the sampling sites to sampling from different countries (Weldon et al. 2015; Linnenbrink et al. 2013).

Wild mice are predominantly colonised by Bacteroidetes, Firmicutes and Proteobacteria – *Lactobacillus* being the most dominant genus (Maurice et al. 2015a). One of the most driving environmental factors in the changing of gut microbiota composition has been shown to be seasonality across two years of study, especially a shift in Bacteroidetes and Firmicutes abundances depending on

a similar shift in the diet from an insect based to seed based diet in winter to spring (Maurice et al. 2015a). Specifically the genetics of the host, transmission rates between individuals and geographical differences have been studied – population structure or the host genetics showed little evidence in influencing the microbiota community; geographical distance did show some significant changes however (Linnenbrink et al. 2013).

Thesis structure

Chapter 1 will detail the gut microbiota composition of wild house mice (*Mus musculus domesticus*) on the Isle of May and specifically focus on the spatial variability of the gut microbiota.

Chapter 2 will investigate the proximal colon and faecal microbiota composition of wild field voles (*Microtus agrestis*) from Kielder Forest. Differences between faecal and colon microbiota composition will be discussed as well as variability of the microbiota in individuals living in captivity for up to 24 hours compared to those in captivity for up to 48 hours.

Chapter 3 considers the importance of relatedness on colon microbiota variability in field voles. Individuals which are closely related, such as dams and their offspring, and their respective microbiota will be compared to one another as well as to the entire population to establish whether closely related individuals have a significantly more similar microbiota to one another than non-related individuals.

Finally, Chapter 4 will detail the haemoparasites present on the Isle of May and investigate whether environmental variables as well as presence of other parasites impacts on the presence or absence of haemoparasites. An association between haemoparasites and gut microbiota composition is also investigated.

Chapter 1: The effects of environmental and host variables on the composition of the gut microbiota in wild house mice (*Mus musculus domesticus*)

(A modified version of this Chapter has been accepted for publication in PLOS ONE, September 2019)

Abstract

The composition of the mammalian gut microbiota can be influenced by a multitude of environmental variables such as diet and infections. Studies investigating the effect of these variables on gut microbiota composition often sample across multiple separate populations and habitat types. In this study we explore how variation in the gut microbiota of the house mouse (*Mus musculus domesticus*) on the Isle of May, a small island off the east coast of Scotland, is associated with environmental and biological factors. Our study focuses on the effects of environmental variables, specifically trapping location and surrounding vegetation, as well as the host variables sex, age, body weight and endoparasite infection, on the gut microbiota composition across a fine spatial scale in a freely interbreeding population. We found that differences in gut microbiota composition were significantly associated with the trapping location of the host, even across this small spatial scale. Sex of the host showed a weak association with microbiota composition. Whilst sex and location could be identified as playing an important role in the compositional variation of the gut microbiota, 75% of the variation remains unexplained. Whereas other rodent studies have found associations between gut microbiota composition and age of the host or parasite infections the present study could not clearly establish these associations. Neither did the present study find any associations between

cytokine expression in the mesenteric lymph nodes and gut microbiota composition. This may be due to data being limited to one single cross sectional time-point rather than being longitudinal sampling. We conclude that fine spatial scales are important when considering gut microbiota composition and investigating differences among individuals.

1. Introduction

The gut microbiota interacts with a variety of fundamental host functions in most animals. It is involved in breaking down complex carbohydrates for energy (Thomas et al. 2011), and immunological functions such as the activation and production of T-Regulatory cells or the induction of cytokines such as FoxP3 or IL10 (Round and Mazmanian 2009; Atarashi et al. 2013). Changes in the composition and microbial abundance of the gut microbiota occur throughout life due to factors such as changes in hormone levels (Clarke et al. 2013), age (Langille et al. 2014), infection with parasites (Holm et al. 2015), diet (Dominianni et al. 2015) and host genetics (Benson et al. 2010a; Bonder et al. 2016). Additionally, vertical transmission from mother to offspring and the transmission of bacteria from the environment, such as the vegetation, can also affect microbial composition (Vaishampayan et al. 2010; Tannock et al. 1990; Thompson, Wang, and Holmes 2008).

One of the most influential variables on gut microbiota composition in wild house mice is their geographical provenance (Weldon et al. 2015; Linnenbrink et al. 2013; Suzuki, Martins, and Nachman 2018). The geographical scale over which this has been studied varies markedly, having up to 100 kilometres between all of the sampling sites (Weldon et al. 2015) or sampling mouse populations from different countries (Linnenbrink et al. 2013). The caecal microbiota of house mice

(*Mus musculus domesticus*) showed differences in alpha diversity across the UK using two farmland sites (in Gloucestershire and Somerset), and the London underground (Weldon et al. 2015). Geographical distance has been identified to account for 16% of gut microbiota variation in wild house mice across Western Europe (Linnenbrink et al. 2013). It has also been suggested that microbiota variation may be limited by the dispersal abilities of the microbes themselves and the level of interbreeding and direct physical contact between the hosts (Linnenbrink et al. 2013). Altitude has also been shown to impact the composition of wild house mouse gut microbiota due to changes in available resources for the bacteria (Suzuki, Martins, and Nachman 2018). As altitude increases, anaerobic bacteria (e.g. *Prevotella* species) increase in the rodent's environment compared to lower altitude habitats whilst other microbes decline due to the limited oxygen available, and this influences the community which can be acquired by the host (Suzuki, Martins, and Nachman 2018).

Geographical location is intimately linked with differences in ecological factors such as local vegetation, which in turn are likely to directly or indirectly affect the diet of an animal (Yatsunenko et al. 2012; Linnenbrink et al. 2013; Kreisinger et al. 2014; Weldon et al. 2015; Suzuki, Martins, and Nachman 2018). Black howler monkeys (*Alouatta pigra*) exhibit changes in their gut microbiota composition depending on which forest habitat type they inhabit (Amato et al. 2016). Fruit availability within the habitat seems to be driving some of the most notable differences in gut compositional changes (Amato et al. 2016). Specifically, a reduction in the abundance of Ruminococcaceae could be detected during periods of low fruit intake due to limited seasonal availability in primary and secondary forests (Amato et al. 2016). Diet driven changes in the gut microbiota occur in wild house mice after being transferred into a laboratory environment

and fed a captive diet. The composition of the microbiota changed from being rich in bacteria of the phylum Bacteroides to one dominated by phylum Firmicutes (Wang et al. 2014). The observed change in dominant phyla present and relative abundance of specific bacterial groups is due to the increase of plant-derived nutrients found in the standard laboratory food and the lower amount of carbohydrates typically consumed in the wild (Wang et al. 2014; Hildebrand et al. 2013).

Sex has been linked to differences in gut microbiota diversity in a few different mammalian species (Org et al. 2016; Dominianni et al. 2015; Bolnick et al. 2014; Yurkovetskiy et al. 2013). In humans, for example, a lower relative abundance of Bacteroidetes was found in women compared to men (Dominianni et al. 2015). When looking at multiple strains of laboratory mice, some sex driven differences in the gut microbiota composition were unique to an individual strain suggesting that genetic variance also plays an important role in the variability of the microbiota in male and female mice (Org et al. 2016).

Age of the host has not been identified to impact the species composition of the microbiota once the individual has reached maturity (Arumugam et al. 2011). Changes in the microbiota composition of lab mouse pups occur through exposure to an increasing number of microbes from the environment, which would suggest that the surroundings play an important role in microbiota establishment and composition (Snijders et al. 2016).

Nematode infections and other gut parasite presence have all been shown to impact microbial diversity (Weldon et al. 2015; McKenney et al. 2015). Infection with the gastrointestinal nematode *Heligmosomoides polygyrus* leads to an increase in the relative abundance of Lactobacillaceae (Walk et al. 2010). Captive

rats infected with rat tapeworm (*Hymenolepis diminuta*) had an increased abundance of *Clostridium* species, whilst *Bacillus* species had a reduced abundance compared to uninfected animals (McKenney et al. 2015). The mouse whipworm (*Trichuris muris*), frequently found in the caecum, is associated with a decrease in the phylum Bacteroidetes whilst increasing the abundance of Lactobacillaceae (Holm et al. 2015; Reynolds, Finlay, and Maizels 2015). Another parasite frequently found in the caecum is the mouse pinworm (*Syphacia oblevata*) which has been associated with a decrease in *Lactobacillus* (phylum Firmicutes) (Kreisinger et al. 2015).

The microbiota in the gut not only interacts with parasite infections but also the immune system and plays an important role in education of the immune system to differentiate between harmful and commensal microbes (Mueller and Macpherson 2006). The intestinal immune system needs to be able to simultaneously maintain a protective immune response against harmful pathogens whilst allowing commensal microbes to co-exist with the host (Mueller and Macpherson 2006). The mesenteric lymph node (MLN) is a very important gut-associated lymphoid structure, draining the gut and holding naive T cells important for antigen tolerization (Macpherson and Smith 2006a; Sakuraba et al. 2009). The MLN directly interacts with the gut bacteria which are carried by dendritic cells (DC) and communicates with the immune system to regulate inflammatory responses (Macpherson and Smith 2006b; Slack et al. 2009). DCs are an important cell type which sample commensal and pathogenic bacteria in the gastrointestinal tract and then present antigens to the lymphoid structures in the gut and prime naive T cells found in the MLN (Sakuraba et al. 2009). Once the DC engages with the naive T cells three distinct steps take place: the T-cell's receptors engage with the peptide-major histocompatibility complex (MHC). Co-stimulation

takes place, especially of the molecules CD80, CD86 and CD40. The DC signalling to the T cells determines the specific differentiation into effector cells through the production of Th1, Th2 or Th17 polarization factors (Sakuraba et al. 2009).

Individuals treated with antibiotics, which eradicate a large number of commensal bacteria, were shown to have an amplified immune response from the MLN when infected with non-pathogenic *Salmonella* (Diehl et al. 2013). Individuals with an intact commensal microbiota (not disrupted by antibiotics) showed little to no inflammatory response from the MLN as fewer antigens were presented to the lymphoid structure (Diehl et al. 2013). It is unlikely that this observation was due to increased epithelial permeability as there was no increase in bacteria reaching the spleen and demonstrates the direct interactions between commensal bacteria and the MLN immune response (Diehl et al. 2013).

Laboratory-based studies often consider the variables which may influence microbiota composition independently from one another; however, using a wild rodent population allows the exploration of how different effector variables combine to influence the composition of the gut microbiota

The present chapter investigated the effect that host variables, environmental variables and immune response have on the gut microbiota diversity and composition of the house mouse, the same species as commonly used in many laboratory studies, on the Isle of May. The mice on the Isle of May offer a unique opportunity to study factors influencing the composition of the gut microbiota in the same species as commonly studied in the laboratory. These mice live wild and not in association with humans in comparison to those on the mainland which largely live a commensal existence.

1. Materials and Methods

The following methods were approved by the University of Nottingham Animal Welfare and Ethical Review Body and comply with the UK's Animals (Scientific Procedures) Act of 1986.

1.1 Field sampling

Wild *M. m. domesticus* were caught on the Isle of May, Firth of Forth, Scotland, in October 2015. A total of 11 sampling sites were used (Fig 1). At each site 15 to 20 traps were laid using 10 separate transect points, each with one or two traps depending on overall size of trapping site. Transect points were between 1 and 3 meters apart. We predominantly used Longworth (Longworth Scientific Instrument Co., Oxford, UK) or Ugglan (Granhab, Gnosjö, Sweden) small animal traps and a small number of home-made "Jordan" traps (Kettel, Perrow, and Reader 2016) constructed from drain pipes with a trapping mechanism inspired by Ugglan traps. Traps were filled with hay and baited with a handful of birdseed and then checked twice a day, morning and late afternoon, across a total of 4 days.



Figure 1. A map of the Isle of May detailing our trapping sites by name along with their respective abbreviations.

Each sampling site was categorised by plant species according to its dominant vegetation, based on the most recent available survey of the island (van der Wal, unpublished). Dominant plant species were Yorkshire fog *Holcus lanatus* and sea campion *Silene maritima*, or the category "Both" was used for sites with equal abundance of these two plant species.

All captured mice were sexed and weighed; females displaying signs of pregnancy or lactation were released. Subsets of animals per site were randomly selected to be culled to ensure an even distribution of samples across the different sampling locations. Individuals selected for culling were euthanized using rising levels of CO_2 and death confirmed by exsanguination, in accordance with Schedule 1 of the

Animals (Scientific Procedures) Act 1986. Immediately following euthanasia, mice were sexed and aged. Males and females were categorised as immature or mature based on the appearance of their external reproductive organs (descended testes in males and perforated vaginas in females). Body weight and snout to vent length were measured for each individual. The mesenteric lymph node was also removed and stored at 4°C in RNAlater overnight, after which the RNAlater was removed and the tissue stored at -80°C. The gastrointestinal tract was removed and stored in 80% ethanol and later used for detection of helminths under a dissection microscope. Eyes were removed and stored in 80% formalin; lenses were later removed, dried and weighed to estimate age in days as described in Rowe et al. (1985). Overall mass of the eye lenses has been previously used studies to estimate age as the lenses grow throughout the animal's lifespan (Augusteyn 2014). Eye lens mass as predictor of age has previously been compared to body mass, body length, tail length, foot length and ear length in corn mice (*Calomys musculinus*) and was found to be the most reliable measure of age compared to the other variables (Carreno et al. 1990).

A single sample of caecum tissue and content was collected from each individual, such that variation among these caecum samples (hereafter, just "samples") is representative of variation among individuals. The sample was immediately snap frozen to preserve DNA yield and stored at -80°C.

1.2 Parasite detection

Parasite screening was carried out for all dissected mice by both examining the fur of the animal and the gastrointestinal tract. A dissection microscope was used to examine the entire fur coat of each mice and manually counting any visible ectoparasites, specifically *Radfordia affinis, Myocoptes musculinus, Myobia*

musculi and *Nosopsyllus fasciatus*. The gastrointestinal tracts of the mice were immediately stored in 80% ethanol after dissection and were later further dissected and inspected under a dissection microscope. Again, the entire gastrointestinal tract was investigated by placing sections of the tract into petri dishes and gently rinsing them with water to dislodge any parasites from the tissue. The petri dishes were then placed under a dissection microscope and following the scored lines of the petri dish parasites were visually identified and counted. To help with the identification printed out images of Trichuris muris and *Syphacia oblevata* were used for comparison.

For further detail on the parasite detection please see Jonathan Fenn's PhD thesis (2019).

1.3 MLN cytokine gene expression

RNA was extracted from the mesenteric lymph nodes using the NuceloSpin® RNA extraction kit (Machery-Nagel) and exactly following the protocol which includes a Precision DNase (Primerdesign) step to remove any unwanted DNA from the samples (see Appendix 4 for full protocols). RNA samples were stored at -80°C. Following RNA extraction samples were synthesised into cDNA using the Primerdesign *Precision nanoScript™ 2* Reverse Transcription Kit. Nanodrop was used to record the cDNA concentration and samples were then stored at -20°C until further use (see Appendix 1 for full protocol). Real-time qPCR was performed using SYBR Green and the Precision FAST qPCR Master Mix (Primerdesign) for a total of 11 genes (Table 1).

Table 1. Immune genes used in qPCR analysis of mesenteric lymph node gene expression levels. Genes were selected covering a range of roles in the immune system, and include cytokines and transcription factors involved in the regulation of cytokine expression.

Gene	Role	Primer Sequences	References
IL-1β	Produced by phagocytes, involved in innate immunity and inflammation, associated with Th1-type responses and TNF induction.	L 5'-CAACCAACAAGTGATATTCTCCAT R 5'- GGGTGTGCCGTCTTTCATT	(Ikejima *et al.*, 1990; Chizzolini *et al.*, 1997; Sansonetti *et al.*, 2000; Sims and Smith, 2010; Cho *et al.*, 2012)
IL-4	Development Th2-type responses against helminth macroparasites, inhibition of IFN and Th1-type immunity.	L 5'-AGCAACGAAGAACACCACAG R 5'- GCAGCTCCATGAGAACATA	(Swain *et al.*, 1990; Vercelli *et al.*, 1990; Kopf *et al.*, 1993)
IL-6	Pro-inflammatory cytokine, involved in both Th17-type inflammation and linking innate and adaptive responses.	L 5'-TCCATCCAGTTGCCTTCTTG R 5'-GGTCTGTTGGGAGTGGTATC	(Jones, 2005; L. Zhou *et al.*, 2007)
IL-12B	Induces Th1 responses by inhibiting IL-4 and increasing IFNγ expression.	L 5'-CACGGCAGCAGAATAAATATGAG R 5'-GAGTTCTTCAAAGGCTTCATCTG	(Manetti, 1993; Magram *et al.*, 1996)
IL-10	Th2-associated immunoregulatory cytokine involved in limiting tissue damage by inhibiting Th1-type inflammation.	L 5'-GGGAAGAGAAACCAGGGAGAT R 5'-GCCACAGTTTTCAGGGATG	(Mosmann and Moore, 1991; D'Andrea *et al.*, 1993; Yang *et al.*, 1999)
IL-13	Development of Th2 cells, and Th2-type protection against helminth macroparasites.	L 5'-GCCAGCCCACAGTTCTACA R 5'-CCACCAAGGCAAGCAAGAG	(Grünig *et al.*, 1998; McKenzie, Bancroft, *et al.*, 1998; McKenzie, Emson, *et al.*, 1998; Wynn, 2003)

Table 1 continued.

IL-17A	Pro-inflammatory cytokine, characteristic of Th17-type inflammatory responses, causes neutrophil recruitment.	L 5'-GTCTGCCCTCCACAATGAAA R 5'-TTAAAGTCCACAGAAAAACAAACA	(Miyamoto *et al.*, 2003; Moseley *et al.*, 2003; Kolls and Lindén, 2004; Lohr *et al.*, 2006)
TNFα	Pro-inflammatory cytokine associated with Th1 and Th17-type inflammation.	L 5'-AGCCAGGAGGGAGAACAGA R 5'-CAGTGAGTGAAAGGGACAGAAC	(Iwamoto *et al.*, 2007; Aravena *et al.*, 2011)
IFNγ	Polarisation and maintenance of Th1-type responses, promotion of IL-12 activity	L 5'-TGATTACTACCTTCTTCAGCAACAG R 5'-CTGGTGGACCACTCGGATG	(Bradley, Dalton and Croft, 1996; Wenner *et al.*, 1996)
Irf5	Promotion of Th1 and Th17 inflammatory responses	L 5'-CTTCCAGCCAGCCCCCTA R 5'-TGATCTCTAGGTCCGTCAAAGG	(Krausgruber *et al.*, 2011)
GATA3	Transcription factor promoting expression of Th2-type cytokines and inhibiting IFN expression	L 5'-GAAGACTTTATTGTACCTGGATAGC R 5'-TGGACATCAGACTTAGTGGTTTC	(Zheng and Flavell, 1997; Yagi *et al.*, 2010)

1.4 Microbiota DNA extraction and sequencing

DNA was extracted from caecum samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.). This included a 20 minute bead beating step utilising the supplied PowerBead tubes. Out of the 100 samples, 80 samples were selected after a two-step PCR amplification was performed for quality control. The first round PCR utilised the bacterial 16S rRNA primers 515F and 806R (16S region V4 primers (Caporaso et al. 2011)). Cycling conditions were 95°C for 3 minutes, followed by 25 cycles of 98°C (20 secs), 60°C (15secs), 72°C (20secs), and a final extension at 72°C for 5 minutes. The second stage PCR used Illumina Nextera PCR library preparation kit containing Illumina adapters. The cycling conditions were 95°C (3mins), followed for 15 cycles at 95°C (30secs), 55°C (30secs), 72°C (30secs) followed by extension at 72°C for 5 minutes. Following the two step PCR, MiSeq sequencing was carried out using MiSeq V3 reagent kit and 500 sequencing cycles. Samples with less than 15,000 sequences were removed to minimise the loss of sequence information associated with sub-sampling the dataset. Sequences were clustered at 97% identity threshold and chimera removal was performed using USEARCH/UCHIME (Edgar et al. 2011). The resulting OTU sequences were classified in Mothur (Schloss et al. 2009) using the Silva reference files (Quast et al. 2013), with a confidence threshold of 80%, and eukaryotic, archaeal, mitochondrial and plastid sequences, along with those which were unclassified at the domain level, were removed. Paired-end reads were quality checked using FastQC ("Andrews 2010 Fastqc," n.d.), low-quality regions trimmed and only sequences with Q30 >90% were kept, and finally, sequences were merged using BBmerge (Bushnell, Rood, and Singer 2017).

All data analysis was carried out using R (R version 3.5.0; 2018-04-23) using the packages Phyloseq (McMurdie and Holmes 2013) and Vegan (Oksanen et al. 2015). Differences in number of reads between caecum samples was accounted for by dividing each sequence count by the total number of reads in that sample, yielding relative abundance measures (McMurdie and Holmes 2013).

1.5 Statistical analysis

Body weight residuals were calculated using the raw body weight measures as the dependent variable in a linear model and including snout to vent length and sex as effector variables. The residual values obtained would either be negative or positive indentifying animals which were lower than average weight for their length and sex or heavier than average respectively.

Alpha diversity, the number of different OTUs within a sample and evenness of the sample, was calculated using both Shannon and Simpson indices. Results obtained from both the Shannon and Simpson indices were qualitatively similar; we only present the Shannon index outputs here. A linear model was used to investigate the associations between Shannon index and host variables: sex, age (based on eye lens weight and converted into age in days), body weight residuals (as a proxy of condition), the trapping location of the host, *T. muris* intensity (log transformed), *S. obvelata* intensity (log transformed) as well as vegetation at trapping site. The overall prevalence of parasites was then calculated island-wide. Furthermore, the potential associations between *S. obvelata* presence and intensity and the phylum Firmicutes was analysed using a linear model, which included the logged number of OTUs belonging to the phylum Firmicutes as dependent variable and *S. obvelata* counts as the independent variable, and

similarly the potential relationship between the phylum Bacteroidetes and *T. muris* was investigated.

Beta diversity, the similarity of OTU composition between caecum samples, was measured using UniFrac distance, a measure which takes phylogenetic distances into account (Lozupone et al. 2005), and Bray--Curtis dissimilarity index. Beta diversity was compared to host and environmental variables using permutational multivariate analysis of variance (Anderson 2001) based on the distance tables. This was done using the Adonis function in R (Oksanen et al. 2015) with sex, age, *T. muris* and *S. obvelata* infection (log transformed), trapping location and health condition of the animals – measured using weight residual as a representative of condition – as predictor variables and Bray-Curtis dissimilarity index or UniFrac distance index as the response. In most instances Bray-Curtis and UniFrac distance measures agreed with each other and in these cases for simplicity only the result from Bray-Curtis is shown. One instance of a difference in outcome between the two measures is highlighted in the results section. P values were estimated by comparing pseudo-F values against 999 random permutations of the data (Anderson 2001). We then followed up the significant results from our analysis of beta diversity by testing for differential expression among OTUs in the relevant variables (trapping location and sex) using the DESeq2 package in R (Love et al. 2014). To identify OTUs driving the difference between males and females a Wald test was performed on the log fold change data. To analyse the differences in OTUs between trapping sites a likelihood ratio test was performed comparing a full model which included both sex and location and a reduced model which only contained sex. An analysis of deviance captures the difference in likelihood between the full and the reduced model.

To investigate the effect of physical distance between traps, a Mantel test was performed comparing distance matrices from the GPS trap location data and the microbiota community data from the caecum samples.

A mantel test was performed to compare two matrices, the beta diversity of the microbiota using Bray Curtis dissimilarity values and the cytokine gene expression of the MLN using log10 of the delta delta CT values, to investigate significant associations between the two matrices. Principal Coordinates Analysis (PCoA) was used as a distance based ordination method to produce PC1 and PC2 using the Bray-Curtis distance to use for plotting the variables.

1.6 Mice sampled in 2016

After completing our initial study of the gut microbiota of mice sampled on the Isle of May in October 2015 we collected another set of data the following year in April and October 2016. The field work, trapping of animals, culling, processing and storing of the samples was carried out the same way as described for the 2015 samples above. DNA extraction of the caecum samples for sequencing analysis was also performed in the same way as with the 2015 samples. Differences only occurred in the processing and sequencing of the caecum DNA samples. The same V4 region of 16s was amplified using the primers described by Caporaso et al. (2011). Instead of 25 cycles of PCR a total of 10 cycles was used. 5 µl of each DNA sample at 1ng/µl was entered into the first-round PCR with conditions 98$_{o}$C for 2 minutes, 20 seconds at 95$_{o}$C, 15 seconds at 65$_{o}$C, 30 seconds at 70$_{o}$C followed by a 5 minute extension at 72$_{o}$C. Second stage of the PCR and the purification steps were the same as the 2015 samples. Instead of using screen-tape to assess the average size and quality of the amplicon library the High Sensitivity DNAKit on either the Agilent Bioanalyzer or the Advanced Analytical

Fragment Analyzer was used. Finally, amplicon libraries were sequenced on the Illumina HiSeq 2500 platform instead of the MiSeq platform. A full report was supplied by the University of Liverpool's metagenomics team and can be found in the appendix (Appendix 2).

As the samples collected in 2016 differ in their metagenomics analysis methods compared to the 2015 data set it is not possible to directly compare or merge the two data frames. Instead the 2016 data serves as a validation to the results and associations found in 2015 and adds further information through the inclusion of seasonality.

2. Results

Following removal of unusable samples, caecal microbiota data were obtained from a total of 79 mice in 2015 and 93 mice in 2016 across the same sampling sites. More detailed information about the data such as the number of females captured each year as well as numbers of males and females captured at the sampling sites can be found in Table 2.

Table 2. More detailed information about the collected data, including the number of females and males sampled each year and split across each sampling site. Also, the number of male, female, ju
mature mice infected with the parasites *Trichuris muris* and *Syphacia oblevata.*

Number of mice	Sampled	Burnet's Leap	Three Tarns Nick	Low Light	Holyman's Road	Main Light	High Tarn	Kettle	Byres	Fluke Street	Priory	South Horn	Infected *Trichuris muris*	In *Sy ob*
2015 Females	21	3	0	0	3	1	1	1	2	4	3	3	10	
2015 Males	58	4	2	5	3	7	8	8	6	3	5	7	16	
2015 Juvenile	28	2	0	2	2	3	4	2	3	2	5	3	4	
2015 Mature	51	5	2	3	4	5	5	7	5	5	3	7	22	
2016 Females	36	3	4	2	2	3	2	2	0	8	3	7	17	
2016 Males	57	9	6	6	2	4	8	2	3	10	7	0	21	
2016 Juvenile	35	6	7	2	3	3	4	2	3	2	2	1	12	
2016 Mature	59	6	3	6	1	4	6	2	0	17	8	6	26	

Sequences obtained were clustered into a total of 3006 OTUs with 579 OTUs having 100 or more reads across all samples, and the highest number of reads being 235268. The mean number of reads per caecum sample/mouse was 68839. Across all samples the phyla Firmicutes, Bacteroidetes and Proteobacteria were the three most abundant at an average of 53%, 30.3% and 9.9% respectively (Fig 2). Sequences from 2016 had some differences to the 2015 sequencing data set with a total number of 661 OTU clusters and a mean of 262307 reads per sample. The most dominant phyla across individuals were still Firmicutes, Bacteroidetes and Proteobacteria.

Investigating the 2015 mice and 2016 mice data sets separately showed that alpha diversity did not vary significantly with sex, age (based on lens weight converted to age in days), trapping location, weight residuals of the host, *S. obvelata* presence or vegetation at trapping site in either year. *T. muris* infection was found to be associated with alpha diversity in 2016 but not in 2015 (Table 3). Overall, these findings suggest a stable alpha composition across our population in 2015 (residual standard error = 0.57, df = 63, F = 0.53, $p > 0.05$) as well as in 2016 (residual standard error = 0.72, df = 73, F = 1.54, $p = 0.11$).

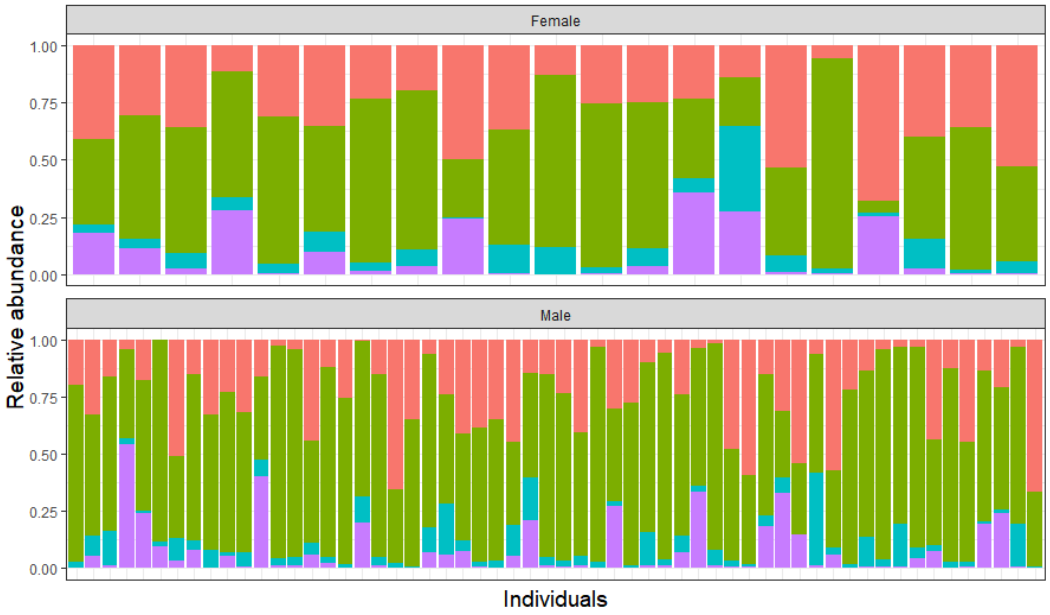


Figure 2. The relative abundance of bacterial phyla in the microbiota of wild mice captured in 2015. Individual labels are individual mice along the x-axis and relative abundance of most common phyla along the y-axis. The figure is divided into two panels containing females in the top panel and males in the bottom panel. The phylum Bacteroidetes is represented in red at the top, Firmicutes are shown as green, Proteobacteria at the very bottom are shown in purple. Any other phyla were grouped together and are represented in blue.

Table 3. Variables of the individual host as well as its environment compared to microbiota alpha diversity using a linear models (separate models for each year). None of the variables tested showed a significant association with alpha diversity measure (Shannon index).

Year	2015			2016		
Variable	Standard Error	p-value	Parameter estimates	Standard Error	p-value	Parameter estimates
Trapping Location	0.89	0.63	-0.41	0.40	0.72	-0.21
Sex (Male)	0.061	0.93	-0.37	0.32	0.40	-0.15
Age	0.046	0.91	-0.027	0.002	0.53	0.001
Weight Residuals	0.016	0.69	-0.41	0.04	0.21	0.05
Trichuris muris intensity (log)	0.12	0.21	-0.14	0.09	0.08	-0.16
Syphacia obvelata intensity (log)	0.032	0.43	0.78	0.12	0.39	-0.1
Dominant Vegetation (*Silene maritima*)	0.45	0.89	0.12	0.17	0.7	0.06
Season (Spring)	N/A	N/A	N/A	0.28	0.33	-0.27

2.1 Trapping location has a significant association with OTU composition

Microbiota OTU composition showed a significant association with trapping location when using a permutational multivariate analysis model based on Bray-Curtis dissimilarity index. The model was used to test associations between the OTU composition of the host, representative of beta diversity, and the predictor variables sex, age, trapping location, *T. muris* and *S. obvelata* infection (log transformed) and overall weight residuals (as a measure of condition) (Table 3). Trapping location accounts for 15% of total microbiota OTU variation ($R^2 = 0.15$, $p = 0.007$, $df = 10$; see Fig 3). Trapping location is the environmental variable that explains the most variance in OTU composition. A further 2% of overall variation can be explained by sex (Table 4).

We used differential expression analysis (DESeq2) to investigate which taxa were driving the observed differences in microbiota among trapping locations in 2015. Trapping location was significantly associated with relative abundance of 123 OTUs. A large number of these OTUs belong to the Lachnospiraceae (48 OTUs) some belonging to the genera *Roseburia, Dorea, Clostridium-XlVa* and *Clostridium-XlVb*. Overall Lachnospiraceae account for 9.9% of all OTUs indentified in 2015, whilst Ruminococcaceae and Clostridiales account for 3% each. The majority of OTUs in this study were grouped as unclassified bacteria (73.3%).

We did not find any significant association between GPS trap location data and the microbiota composition of the caecum (Mantel statistic $r = 0.03$, $p = 0.27$). These results are partially supported by separate analysis of 2016 individuals which similarly show that trapping location and OTU composition are significantly

associated (R^2 = 0.13, p = 0.0002, df = 9; Fig. 3B). In the 2016 individuals no significant association was detected between beta diversity and sex of the host (R^2 = 0.013, p = 0.15, df = 1).

Table 4. Environmental and host variables were compared to OTU composition (Bray-Curtis index) using a permutational multivariate analysis (Adonis) for individuals captured in 2015.

Variable	R^2	p-value	Degrees of freedom
Trapping Location	0.15	0.009 *	10
Sex	0.018	0.034 *	1
Age (in days)	0.01	0.42	1
Weight Residuals	0.01	0.89	1
Trichuris muris infection (log)	0.013	0.51	1
Syphacia obvelata infection (log)	0.012	0.58	1

Table 5. The bacterial families identified using a likelihood ratio test driving the difference between trapping sites and the number of OTUs belonging to each family.

Family	No. of OTUs
Bacteroidaceae	3
Clostridiales	11
Firmicutes	6
Helicobacteraceae	2
Lachnospiraceae	48
Porphyromonadaceae	5
Ruminococcaceae	10
Rikenellaceae	3
Others	7
Unclassified	29

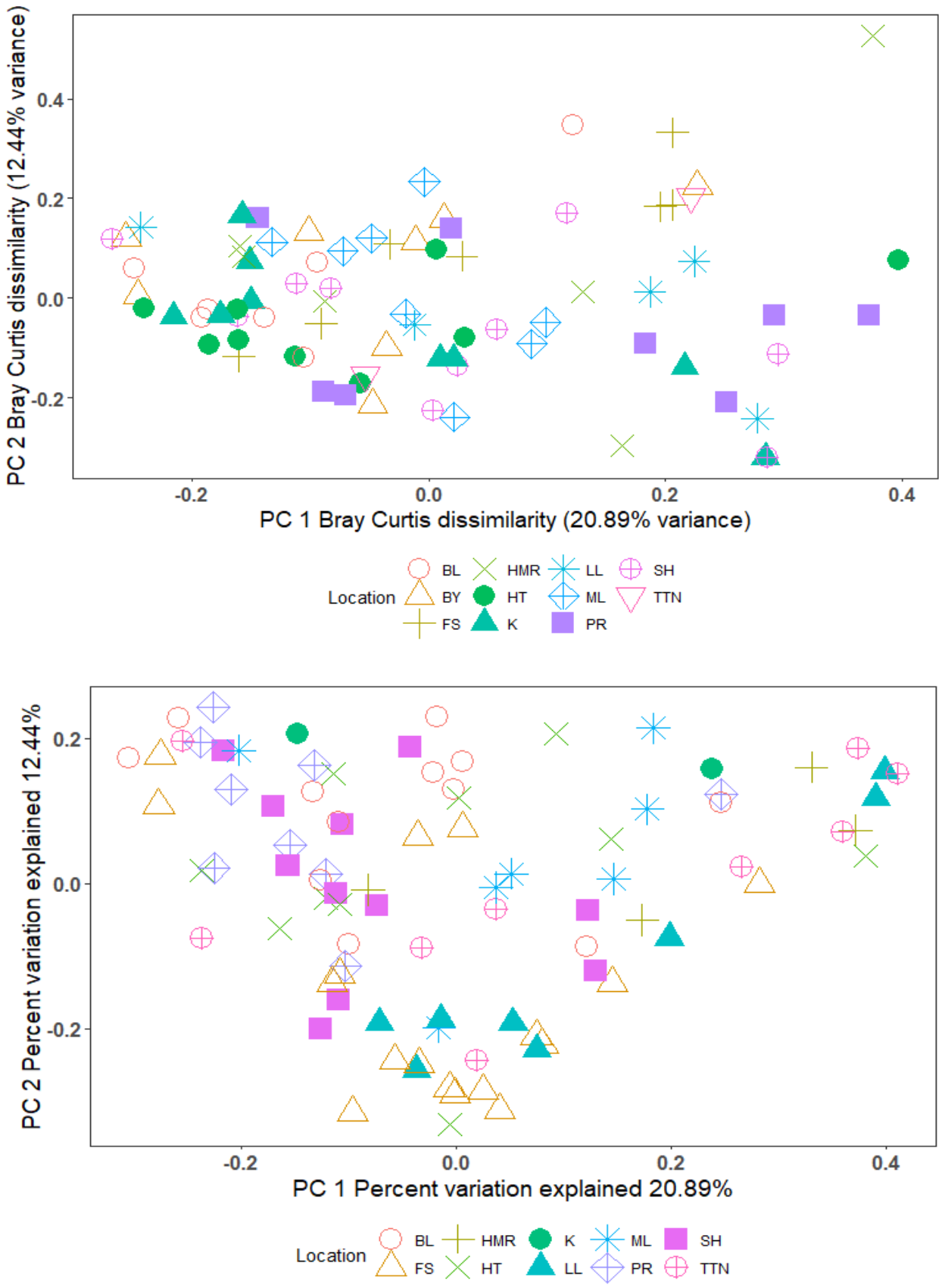


Figure 3. Bray Curtis dissimilarity was used to calculate the distance between individuals' OTU composition using PC1 and PC2 for plotting ordination. PC1 contributes 20.89% of variance while PC2 contributes 12.44% to variance. Locations are shown as separate colours and shapes to show differences between the sites but also visualise the differences and similarities of individuals within sites. The OTU composition varies significantly between trapping locations across the island. The top panel shows trapping location differences for mice caught in 2015, the bottom panel for mice from 2016.

2.2 Seasonality has significant association with microbiota composition

As sampling in 2016 was carried out over two separate months, April and October, we analysed seasonal differences by comparing beta diversity (using Bray Curtis dissimilarity) to "Spring" and "Autumn". This was carried out as part of a permutational multivariate analysis model based on Bray-Curtis dissimilarity measures which included sex, age and weight residuals of the host along with *T. muris* and *S. obvelata* infection (log value) and trapping location (Table 5). Seasonality was found to be significantly associated with beta diversity of the caecum microbiota in 2016 (R^2 = 0.03, p = 0.045, df = 1).

We used differential expression analysis (DESeq2) to investigate which taxa were driving the observed differences in microbiota between spring and summer trapping sessions in 2016 (Table 7). Seasonality was significantly associated with relative abundance of 163 OTUs more commonly found in summer and 35 OTUs more abundant in spring. A large number of these OTUs belong to the Lachnospiraceae (75 OTUs in summer and 8 OTUS in spring), some belonging to the genera *Roseburia, Dorea, Coprococcus* and the *species Ruminococcus gnavus.* Ruminococcaceae OTUs were also very abundant, especially in summer (42 OTUS) compared to spring (3 OTUs). Overall, Lachnospiraceae account for 27.8% of all OTUs indentified in 2016, whilst Ruminococcaceae account for 15.4%. In the 2016 data frame 29.9% of OTUs were unclassified bacteria.

Table 6. Environmental and host variables were compared to OTU composition (Bray-Curtis distance) in individuals sampled in 2016 using a permutational multivariate analysis (Adonis).

Variable	R^2	p-value	Degrees of freedom
Trapping Location	0.12	0.0026**	9
Sex	0.013	0.15	1
Age	0.012	0.22	1
Weight Residuals	0.013	0.18	1
Trichuris muris infection (log)	0.014	0.10	1
Syphacia obvelata infection (log)	0.011	0.47	1
Season	0.032	0.045*	1

Table 7 . DESeq2 analysis was performed to identify OTUs at Family level which had higher relative abundance in spring compared to summer trapping sessions and vice versa.

More abundant in summer		More abundant in spring	
Family	No. OTUs	Family	No. OTUs
Clostridiaceae	3	Bacteroidaceae	13
Enterobacteriaceae	4	Clostridiaceae	3
Helicobacteraceae	4	Lachnospiraceae	8
Lachnospiraceae	75	Lactobacillaceae	2
Lactobacillaceae	4	Porphyromonadaceae	3
Rikenellaceae	7	Ruminococcaceae	3
Ruminococcaceae	42	Other	3
S24-7 (Bacteroidales)	12		
Unclassified	12		

2.3 Cytokine gene expression in the mesenteric lymph node is not significantly associated with beta diversity of the caecal microbiota

A mantel test, which compares two matrices, based on Bray-Curtis index was performed on the gene expression data from the MLN and the microbiota data from the caecum for both the 2015 data set and the 2016 data set respectively. 11 genes were used but no significant association was found between MLN gene expression and microbiota composition in either 2015 (Mantel statistic r = 0.05, p=0.176) or in 2016 (Mantel statistic r = -0.087, p=0.983).

2.4 OTU composition of individuals shows potential differences between the sexes and age

Using the Bray-Curtis dissimilarity index a significant difference in OTU composition was found between male and female mice in 2015 (Adonis, $R^2 = 0.02$, p = 0.04, df = 1; see Fig 3B). However, utilising a weighted UniFrac distance instead of the Bray-Curtis index no significant association was found in the model using the same variables (Adonis, $R^2 = 0.014$, p = 0.17, df = 1). Age, parasite burden and weight residuals all showed no significant association with the beta diversity (Table 3).

Differential expression analysis was utilised to establish which OTUs occurred at significantly different proportions in females compared to males in 2015 (Table 8). Out of 14 OTUs which were found to be significantly more abundant in males compared to females, 7 belonged to the family Lachnospiraceae, specifically belonging to the genera *Robinsoniella, Blautia, Clostridium-XlVb* and *Clostridium-XlVa*. Two of the OTUs belonged to the Ruminococcaceae family, one of which was identified as part of the genus *Pseudoflavonifractor*. Females had a total of 31

significantly more abundant OTUs compared to males, 5 of which belonged to the family Lachnospiraceae. Four of the OTUs which were more abundant in females belonged to the family Clostridiaceae and another 4 OTUs belonged to the Porphyromonadaceae (Table 8).

Individuals captured in 2016 showed no significant association between sexes utilising both Bray Curtis and UniFrac distance measures (Bray Curtis: $R^2 = 0.013$, $p = 0.22$, $df = 1$). The age of the host was not significantly associated with OTU composition either in individuals captured in 2016 ($R^2 = 0.013$, $p = 0.22$, $df = 1$; Table 6).

Table 8. DESeq2 analysis was performed to identify OTUs at Family level which had higher relative abundance in males compared to females and vice versa.

More abundant in males		More abundant in females	
Family	No. OTUs	Family	No. OTUs
Lachnospiraceae	7	Bacteroidaceae	4
Ruminococcaceae	2	Clostridiaceae	4
Erysipelotrichaceae	1	Lachnospiraceae	5
Clostridiales	1	Porphyromonadaceae	4
Peptostreptococcaceae	1	Proteobacteria	1
Rikenellaceae	1	Unclassified	7
Unclassified	1	Other	4

2.5 Infection with parasites does not significantly associate with microbiota composition

The most commonly found endoparasite species were *T. muris* and *S. obvelata* and commonly found ectoparasites were the fur mites *Radfordia affinis, Myocoptes musculinus* and *Myobia musculi* and the northern rat flea (*Nosopsyllus fasciatus*) (Table 9).

Table 9. The percentage of infected mice (prevalence) for the different, commonly found parasites.

	2015	**2016**
Parasite species	**Prevalence (%)**	**Prevalence (%)**
Trichuris muris	40.5	51
Syphacia obvelata	57	14.6
Radfordia affinis/ Myobia musculi	97.5	95.8
Nosopsyllus fasciatus	22.8	55.2

Using a linear model we specifically tested for changes in the abundance of OTUs belonging to Firmicutes against observed *S. obvelata* presence, sex and age of the host but found no significant associations (residual standard error = 0.124, df = 73, $F = 1.38$, $p > 0.05$). We similarly tested for an association between Bacteroidetes abundance and *T. muris* prevalence but found no significant associations between variables (residual standard error = 0.13, df = 73, $F = 1.38$, $p > 0.05$).

3. Discussion

Here, we report a significant association between the beta diversity of the wild mouse gut microbiota and the trapping location of the individuals. Dominant vegetation species at trapping locations did not directly impact the composition of the gut microbiota; however, the exact vegetation composition may differ among our trapping locations and could potentially lead to differences in dietary profiles of individual mice. Differences in the microbiota composition of the gut in wild mice have commonly been shown to be associated with geographical location. However, sampling distances in wild studies range from up to 100km between trapping sites (Weldon et al. 2015) to different countries (Linnenbrink et al. 2013). This means results will be confounded by local differences, such as host genetics, local infections and vegetation, among mouse populations (Weldon et al. 2015; Linnenbrink et al. 2013; Maurice et al. 2015b). The furthest distance between our trapping sites on the Isle of May was 1.5km, on average our sites were spaced 100m apart and the mouse population is believed to be able to roam freely across the whole island with male and female territories spanning between $100m^2$ up to $400m^2$ (Berry et al. 1991). Despite the small spatial scale of the present study, trapping location was significantly associated with gut microbiota composition in our mouse population. There was, however, no significant association between the geographical distance among the individual traps and the microbiota composition. Therefore, while we observe geographical variation in gut microbiota, this variation is not directly related to distance.

There are several potential explanations to account for the small-scale locational differences and the observed variation in caecal microbiota composition. Animals living in close proximity are likely to be both genetically related and exposed to

similar bacteria in the environment. Both male and female mice tend to remain within their home ranges with close overlap in ranges between small groups or pairs of mice (Triggs 1991).

Vertical transmission can occur from mothers and close litter mates (Benson et al. 2010a). Similarly, individuals which are directly related to one another may share a greater proportion of gut microbiota than unrelated animals (Eckburg et al. 2005).

As we did not observe a significant association between inter-trap distances and microbiota composition, it seems unlikely that either genetic relatedness or vertical transmission could explain our observation. Instead, the explanation that best fits our data is that environmental factors, such as subtle differences in local vegetation and therefore the diet of the mice across the island, are driving these differences.

OTUs belonging to the families Lachnospiraceae and Ruminococcaceae were identified as potentially shaping differences in microbiota composition among trapping sites. Both Ruminococcaceae and Lachnospiraceae are involved in the breakdown of complex plant materials (Biddle et al. 2013), such as cellulose, and are able to process plant polysaccharides (Flint et al. 2008) and have been associated with changes in diet in response to high plant and fibre content (Amato et al. 2016; Love et al. 2014; Maurice et al. 2015b). The specialised role of these bacterial families could suggest a significant difference in available fibre and vegetation among the trapping sites of the present study which drives the difference in microbiota communities observed. Another study on wild house mice identified *Bacteroides* (Bacteroidaceae), *Robinsoniella* (Lachnospiraceae) and *Helicobacter* (Helicobacteraceae) as being significantly impacted by geographical

variation of trapping site (Linnenbrink et al. 2013). OTUs identified as being influenced by sampling site in the present study also belong to these same families.

The gut microbiota in wild mice captured on the Isle of May is made up of the same common phyla as other rodent studies: Bacteroidetes, Firmicutes and Proteobacteria predominated and are the most common bacterial phyla across most mammalian species (Linnenbrink et al. 2013; Wang et al. 2014; Weldon et al. 2015). Firmicutes have been found to be the most common phylum within wild rodent microbiota compared to laboratory-reared individuals which often display higher levels of Bacteroidetes (Rogers et al. 2014; Weldon et al. 2015). This difference in dominant phylum has been shown to be associated with differences in available diet or even access to food or periods of fasting which have been shown to potentially increase the relative abundance of Bacteroidetes (Kotzampassi et al. 2013). The diet of many wild rodents changes with season, from plant- and seed-based to insect-based depending on food availability (Maurice et al. 2015b). In particular *Lactobacillus* was found to be significantly increased in a population of wild wood mice during spring compared to autumn and winter, which corresponds to an increase in available insects, whilst *Alistipes* and *Helicobacter* were shown to be more abundant in autumn than in spring, which coincides with an increase in available seeds and a possible shift in the mouse diet (Maurice et al. 2015b). It was found that the amount of food consumed differed between spring and autumn may also impact on the composition of the gut microbiota (Maurice et al. 2015b). The present study found a significant increase in the abundance of OTUs belonging to *Helicobacter* during summer sampling compared to spring sampling whilst Bacteroidaceae was found to be more abundant in the spring samples. The present study also found a

significant effect of seasonality on the beta diversity of the caecal microbiota when comparing mice sampled in April and those sampled in October. This finding is supported by other captive and wild rodent studies finding similar patterns (Maurice et al., 2015; Li et al., 2016; Stevenson et al., 2014). A survey on wild living wood mice (*Apodemus sylvaticus*) showed a significant decrease in *Lactobacillus* levels whilst *Alistipes* and *Helicobacter* increased during autumn (Maurice et al., 2015). A possible explanation for this observation could be the changes in diet as it shifts from a predominantly insect based diet to mostly grain based during colder months (Maurice et al., 2015). Similar seasonal shifts in the microbiota composition were identified in black howler monkeys (*Alouatta pigra*) when diet shifted from a predominantly fruit based diet to reduced fruit intake and higher leaf consumption in colder months (Amato et al., 2015; Amato et al., 2016).

The age of the host did not show any significant association with microbiota composition, which is unsurprising as both human and murine studies have suggested stability of the microbial composition over time within these host species (Eckburg et al., 2005; Benson et al. 2010b). Significant differences in microbiota compositions are only noted during infancy in the first few weeks and months of life (Palmer et al. 2007). However, as we only sampled individuals post-weaning it is unlikely that we would have sampled individuals young enough to note a difference with age.

Sex driven differences in the composition of the gut microbiota have been found in mice but are often reported as weak (Maurice et al. 2015b) to non-existent or negligible (Weldon et al. 2015; Kreisinger et al. 2015). Whilst we observed no differences in the alpha diversity on the Isle of May associated with sex, the OTU

composition – the beta diversity – of the gut microbiota does show a weak association. The difference in OTU composition between male and female mice might be explained by differences in their diet. Though there is no evidence that male and female mice exhibit different foraging behaviour, there are differences in the effect diet has on the microbiota between sexes in both fish and mice (Bolnick et al. 2014). Males showed an overall greater change in OTU abundance after a change in their diet than females (Bolnick et al. 2014). These findings are, however, based on the assumption that males and females consume the same diet. Wild plateau pikas (*Ochotona curzoniae*) were found to show distinctly individualised foraging preferences within the same sampling location which impacted their gut microbiota composition (Li et al. 2016). A possible explanation could be differences in food preference between male and female wild rodents (Li et al. 2016; Bolnick et al. 2014).

Another possible explanation for compositional differences between the sexes would be hormone-driven changes leading to differences in the microbiota (Markle et al. 2013; Org et al. 2016). Hormonal effects are difficult to establish as they were shown to be obscured by variation in host genetics and could only clearly be established in individuals belonging to the same genetic strain (Org et al. 2016). The present study cannot conclusively establish a significant association between beta diversity and sex as this association was not observed when utilising weighted UniFrac distance. Whilst Bray-Curtis dissimilarity compares the differences in relative abundance between two sites, or two individuals, weighted UniFrac also considers phylogenetic distances. The lack of significant association with UniFrac distances may indicate that while OTU counts differ between males and females, the taxa present are similar and therefore phylogenetic distances are not large.

No significant interaction was found between caecal microbiota composition and the immune gene expression in the mesenteric lymph node (MLN) in the present study. The lack of significant association could be due to the relatively small number of immune genes (N = 11) utilised. As the MLN closely interacts with live commensal bacteria being carried by dendritic cells (Macpherson et al., 2006) a relationship between resident microbes in the gut and the MLN would be expected but has not been extensively studied, especially in wild living rodents.

Based on the results from other studies (Hayes et al. 2010; Maurice et al. 2015b; Kreisinger et al. 2015) we expected to see that individuals testing positive for the presence of parasites – in particular *S. obvelata* and *T. muris* – would show differences in their microbiota composition compared to non-infected individuals. The presence of *T. muris* infection in mice has also been associated with an increase in the abundance of the *Lactobacillus* genus in the gut (Holm et al. 2015). Presence of bacteria in the large intestine has been positively associated with the hatching of *T. muris* eggs suggesting that this gastrointestinal parasite requires the chemical stimuli provided by a number of different bacteria (Hayes et al. 2010). Though the present study did not find an increase in the relative abundance of *Lactobacillus* associated with *T. muris* infection, an increase in the total number of bacteria present may still occur but was not investigated as part of this study.

We did not find an association between *T. muris* and *S. obvelata* infection and the alpha or beta composition of the microbiota in Isle of May mice. This may be due to sampling only one site of the gastro-intestinal tract. The presence of nematodes in the gut has been shown to influence the gut microbiota in a very gut site specific manner (Kreisinger et al. 2015). One of the most notable interactions between parasite and microbiota composition occurred in the small

intestine of individuals infected with the tapeworm *Hymenolepis nana* where there was an increase of the *Bacteroidetes* species S24-7 (Kreisinger et al. 2015). Similarly, laboratory reared C57BL/6 mice showed significant shifts in their microbiota composition when artificially infected by *Heligmosomoides polygyrus bakeri* (Walk et al. 2010). The changes in the microbiota composition could, however, only be identified in the ileum which is the helminth's typical niche in its host (Walk et al. 2010). Microbiota samples from the caecum of the infected host did not show any compositional changes suggesting that helminth infection affects the immediate surrounding gut microbiota but not necessarily throughout the gastrointestinal tract (Walk et al. 2010; Kreisinger et al. 2015). By sampling multiple sites within the mouse gut we may in the future be able to establish associations between different gut endoparasite infections and the gut microbiota composition.

Collecting detailed vegetation data at our sites as well as analysing gut contents to identify the diet of mice would help in establishing causality between dietary differences and microbiota compositional changes. Additionally, collecting longitudinal data would give a greater insight into variation over time allowing observation of changes occurring in host's biology such as in parasite infections or body condition and comparing them to differences in microbiota composition. As well as determining causality for geographical differences in the microbiota, longitudinal monitoring could offer an insight into fitness benefits of microbiota composition when considering reproductive success and longevity of the host.

In the present study the relative abundance of bacterial OTUs was utilised to investigate associations with environmental and biological variables. In some contexts, analysing the absolute abundance of bacterial taxa can give greater ability to distinguish among individuals. For example, differences between

patients suffering from Crohn's disease and healthy individuals were associated with absolute changes in bacterial numbers (Vandeputte et al., 2017). However it was not possible to examine differences in absolute abundance of bacteria with the data gathered in this study as cell counts and qPCR assessment of bacterial loads would have been needed for this type of analysis. Furthermore, by focusing on relative abundance we have been able to compare our results more directly to similar studies as it is currently a more common approach.

In summary, we found that there is significant variation in gut microbiota diversity even over very small distances which highlights geographical location and relative distance between sampling sites as an important consideration for future microbiota studies.

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Chapter 2: The gut microbiota composition in wild voles (*Microtus agrestis*) varies among tissue sampling types, sexes, time spent in captivity and is associated with pro-inflammatory immune expression in the mesenteric lymph node

Abstract

In this study we explore how variation in the proximal colon and faecal microbiota of *Microtus agrestis* in Kielder Forest, in Northumberland, is associated with intrinsic and extrinsic factors, especially the immune response of the host. The present study focuses on the interactions between gut microbiota composition and the immune gene expression of the cells of the mesenteric lymph node. The interactions are tested utilising two different microbiota samples: faecal pellets and proximal colon samples from the same individual. The difference in beta diversity was shown to be significantly correlated with the sample type, either faecal pellet or proximal colon, taken from the same individual. The time of collecting the samples was also found to be significantly associated with microbiota composition when comparing voles sampled within 24 hours of capture and voles kept in the laboratory for up to 48 hours. Alpha diversity of the gut microbiota was not found to be significantly associated with host variables, namely sex, age, sample tissue type and weight residual of the animal nor was it associated with the trapping season. The beta diversity of the proximal colon of individuals sampled within 24 hours was found to be significantly associated with the cytokine gene expression of cells in the mesenteric lymph node (MLN). Faecal samples and the colon samples taken from individuals being housed in the laboratory for up to 48 hours were not found to be associated with the gene expression of the MLN. When investigating OTUs driving the observed differences

between the tissue samples and the time at which samples were collected, a number of different families were identified as significantly driving the difference especially Lachnospiraceae and Ruminococcaceae. Sex of the host was also shown to be significantly associated with the beta diversity of the proximal colon whilst the effect of sampling season could not be conclusively determined.

1. Introduction

The mammalian microbiota has evolved alongside its host to perform a variety of essential functions which include the digestion of complex carbohydrates (Fabich et al. 2008), the production of vitamins (LeBlanc et al. 2013), stimulation of the intestinal immune system (Round and Mazmanian 2009) and most importantly protection of the intestines and gut from colonisation by pathogenic microbes (Kamada et al. 2013). Maintaining a healthy gut microbiota is therefore essential for the host's health. This study aims to identify firstly which environmental and host-specific variables are associated with changes in the gut microbiota composition and diversity and secondly any associations between the gut microbiota composition and immune response in the host.

1.1 Variables affecting the gut microbiota

Changes in seasonality, which affect vegetation and can affect available food sources have been associated with notable differences in gut microbiotal composition (Amato et al. 2016; Maurice et al. 2015). Limited availability of fruit was shown to drive a reduction in the abundance of *Ruminococcaceae* in black howler monkeys (*Alouatta pigra*) (Amato et al. 2016). The energy intake from the diet was reduced due to shifts in food availability. For example, a change in

energy production in the gut microbiota, specifically an increase in the production of short-chain fatty acids, was observed (Amato et al., 2015; Amato et al. 2016).

It has been suggested the microbiota acts as a buffer during periods of low food intake by increasing those bacterial groups capable of harvesting more energy from the limited food supply (Amato et al. 2016). The same study also suggests that changes in the microbiota were driven by seasonality. Animals, which exhibited a significant change in their gut microbiota composition over the course of 10 months, did not show any changes in their habitat and space use. Nor were there any other alterations in their overall behaviour leaving seasonality and potential changes in diet as the only remaining explanatory, variables influencing the gut microbiota (Amato et al. 2016). Seasonality was also identified as a significant driver of changes in gut microbiota composition in wild wood mice (*Apodemus sylvaticus*) (Maurice et al. 2015).

Housing a wild caught animal in a laboratory setting has been shown to impact the composition and reduce the diversity of the gut microbiota as shown by a study on woodrats (*Neotoma lepida*) which lost 19% of their microbes after being housed in a laboratory for two weeks (Kohl and Dearing 2014). This change in diversity and composition may be due to the change in diet from a wild seed-based diet to laboratory chow pellets, as well as other differences in their environment (Kohl and Dearing 2014). The short-term effect of housing wild caught animals for a period less than 48 hours has not been explicitly investigated. However, investigating the possible effects of short-term changes in environment would be potentially interesting as changes may occur due to changes in the diet (Carmody et al., 2015) or possibly by increased levels of stress (O'Mahonay et al., 2008; Cryan and Dinan, 2012). Stress is associated with increased levels of cortisol

which in turn can alter gut permeability and barrier function, and change gut microbiota composition (Cryan and Dinan, 2012).

Sex has been linked to differences in gut microbiota diversity in a few different mammalian studies (Org et al. 2016; Dominianni et al. 2015; Bolnick et al. 2014; Yurkovetskiy et al. 2013). Male and female laboratory mice showed a distinctly different microbiota composition when being fed different types of diet (Org et al. 2016; Bolnick et al. 2014). A standard chow diet was compared to a high fat and sugar diet (Org et al. 2016). A clear separation could be observed between the two diets when using Bray-Curtis dissimilarity index and males and females within each diet type also showed a distinct separation in their microbiota compositions (Org et al. 2016). A similar sex and diet dependent difference in microbiota composition was found in laboratory stickleback (*Gasterosteus aculeatus*) (Bolnick et al. 2014). The diet-dependent differences in microbiota composition may be due to differences in the genetic composition of the host as shown by a human study which found an association between host genetics and diet in regulating microbiota composition (Bonder et al. 2016).

A study on different genetic strains of laboratory mice showed a difference in the composition of gut microbiota between males and females within the same genetic strain (Org et al. 2016). *Actinobacteria* and *Tenericutes*, for example, were shown to be more abundant in males compared to females. However, these findings were strain specific and may also be due to genetic differences between the mice (Org et al. 2016). A study on human subjects did identify several genetic markers associated with microbial pathways, which would suggest that the composition of the microbiota can at least in part be a heritable trait influenced by the individual's genetic make-up (Bonder et al. 2016; Goodrich et al. 2014).

Molecular profiling of laboratory mice has revealed some candidate genes which play a part in the modulation of microbial abundance either specifically linked to a taxon or influencing the variation of taxa across an entire phylum (McKnite et al. 2012). Using a Quantitative Trait Loci (QTL) approach, a region on chromosome 4 which overlaps with a number of interferon genes has been associated with variation in the abundance of *Bacteroides* whilst Irak4, a signalling molecule in the Toll-like receptor pathways has been identified to influence Rikenellaceae (McKnite et al. 2012). Tgfb3, a cytokine modulating the barrier function of the intestine and tolerance to commensal bacteria, was identified as potentially playing a part in the modulation of Prevotellaceae (McKnite et al. 2012).

Another variable impacting the composition and diversity of the gut microbiota is parasite infection. An example in mice is *Heligmosomoides polygyrus* infecting the gut and leading to changes in the conditions of the ileum (Walk et al., 2010). The production of FoxP3 and T cells in the lamina propria and ileum were shown to increase due to the infection with the helminth and lead to a decrease in inflammatory responses (Walk et al. 2010). In turn, a lowering of inflammation in the ileum has been suggested to lead to an increase in *Lactobacilliaceae* as it becomes a more favourable habitat for the bacterium which itself has been associated with anti-inflammatory responses in the gastrointestinal tract (Walk et al., 2010). This would imply microbial-helminth mutualism taking place in the mouse gut as the presence of the helminth creates a favourable environment for the microbes (Walk et al. 2010). A high level of helminth infection in rats resulted in an increase of *Clostridia*, whilst *Bacilli* species declined (McKenney et al. 2015). *Trichuris muris*, frequently found in the caecum, has been shown to create favourable conditions for gram-positive bacteria, such as *Firmicutes* and *Lactobacillaceae*, but decreased abundance of gram-negative bacteria such as

Bacteroidetes (Holm et al., 2015). Treating mice with *Lactobacillaceae* was shown to increase susceptibility to nematodes such as *T. muris* (Dea-Ayuela, Rama-Iñiguez, and Bolás-Fernandez 2008) and *H. polygyrus* (Reynolds, Finlay, and Maizels 2015), potentially due to the down-regulation of IFNγ which had a negative correlation with the presence of *Lactobacillaceae* (Holm et al. 2015). The increase in *Lactobacillaceae* along with the decreased IFNγ response allows for increased *T. muris* infection which in turn gives rise to increased *Lactobacillaceae* infection, further evidence of potential microbiota and helminth mutualism in murine systems (Holm et al. 2015).

1.2 Influence of gut microbiota on host variables

The microbiota in the gut not only potentially interacts with parasite infections or vice versa, but also the immune system and plays an important role in education of the immune system to differentiate between harmful and commensal microbes (Mueller and Macpherson 2006). Toll-like receptors (TLRs) are important. They react to surface components found on bacteria (Mueller and Macpherson 2006; Sansonetti 2004; Backhed et al. 2004). Specific TLRs react to molecules present in specific bacteria; for example TLR4 recognises the LPS produced by gram-negative bacteria, whereas TLR2 responds to gram-positive bacteria (Schiffrin and Blum 2002).

The epithelial barrier and its structure play an important part in preventing disease in the gut by stopping gut microbes from entering the blood stream (Fasano et al. 2000; Moreira et al. 2012). T-box transcription factors, such as Tbx21, regulate the pro-inflammatory response in the gastrointestinal tract and regulate TNF-alpha production which influences the integrity of the epithelial barrier (Garrett et al. 2007). Other important structures in the gut which

contribute to the immune response and interact with commensal bacteria are the mesenteric lymph nodes (MLN) (Macpherson and Smith 2006b) and Peyer's Patches (PPs) (Cebra, 1999).

Gram-positive bacteria, such as *Clostridia*, were found to play an important part in immune response changes, specifically the increased presence of T helper 17 (Th17) cells, a subset of CD4 T helper cells (Kamada et al., 2013). Th17 cells are important in the defence against microbes and are especially densely accumulated in the intestines which would suggest that a gut-intrinsic mechanism might be involved in the production and development of Th17 cells (Kamada et al. 2013). Germ-free mice, which do not have an established gut microbiota and had recently been treated with antibiotics, showed a significant reduction in Th17 cells compared to mice with an intact microbiota (Kamada et al., 2013). Specifically the absence of segmented filamentous bacteria (SFBs) was found to influence the levels of Th17 cells in mice (Ivanov et al. 2009). The presence of SFBs induces the increased production of pro-inflammatory Th17 cells which in turn produce a number of different cytokines such as IL-17 and IL-22 (Ivanov et al. 2009). This up-regulation of pro-inflammatory cytokines can aid in the elimination of pathogens but if misregulated it can also lead to the development of auto-immune diseases (Ueda et al. 2003).

T regulatory cells (Tregs) specifically respond to polysaccharide A which is a component of the cells wall of *Bacteroides fragilis* (Round and Mazmanian 2009), as well as waste products such as short chain fatty acids which are generated by anaerobic respiration of a number of different bacteria. Anti-inflammatory cytokines such as IL-10 are produced by the Treg cells in response to detecting these waste products of cell wall components (Round and Mazmanian 2009). By

interacting with the immune system the microbiota can reduce the development of disease such as colitis by increasing the production of anti-inflammatory cytokines and Treg cells (Manichanh et al. 2012). Natural Tregs are specialised for immune suppression (Sakaguchi et al. 2008), and actively suppress immune responses to pathogens and commensals thus contributing to immune homeostasis in the host (Miyara and Sakaguchi 2007). They act by suppressing the activation, expansion and cytokine formation of CD4+ T cells and CD8+ cells (Miyara and Sakaguchi 2007). The expansion of CD4+ and CD8+ T cells has been associated with autoimmune disease in animals and humans and is linked to disruption of Tregs function and development (Sakaguchi et al. 2008).

Naturally occurring Treg cells express the transcription factor FoxP3 which in turn is necessary for regulating Treg development and function in the host (Ohkura, Kitagawa, and Sakaguchi 2013). To become activated Treg cells will migrate to the lymph nodes where self-antigens or microbial antigens are presented (Sakaguchi et al. 2009). Tregs will suppress inflammatory responses of the lymph structures to commensal- and self-antigens and therefore contribute to immune tolerance (Sakaguchi et al. 2008).

Commensal bacteria found in the gut can potentially become pathogenic when entering the blood stream or if the individual is immune-compromised by lacking an inflammatory response (Chinen and Rudensky 2012). Colonisation with *Escherichia coli* of germ-free mice deficient in IL-10, an anti-inflammatory cytokine, led to development of colitis, whereas wild type mice, normal levels of IL-10, did not develop disease (Kim et al. 2005, 2007). Similarly, the colonisation with non-pathogenic *E. coli* of germ-free mice which had reduced numbers of anti-inflammatory FoxP3 Treg cells, also led to development of colitis in contrast to mice which had normal levels of Treg cells (Waidmann et al. 2003).

1.3 Study site and species of present study

For details on the present study's sampling site and species, field voles (*Microtus agrestis*) from Kielder Forest, please see Introductory Chapter.

2. Aims and hypotheses

We aimed to investigate the diversity and composition of both faecal and proximal colon microbiota in wild living field voles (*Microtus agrestis*) for a comparison of microbiota compositions between different tissue types. We also aimed to identify host and ecological variables influencing changes in the microbial composition. We hypothesised that the sex of the host will have a notable impact on the composition of the microbiota with males and females showing distinct differences in their compositions and relative abundances. Seasonality and the resulting differences in available food for foraging were hypothesised to also have a significant impact on the beta diversity of the microbiota in both the faecal and proximal colon samples. We aimed to identify any significant differences in the composition and taxonomic groups found in faecal samples and proximal colon samples within the same host. Finally, we aimed to investigate the associations between the composition of the microbiota and gene expression of the mesenteric lymph node (MLN). As the mesenteric lymph node plays an important role in the immune response towards commensal bacteria found in the gut we hypothesise that differences in microbiota composition of the proximal colon will be associated with variation in the gene expression of cytokines from the MLN.

3. Methods

3.1 Field work and dissection

The data used in this study were gathered as part of collaboration between the University of Liverpool, Salford University and University of Nottingham. Field work was carried out by a team of technicians from the University of Liverpool whilst the culling and obtaining of cross sectional tissue samples was carried out by individuals based at the University of Nottingham.

Wild-living field voles (*M. agrestis*) were captured in Kielder forest, Northumbria. The animals in this study were all taken from one sampling site. The sampling site consisted of a central trapping grid of approximately 0.375 hectare with 150 Ugglan small mammal live traps laid out and spaced 3-5m apart. A further 100 live traps were spaced at 5-10m apart along the margins of the sample site (Jackson et al. 2011a; Wanelik et al. 2018). Trapping sessions were conducted every two weeks and traps were checked 5 times in 3 days with newly caught individuals receiving a Passive Integrated Transponder (PIT) tag (AVID, UK) for future identification (Taylor et al. 2018). Individual voles caught in the field were sexed, body length was measured and a small blood sample was taken from tail and ear bleeds into 5ml of RNAlater (Fischer Scientific, UK). A faecal pellet was collected from each vole (whenever possible) including re-captured individuals. These faecal samples were stored at -80°C. Lactating or obviously pregnant females were released for ethical reasons (Jackson et al. 2011b). Captured animals randomly selected for destructive sampling were taken to the laboratory and housed in identically set up cages, with fresh water, oats and carrot pieces provided ad

lib. Wood chippings as well as soft bedding for nesting were also provided (Jackson et al. 2011a). Approximately half of the voles were dissected by the next day, after housing them for approximately 24 hours, whilst the rest were processed two days post-capture keeping them in laboratory housing for approximately 48 hours in total. Voles were killed in accordance with HO licence protocols using rising CO_2 levels followed directly by exsanguination. Males and females were categorised as immature or mature based on the appearance of their external reproductive organs (descended testes in males and perforated vaginas in females). Body weight and snout to vent length were measured for each individual. A small piece, approximately 10mm in size, of the proximal colon was removed, snap-frozen in liquid nitrogen, and then stored at -80°C. The mesenteric lymph node was also removed and stored at 4°C in RNAlater overnight, after which the RNAlater was removed and the tissue stored at -80°C. A faecal sample post-dissection was also collected and stored at -80°C. Both females and males were included to maximize sample size (Wanelik et al. 2018). All animal procedures carried out were performed with approval from the University of Liverpool Animal Welfare Committee and under a UK Home Office licence.

3.2 Proximal colon and faecal DNA extraction, sequencing and metagenomics

DNA from both the 10 mm proximal colon samples and one faecal pellet per individual were extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.; full protocol in Appendix 3). This included a 20 minute bead beating step utilising the supplied PowerBead tubes. Extracted DNA was sent to the Centre for Genomic Research at the University of Liverpool for further

purification and next generation Illumina sequencing. A full protocol of all steps carried out by the Liverpool team can be found in Appendix 2.

A two-step PCR was carried out using the primers described by Caporaso et al. (2011) to amplify the V4 region of 16S. Conditions of the PCR were 10 cycles of 98°C for 2 mins, 20sec at 95°C, 15secs at 65°C, 30sec at 70°C followed by a final 5 min extension at 72°C. Samples were purified using AMPure SPRI beads before the second PCR round which was done under the same conditions for 15 cycles. Another purifying step was carried out before generating amplicon libraries. The amplicon libraries were sequenced on an Illumina® HiSeq 2500 platform with version 2 chemistry using sequencing by synthesis (SBS) technology to generate 2 x 300 base-pair paired-end reads. Reads were quality filtered and Illumina as well as PCR primer sequences were removed. A custom pipeline base in QIIME1.9.1 (Caporaso et al., 2010) was used for the metagenomics analysis to form operational taxonomic units (OTUs) and add taxonomic identifications. The GreenGenes database was used throughout the analysis.

3.3 Mesenteric lymph node qPCR

Host RNA was extracted from the mesenteric lymph nodes using the NucleoSpin® RNA extraction kit (Machery-Nagel) and exactly following the protocol which includes a Precision DNase (Primerdesign) step to remove any unwanted DNA from the samples (see Appendix 4 for full protocols). RNA samples were stored at -80°C. Following RNA extraction samples were synthesised into cDNA using the Primerdesign *Precision nanoScript™ 2* Reverse Transcription Kit. A nanodrop was used to record the cDNA concentration using the 240/260 ratio and samples were then stored at -20°C until further use. Real-time qPCR was performed using SYBR Green and the Precision FAST qPCR Master Mix (Primerdesign) for a total of 21

genes including housekeeping genes (Table 1). GeNorm analysis was performed on the qPCR output files using the qbase+ software (Hellemans et al., 2007) to identify the most appropriate housekeeping gene.

Table 1. List of genes and their function used for real-time qPCR

Gene	Function	Sequence
CD4	A protein coding gene which encodes membrane glycoprotein of T lymphocytes	F 5'- TCCTCTGCTGTCTCAAGTGC R 5' -TCTTCTCACTCAGGAGCCTCT
CD8A	CD8 antigen is cell surface glycoprotein found on cytotoxic T lymphocytes	F 5' - CAGTGGAGAAGAGGGGATTGG R 5' - GCAGCAGATGAGAGTGATGAC
COL1A1	Collagen encoding gene found in most connective tissues and bone, cornea, dermis and tendon. Fibrillar forming collagen	F 5' – CTCGCTTCCGTACTCGAACT R 5' - GAAGAACTGGTACATCATCAGCACAA
CTGF	Connective tissue growth factor associated with vascular epithelial cells	F 5'- CGGACAGTGAGGTTACCACAT R 5' - GGCAGCCTGAGTGATGGAG
DEFB1	Beta defensin an antimicrobial peptide implicated in the resistance of epithelial surfaces to microbial colonization	F 5' – TGTAGGTGCCGGTACTCTCA R 5'- CTGGAGCGGAGACAGAATCC
FIZZ1	Induced in AECs and functions as an inducer of myofibroblast differentiation it plays a significant role in mediating such cross-talk between epithelial cells and fibroblastic cells	F 5' – ATGATCCACAACCATAGCCACA R 5' - GCTGATGGTCCCAATGAATACTG
FoxP3	Maintaining homeostasis of the immune system by allowing the acquisition of full suppressive function and stability of the Treg lineage, and by directly modulating the expansion and function of conventional T-cells	F 5' – TCTGAACACAGTCATGGGGAGA R 5' - CCAGGATGAGCTGACTTTCCA
GATA3	Transcriptional activator required for the T-helper 2 (Th2) differentiation process following immune and inflammatory responses	F 5' – GGTCAAACTATCCCACAAAGATTTT R 5' - CGTCGGAAGCCCAGTAGAG
IFNγ	Activator of macrophages as well as stimulating natural killer cells and neutrophils	F 5' – CAGGTCCAACGCAAAGCTG R 5' – TTCCGCTTCCTTAGGCTTGAT
IL1B	Pro-inflammatory. Promotes activation and release of other cytokines and acute-phase proteins. Associated with Th17 response	F 5' – TGCCTTGTGCTGTGTGACC R 5' - CATGGAGAAGATCACTTGTTGGTT
IL2	Promote development of Treg cells. Important in the activation, growth and death of T lymphocytes. Pro-inflammatory role	F 5' - TGAGCAGGATGGAGAATTACAGG R 5' - GTCCAAGTTCATCTTCTAGGCAC

Table 1 continued.

Gene	Function	Sequence
IL6	Pro-inflammatory. Synergises with TGFb to drive a Th17 response	F 5' – CAGGACTGTTATCACACAACTCTT R 5' - GGAGACTATACAGAGGACACCAC
IL10	Immune suppression as it down-regulates antigen presentation and expression of dendritic cells. Down-regulated pathogenic Th1, Th2 and Th17 response	F 5' – GTTGCCAAACCTTGTCAGAAAT R 5' - CCCAGGGAGTTCAAATGCT
IL17a	Pro-inflammatory. Protective immunity of the lung. Promotes cytokine and neurophil production by epithelial cells	F 5' – TGTGGTGAAGGCAGGGTTA R 5' – TCGGGCTAAGGGAGTTCAG
MS4A1	Involved in the regulation of B-cell activation and proliferation	F 5' – ACCTGTACGTTGATATCTACAACTG R 5' - GCTTCCGTGCTTACCAGGA
NLRP3	Regulates the differentiation of T helper 2 (Th2) cells	
ORAI1	Calcium Release-Activated Calcium Modulator Primary channel for calcium influx for T cells	F 5' – GTTGGCCTGAGTGTCTCTTGA R 5' - GCCTTCTCCACTGTCATCGG
TBX21	Activates Th1 related genes and suppresses Th2 and Th17 related genes	F 5' – CCAGGAAGGTCGGGGGTAGA R 5' - CTGTCAACTGCTCGGAGGA
TGFB1	Can promote either T-helper 17 cells (Th17) or regulatory T-cells (Treg) lineage differentiation in a concentration-dependent It is a potent stimulator of osteoblastic bone formation	F 5' – TCCCAGAGAGAGCGTTCATC R 5' - GAGAAAGCAGCAGGAGTCAC
TNF	Tumour necrosis factor Pro-inflammatory cytokine involved in regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation	F 5' – GTCTACTCCCAGGTTCTCTTCA R 5' - GCAGACAGGAGGTTGACTTTG
YM1	Enhances Th2 cytokine production Expressed by MLN cells and promotes locally Th2 response	F 5' – GCTCCTTAGACTACATCCAGGTC R 5' - CACATTGAGGAAGGTGTTGGTG
YWHAZ	Housekeeping gene	F 5' – GTTAAGTTCTGTGCTTCCAAATCG R 5' - GCCAACAAGTCGGAATGGTG

All data analysis was carried out using R (R version 3.5.0; 2018-04-23) using the packages Phyloseq (McMurdie and Holmes 2013) and Vegan (J. Oksanen et al. 2015).

Alpha diversity, the number of different OTUs within a sample and evenness of the sample, was estimated using both Shannon and Simpson indices. Results obtained from both the Shannon and Simpson indices were qualitatively similar. We only present the Shannon index outputs here. A general linear model was used to investigate the associations between Shannon index and host variables: sex, age, body weight residuals (as a proxy for condition), sampling season and the tissue type used (faecal or proximal colon). Body weight residuals were calculated using the raw body weight measures as the dependent variable in a linear model and including snout to vent length and sex as independent variables. The residual values obtained would either be negative or positive identifying animals which were lower than average weight for their length and sex or heavier than average respectively.

Beta diversity, the similarity of OTU composition between individuals, was measured using weighted UniFrac, a distance measure which takes phylogenetic distances into account (Lozupone et al. 2005), as well as the Bray-Curtis dissimilarity index (Bray and Curtis 1957) which calculates the dissimilarity of counts between individuals. Multidimensional scaling was then performed to determine a set of points so that the distances between the points are approximately equal to the dissimilarities and to effectively associate one distance point with one individual for further analyses. Beta diversity dissimilarity points for each individual were then compared between sex, age, trapping season and condition of host and using permutational multivariate analysis of variance

(Anderson 2001) based on the distance tables. This was done using the Adonis function in R with sex, age, trapping season and weight residuals as predictor variables and Bray-Curtis dissimilarity index as the response. P values were estimated by comparing pseudo-F values against 999 random permutations of the data (Anderson 2001). A paired Wilcoxon test was perfumed to compare OTU composition between tissue types using Bray-Curtis dissimilarity distance.

To follow up the significant results from the analysis of beta diversity, differences among quantities of OTUs was investigated in association with the relevant variables (tissue sample type, sex and season) using the DESeq2 package in R (Love et al. 2014). To identify OTUs driving the difference between faecal and proximal colon samples a Wald test was performed on the log2 fold change data. The same was carried out for the difference between males and females. To analyse the differences in OTUs between trapping season a likelihood ratio test was performed comparing a full model which included both sex and season and a reduced model which only contained sex. An analysis of deviance captures the difference in likelihood between the full and the reduced model.

Mantel tests (Mantel 1967) were performed comparing generated matrices from the MLN gene expression data of the cross-sectional voles and the microbiota sequence data from the same individuals, once using proximal colon samples followed by using the microbiota data from faecal samples. The test was again repeated splitting the data matrices according to time spent in captivity prior to dissection.

4. Results

Faecal microbiota sequences and proximal colon microbiota sequences for culled voles were combined to give a total of 105 cross-sectional microbiota samples. Of

these samples, 42 had sequencing data from their proximal colon microbiota as well as gene expression data from their mesenteric lymph node. Sequences obtained were clustered into a total of 1585 OTUs and the mean number of reads per individual vole in our data was 240884. Across all 105 microbiota samples the dominant phyla found were *Bacteroides, Firmicutes* and *Proteobacteria* (Figure 1).

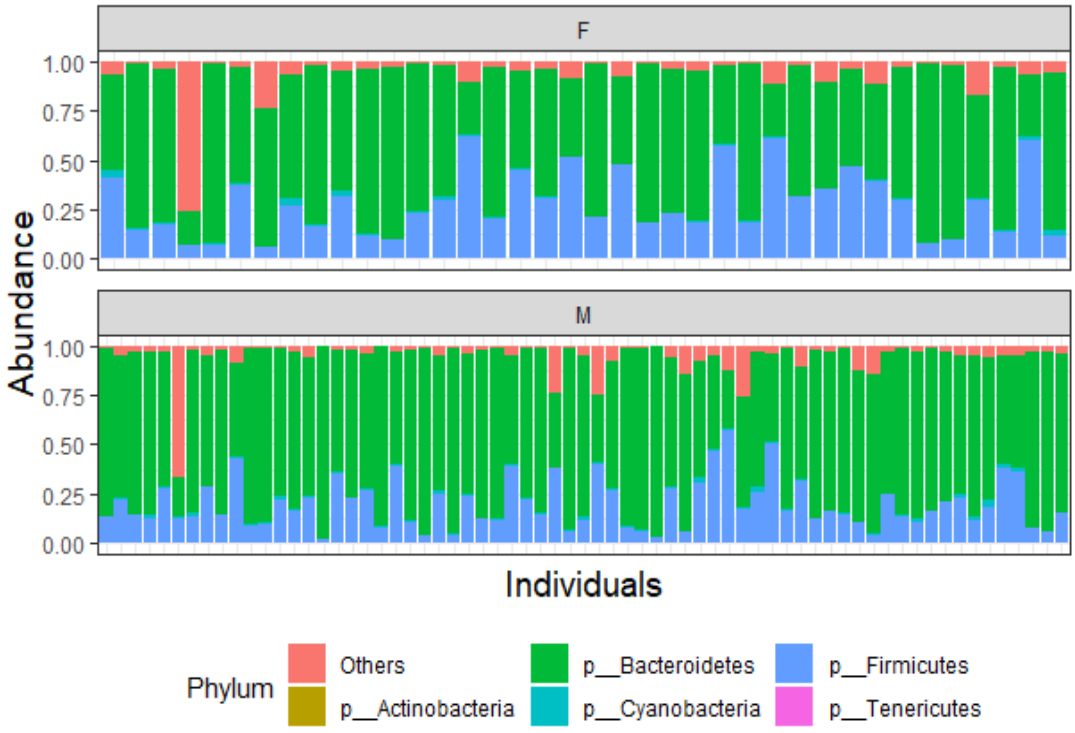


Figure 1. Abundance of phyla across all cross-sectional microbiota samples, including both faecal and proximal colon samples. Plot is divided by sex, females are displayed in the top and males in the bottom plot. Composition is divided into the six most dominant phyla.

As UniFrac and Bray-Curtis index analyses yielded very similar outputs, only the Bray-Curtis index results are reported in this chapter with exceptions only if Bray-Curtis index and UniFrac measures gave significantly different results. The alpha diversity, calculated using the Shannon index, of the proximal colon and faecal

microbiota was not significantly associated with any of the host or environmental variables tested (Table 2).

Table 2. Alpha diversity (Shannon index) of the microbiota compared to host and environmental variables. None of the variables were found to have a significant association with microbiota alpha diversity.

Variable	Standard error	t-value	p-value
Age (mature)	0.21	1.24	0.22
Sex (male)	0.12	-1.52	0.13
Tissue (faeces)	0.11	-0.45	0.65
Sampling season (summer)	0.12	0.47	0.49
Weight residual	0.01	0.69	0.49

4.1 The OTU composition is significantly different between sample types

A significant difference was found between the OTU composition of faecal samples and proximal colon samples within the same individuals ($V=1015$, $p<0.001$) using the Bray-Curtis dissimilarity index. A paired Wilcoxon test was performed to identify differences in the beta diversity of the microbiota between faecal and proximal colon samples. Principal Coordinates Analysis (PCoA) shows that some OTUs were shared between the two sample types and a number of OTUs are exclusive to the faecal samples and others exclusive to the proximal colon (Figure 2).

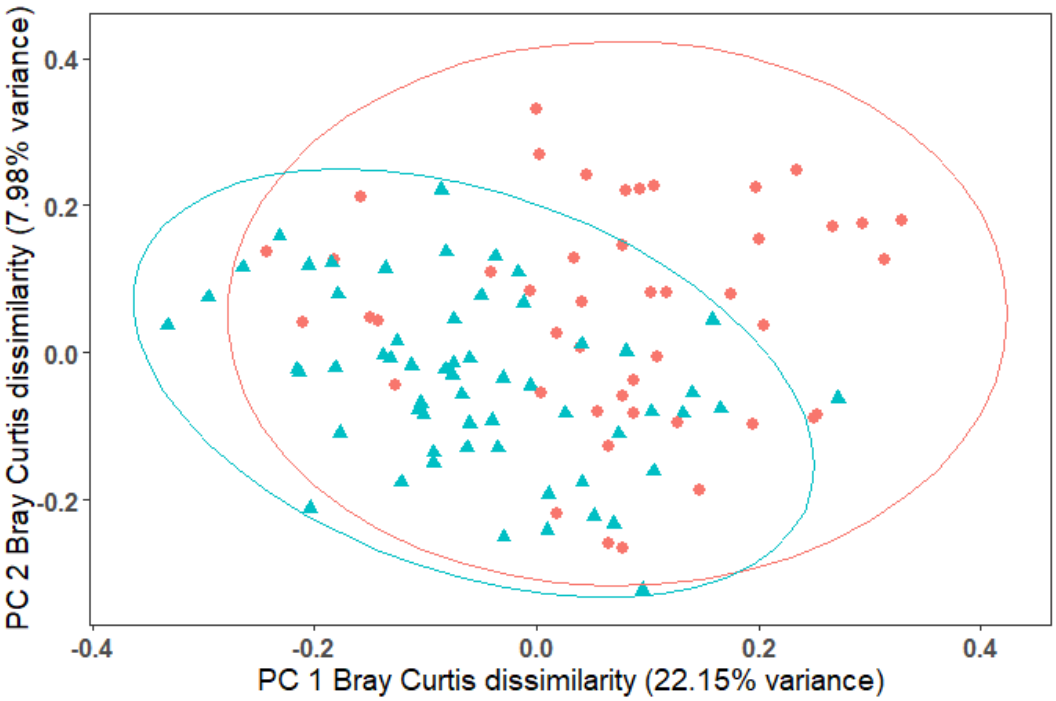


Figure 2. Principle Coordinate Analysis shows the differences in OTU composition between faecal samples (blue triangles) and proximal colon samples (red circles). Bray Curtis dissimilarity index was used for the ordination with PC1 explaining 22.15% of variance and PC2 is explaining 7.98% of variance. OTU composition is significantly different between tissue types (Paired Wilcoxon test: $p < 0.001$).

Using DESeq2 analysis, which identifies differential expression - or in this case differences in OTU quantities - in the data set by use of negative binomial generalized linear models, OTUs which were significantly different between the two sample types were identified (Table 3). OTUs more commonly found in the faecal samples mostly belong to the phylum Bacteroidales and account for 66.7% of OTU groups in the faeces. Proximal colon samples contained OTUs predominantly belonging to the phylum Clostridiales accounting for 88.9% of OTU groups as well as a small number of OTUs belonging to *Desulfovibrionales* (3.17% of OTUS).

Table 3. OTU phyla which were quantitatively more common in faecal microbiota samples compared to proximal colon samples mostly belonged to the Bacteroidales phylum. Proximal colon OTUs which were found in colon but not faecal samples mostly belonged to the Clostridiales phylum.

Proximal colon		Faeces	
Order/family	**No. of OTUs**	**Order/family**	**No. of OTUs**
Clostridiales (unclassified)	54	Bacteroidales (S24-7)	11
Desulfovibrionaceae	6	Bacteroidales (unclassified)	5
Helicobacteraceae	4	Ruminococcaceae	2
Lachnospiraceae	42	Mycoplasmataceae	1
Ruminococcaceae	66	Helicobacteraceae	1
Others/Unclassified	10	Others/Unclassified	2

4.2 Beta diversity of the microbiota is associated with sex and seasonality

Using a permutational analysis model the associations between selected environmental and host variables and the OTU composition, or beta diversity distance points, were investigated. UniFrac distance was used as a measure of beta diversity for both proximal colon and faecal samples. Sex of the host and the overall weight residuals, as a representative of condition, both showed significant associations with OTU composition (Table 4). Sampling season was also significantly associated with beta diversity, whilst age of the host did not show a strong association with beta diversity (Table 4). When utilising Bray-Curtis dissimilarity index as the beta diversity distance measure similar results were found with the exception of weight residuals which no longer showed a significance (R^2 = 0.013, F = 1.19, p = 0.17).

Table 4. Permutational analysis model (Adonis) comparing beta diversity (UniFrac distance and Bray Curtis distance) of the microbiota with environmental and host variables. Sex, trapping season and overall weight residuals were significantly associated with the beta diversity of the proximal colon.

Variables	UniFrac distance			Bray Curtis distance		
	Degrees of freedom	R^2	p-value	Degrees of freedom	R^2	p-value
Sex	1	0.016	0.014*	1	0.014	0.018*
Age	1	0.014	0.08	1	0.018	0.11
Trapping Season	1	0.023	0.0001***	1	0.022	0.0016**
Weight Residuals	1	0.015	0.04*	1	0.013	0.17

Differential expression analysis (DESeq2) was used to investigate which taxa were driving the observed differences in microbiota between the sexes and the seasons. The likelihood ratio test comparing a full model including both season and sex showed that season was not a significant variable compared to the reduced model which only included sex.

The Wald test showed that 19 OTUs were significantly more abundant in males compared to females, most of which belonged to the order Bacteroidales, and the family S24-7, with only two exceptions, one belonging to the family Ruminococcaceae the other an unclassified Firmicutes OTU. Females were shown to have 37 OTUs that were more abundant than in males, seven belonging to the family Lachnospiraceae, another seven OTUs belonging to Ruminococcaceae and nine OTUs which are part of the Bacteroidales and the family S24-7. Three OTUs were part of the family Lachnospiraceae, two OTUs belonging to Rikenellaceae, the rest were unclassified bacteria OTUs (Table 5).

Table 5. OTU orders and families identified to be more abundant in females compared to males and vice versa. 37 OTUs were identified as being more abundant in females compared to males and 19 OTUs were more abundant in males compared to females.

Females		Males	
Order/family	**No. of OTUs**	**Order/family**	**No. of OTUs**
Lachnospiraceae	7	Bacteroidales (S24-7)	17
Ruminococcaceae	7	Ruminococcaceae	1
Bacteroidales (S24-7)	9	Paraprevotellaceae	1
Rikenellaceae	2	Other/Unclassified	1
Helicobacteraceae	1		
Staphylococcaceae	1		
Others/unclassified	9		

4.3 Gene expression in the mesenteric lymph node (MLN) is significantly associated with the composition of the microbiota

A Mantel test, which compares two matrices, based on the Bray-Curtis index was performed on the gene expression data from the MLN and the microbiota data from the proximal colon. Twenty four genes were used and a significant association was found between MLN gene expression and microbiota composition (N=42, p=0.029). Specifically four MLN genes could be identified using a function (bioenv) to subset the MLN expression data and identify the variables which have the maximum (rank) correlation with the microbiota data. IL2, Tbx21, CD8A and CTGF were identified as being the most relevant genes associated with microbiota composition.

4.4 MLN gene expression is only significantly associated with proximal colon microbiota composition but not the faecal microbiota

Another mantel test was performed comparing the MLN gene expression data matrix to the faecal microbiota matrix (using Bray-Curtis index). No significant association was found between the faecal microbiota and the gene expressions of

the MLN (N=55, p=0.24). To ensure this difference in result was not due to faecal samples having been sampled at several different time points and possibly having changed due to external temporal factors, the mantel test was rerun using only the faecal samples on the last day pre-cull but still no relationship was found between faecal microbiota and MLN gene expression (N=39, p=0.77).

4.5 Beta diversity of the microbiota is different in animals kept captive for one day and those kept captive for two days

To further investigate the potential impact of temporal changes on the relationship between MLN gene expression and microbiota beta composition the Mantel test was repeated once more to differentiate between proximal colon samples taken from voles which had been in captivity for up to 24 hours and those kept for 48 hours. The proximal colon of voles housed in captivity and fed on a standardised diet was significantly associated with the MLN gene expression matrix after 24 hours (N=18, p=0.03). However, after being in captivity for 48 hours the association between proximal colon microbiota and MLN gene expression became non-significant (N=24, p=0.08).

Differential expression analysis (DESeq2) was used to further investigate which taxa were driving the observed differences in microbiota between animals culled within 24 hours and those culled within 48 hours. Thirty-four OTUs were found to be more abundant in voles which stayed in captivity for one day compared to individuals in captivity for two days. Of these OTUs, 8 belonged to the order Bacteroidales and the family S24-7, 5 OTUs belonged to the family Lachnospiraceae, another 5 OTUs to the family Ruminococcaceae and 3 OTUs belonging to Spirochaetaceae. After two days in captivity samples collected from voles showed a greater abundance in 30 OTUs compared to individuals sampled

within 24 hours. Eleven OTUs belonged to the Bacteroidales S24-7 family and 6 OTUs belonged to the Lachnospiraceae. The families Ruminococcaceae, Erysipelotrichaceae and Paraprevotellaceae each had two OTUs more abundant in samples taken after 48 hours in captivity (Table 6).

Table 6. OTU orders and families identified to be more abundant in animals sampled within 24 hours and those sampled after spending 48 hours in captivity.

24 hours samples		48 hours samples	
Order/family	**No. of OTUs**	**Order/family**	**No. of OTUs**
Bacteroidales (S24-7)	8	Bacteroidales (S24-7)	11
Lachnospiraceae	4	Lachnospiraceae	6
Ruminococcaceae	5	Erysipelotrichaceae	2
Spirochaetaceae	3	Paraprevotellaceae	2
Others/unclassified	14	Other/unclassified	9

5. Discussion

The present study demonstrates that the compositions of the microbiota from two different tissue sample types, namely the proximal colon and faecal pellets taken from the same individual, are significantly different. The faecal samples in the present study were more abundant in the number of Bacteroidales present, specifically the family S24-7. In comparison, proximal colon samples had a greater abundance of OTUs belonging to the families Lachnospiraceae and Ruminococcaceae as well as OTUs belonging to Desulfovibrionaceae and Helicobacteraceae. Bacteroidetes are associated with the breakdown of nutrients, especially carbohydrates (Thomas et al. 2011), and may therefore be more commonly found along discarded food particles in the faecal matter. Faecal matter has been estimated to contain up to 60% bacteria (Stephen et al. 1980) which could originate from across the gastrointestinal tract. The gastrointestinal tract is made up of multiple sites, each with a different composition and density of

bacteria. It is impossible to tell exactly which gastrointestinal site the bacteria found in faecal pellets have originated from and how representative they are of the overall gut microbiota. The stomach and small intestines, for example, are scarcely colonised by any bacteria due to the presence of acid, bile and pancreatic secretions which kill most microorganisms (Guarner and Malagelada 2003). The proximal colon and caecum, in contrast, contain a large number of live bacteria as they offer a variety of nutrients, high levels of fermentation and high production of short-chain fatty acids, all aiding the establishment and rapid growth of bacteria (Guarner and Malagelada 2003).

Lachnospiraceae and Ruminococcaceae are associated with the reduction of Th17 inflammatory response when introduced to the gastrointestinal tract of ulcerative colitis suffers (Jiang et al. 2014; Natividad et al. 2015), which may suggest the importance of these two bacterial families as part of the anti-inflammatory response, and the direct interaction with the gut lymphoid structures such as the MLN.

Another significant variable affecting the relationship between MLN gene expression and gut microbiota composition was the time of sampling after capture. This was potentially due to changes in available diet after transferring the host to a laboratory facility for a period of either 24 or 48 hours and may have potentially lead to changes in the beta diversity level of the proximal colon microbiota. A study on woodrats (*Neotoma lepida*) demonstrates that moving wild caught individuals into captivity alters the OTU composition of the gut noticeably (Kohl et al. 2014). The rats lost 19% of their original microbe composition after 2 weeks in captivity and a total of 24% of OTUs after 6 month

(Kohl et al. 2014). These changes may have been due to differences in their diet, changes in their physiology due to limited movement or differences in their environmental microbes (Kohl et al. 2014). Our results demonstrate that even short-term changes in diet and environment can have a noticeable impact on microbiota composition. Samples taken within the first 24 hours of capture had a greater abundance of OTUs belonging to the Lachnospiraceae, Ruminococcaceae, S24-7 and Spirochaetaceae. After 48 hours in captivity samples showed an increased abundance in OTUs also belonging to Lachnospiraceae, Ruminococcaceae and S24-7, however, different OTU clusters, as well as Erysipelotrichaceae and Paraprevotellaceae. It is not possible to identify the definite cause of this observed change within the scope of the study and available data. However, it can be hypothesised that changes of the microbiota within such a short time frame could be due to a number of factors. One potential influence could be changes in stress levels of the captured animals, which has been shown to impact the microbiota composition in rodents (Tannock et al., 1974; Rhee et al., 2009; O'Mahony et al., 2009). A study conducted on laboratory mice showed a change in beta diversity composition in the microbiota after exposing mice to a two-hour long stressor (Galley et al., 2014). As the rodents in the current study were exposed to potential stress due to removal from their homes and transfer to a laboratory for up to 48 hours it is reasonable to assume that the stress response may have caused changes in the microbiota composition.

A study on wild caught woodrats introduced into captivity showed an increase in OTUs belonging to the Firmicutes (Kohl et al., 2014) and similarly the present vole study identified an increase in Firmicutes. These observations are supported by

the findings in other rodent studies and suggest that captivity has a significant impact on the composition of the microbiota.

There was a significant association between the OTU composition of the proximal colon in wild living voles and the host sex. Sex of the host has previously been identified as influencing the composition of the microbiota (Dominianni et al. 2015; Org et al. 2016; Bolnick et al. 2014). A study on a large number of laboratory mouse strains found that sexual differences in the microbiota composition are genetic strain dependent and only occur in some but not all genetic strains (Org et al. 2016). Additionally, an association between microbiota compositional changes and the sex of the host could also be detected when feeding the mice different diets either in the form of standard chow of a high-fat diet (Org et al. 2016). The differences in microbiota composition between male and female voles in the present study may therefore be due to a number of reasons including genetic variations of the host, hormonal differences or slight differences in their diet. The present study found a significantly greater abundance of OTUs belonging to the order of Bacteroidales and specifically the family S24-7 in male voles compared to female voles. Similarly, a study on human subjects found decreased levels of Bacteroidetes (phylum which the order of Bacteroides belongs to) in women compared to men (Dominioanni et al., 2015). Actinobacteria, a gram-positive bacteria, was also found to be more abundant in women compared to men in the human microbiota study (Dominianni et al., 2015). The present vole study did not find more abundant OTUs belonging to the Actinobacteria in female voles. However, many of the OTUs identified as more abundant in females belonged to the Firmicutes, which belong to the gram-positive bacteria, for example Staphylococcaceae. Other studies on mice have made contradictory findings when

investigating differences between male and female individual OTUs or bacterial families and species (Rizetto et al., 2018; Org et al., 2016). Whilst one study found an increased abundance of Lachnospiraceae, specifically *Dorea, Coprococcus* and *Ruminococcus,* in female mice (Org et al., 2016) the other murine study found an increase in Lachnospiraceae in males (Rizetto et al., 2018). In the present study we found an increased abundance of Lachnospiraceae, belonging to the Firmicutes, in females only, whilst all more abundant OTUs indentified in males belonged to the Bacteroidetes.

The present study identified a potential association between the gut microbiota and trapping season. Other rodent studies have observed changes in gut microbiota composition due to seasonality, for example in wild *Apodemus sylvaticus* populations which showed a decrease in *Lactobacillus* species along with an increase in *Alistipes* and *Helicobacter* between summer and autumn sampling (Maurice et al. 2015). The change in gut microbiota composition is suggested to stem from changes in available food sources and therefore diet of the host (Maurice et al. 2015). Changes in diet have been shown to directly influence the composition of the gut microbiota (Li et al. 2016; Amato et al. 2016; David et al. 2014). Changes in the gut microbiota of the earthworm *Lumbricus rubellus* were directly associated with the type of soil and vegetation the worm was placed in (Knapp et al. 2009). By placing worms in either grass litter or dwarf shrub litter the ingested food would differ between the two worm groups and their gut microbiota clearly clustered according to the food source available (Knapp et al. 2009). Vole diet has been found to remain relatively stable throughout the year and does not appear to be influenced by changes in available fresh plant material as grass shoots and dead material from the same grass species could be found in faeces throughout the year (Wheeler 2005). This could

potentially explain why the present study did not find clear evidence of season impacting the gut microbiota as diet in the voles remained stable throughout the trapping sessions with only minimal changes. Voles show no clear preferences in their food selection but will prioritise the most palatable and nutrient rich foods available (Wheeler 2005).

We report a significant association between the proximal colon microbiota composition, at the beta diversity level, and the immunological gene expression levels of the mesenteric lymph node in a wild population of voles. The gene expressions most correlated with microbiota dissimilarity belonged to pro-inflammatory immune responses, namely the expression of CD8, IL2 and Tbx21 (or T-bet) (de Rham et al. 2007; Lau et al. 2017; Hodge et al. 2017). Transcription factor T-bet is necessary for activation and transcription of a number of genes associated with a pro-inflammatory Th1 response, for example the production of IFNγ in CD4+ cells (Koch et al. 2009). As Tregs play a fundamental role in maintaining mucosal homeostasis in the host it has been suggested that a large number of commensals will interact and increase the frequency of Tregs in the host rather than just a select few (Ahern, Faith, and Gordon 2014; Geuking, McCoy, and Macpherson 2013). This could explain the increased expression of Tbx21 as commensals will interact with Tregs to maintain homeostasis in the colon.

The association between microbiota beta composition and MLN gene expression in the present study was only evident between proximal colon microbiota samples and the MLN but not the faecal microbiota. This may be due to the physiological proximity of the proximal colon to the MLN and the bacteria found in the colon reflecting more directly which microbes the MLN directly interacts and responds to.

In summary the present study found that there was a significant association between the proximal colon microbiota and the immune response expressed by the mesenteric lymph node in wild living field voles. This association is not evident when comparing faecal microbiota samples of the same individuals. The differences in microbiota compositions between tissue types are important to consider when working on longitudinal samples consisting solely of faecal pellets for microbiota analysis.

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Chapter 3: Relatedness does not significantly influence the composition of the proximal colon microbiota in wild living voles (*Microtus agrestis*) in Kielder Forest

Abstract

In this study we explore if variation in the faecal microbiota of *Microtus agrestis* in Kielder Forest, in Northumberland, is associated with relatedness of the host to other individuals. The study investigates whether offspring will show greater similarities in their microbiota composition with their dams and sires or if gut microbiota composition is independent of inheritance and relatedness. The beta diversity of the faecal microbiota was not significantly associated with relatedness: faecal microbiotas of two random individuals were shown to be just as similar or dissimilar as faecal microbiota of offspring and dam. The relative proximity of individual traps which samples were collected from also showed no significant association with microbiota composition. Finally, the days between collecting faecal samples from individuals did not show a significant impact on the gut microbiota. Only a small subsample of faecal pellets could be used for the analyses presented here to include information about pedigree and relatedness of the animal.

1. Introduction

The mammalian microbiota is first established during the birthing process when the offspring comes in direct contact with the microbes living in their mothers' birthing canal (Dominguez-Bello et al. 2010; Vaishampayan et al. 2010; Bäckhed et al., 2015). During gestation the foetus develops in an essentially germ-free

environment within the amniotic fluid (Palmer et al., 2007). Microbes in the birthing canal, the vagina and the skin of the mother are the first an infant is typically exposed to during a natural delivery (Dominguez-Bello et al. 2010). These first microbes form the foundation of the developing skin and gut microbiota of the offspring and play an important role in the development of the infant's immune system as the microbiota directly interacts with the immune response in humans (De Agüero et al. 2016).

In preparation for the seeding of their offspring's' initial microbiota the mothers' vaginal microbiota in humans changes during pregnancy from a highly diverse microbiota to a microbiota mostly dominated by *Lactobacillus* (Romero et al. 2014; MacIntyre et al. 2015). *Lactobacilli* are involved in the production of lactic acid which helps to maintain a low pH in the vagina to create a hostile environment for most microbes and serve as a protective mechanism during pregnancy (MacIntyre et al. 2015; Reid et al. 2011). They are also believed to be involved in the reduction of pathogen growth by secreting antibacterial bacteroicins (MacIntyre et al. 2015). The increase in Lactobacillus and lowered overall diversity in the vaginal microbiota help to lower the number of potentially pathogenic microbes transmitted to the offspring during birth.

The initial microbiota that colonises the infant is closely matched to their mother's gut, vaginal, and even faecal microbiota composition after birth (Palmer et al. 2007; Yatsunenko et al. 2012; Dominguez-Bello et al. 2010). The colonisation of the gastrointestinal tract in the offspring is highly variable according to method of delivery (Rutayisire et al. 2016; Dominguez-Bello et al. 2010) and the feeding habits after birth (Koenig et al. 2011). Infants delivered by caesarean section were found to have a lower abundance and diversity of Actinobacteria and Bacteroidetes and higher abundance of Firmicutes compared to vaginally

delivered babies (Rutayisire et al. 2016). Studies on the seeding of the microbiota from mother to offspring and initial establishment of the gut microbiota are often conducted in human models or in captive bred mice populations (Bäckhed et al. 2015; Vaishampayan et al. 2010; Dominguez-Bello et al. 2010; Koenig et al. 2011). Understanding the role of the mother's microbiota on the offspring's microbiota and the associated development of the immune system in a wild rodent system is challenging. Identifying dams and their respective offspring is only possible through genetic analysis and samples collected from wild individuals are often taken many days or even weeks after the birth process allowing the offspring to mature and start consuming solid foods.

As solid foods are introduced and the human infant, for example, is exposed to a wider range of pathogens over time the infant microbiota changes to resemble that of a typical "adult" microbiota, specifically increasing in the abundance of *Bifidobacterium* (Vaishampayan et al. 2010). When sampling the infant microbiota at 1 month of age the dominant phyla present were *Escherichia* at 45% and *Bacteroides* at 21% (Vaishampayan et al. 2010). A noticeable shift in the dominant phyla occurred over the next ten months and when sampling infants' microbiota at 11 months of age Bifidobacterium and Collinsella were the most dominant (38% and 22% respectively) whilst Escherichia and Bacteroides are reduced to 6% and 0.4% respectively (Vaishampayan et al. 2010). The presence of Bifidobacterium and Bacteroides is typical for an "adult"-like microbiota as these microbes are important in the metabolism of carbohydrates in the gut (Vaishampayan et al. 2010). The species of Bifidobacterium acquired by the offspring at 11 months were shown to be different species from the mother and Bacteroides species which were originally passed down from the mother after birth were replaced by novel *Bacteroides* species (Vaishampayan et al. 2010).

Samples collected 11 months after birth showed that the maternal and infant microbiota compositions started to diverge from one another (Vaishampayan et al. 2010). By 12 months of age the human infant functional microbiota composition had transitioned to resemble the functional microbiota of adults, most noticeably an increase in energy utilisation species for carbohydrate uptake whilst lactose specific transporters decreased in abundance (Bäckhed et al. 2015).

The gastrointestinal microbiota in adults remain relatively stable over time once it is established major changes in abundance and composition mostly happen within the first few years of life (Koenig et al. 2011; Bäckhed et al., 2015). Over the period of 2.5 years the diversity and community composition gradually increased and changed over time whilst major taxonomic groups showed abrupt shifts in their abundance when diet and health changed (Koenig et al. 2011). The human infant gut microbiota after birth was found to be enriched in lactate utilising microbes after birth but functional genes involved in plant polysaccharide metabolism were present shortly after and even before solid foods were introduced to the diet, suggesting that the microbiota is primed for food changes before they actually occur (Koenig et al. 2011).

Changes in the gut microbiota have been suggested to influence changes in the scent of an individual which would have effects on kin recognition and mate choice (Lizé, McKay, and Lewis 2013). Specifically, the bacterial metabolites have been identified as contributing to the odour of individuals which allows for kin and nest mate recognition (Minkley et al. 2006). Microbes will be shared and passed on to other individuals in species which live in close proximity, for example in nest building species such as the termite (Lizé, McKay, and Lewis 2013). This sharing of microbes in social species can potentially be used to identify nest mate identity as individuals can be matched to the microbe composition of their nest

(Lizé, McKay, and Lewis 2013). A study on termites (*Hodotermes mossambicus*) showed high consistency in gut microbiota composition among nest mates and distinct differences to individuals from other nests (Minkley et al. 2006).

Nest specific differences in microbiota composition could also be detected in Chinstrap penguins (*Pygoscelis antarctica*); however, the difference was only obvious between chicks not adult penguins (Barbosa et al. 2016). This might suggests that environmental variables are more important in driving microbiota differences in chicks than genetic factors (Barbosa et al. 2016). After controlling for nest differences the most important variable affecting microbiota composition was age showing a significant difference between chicks and adult animals regardless of whether they were related to one another or not (Barbosa et al. 2016). Chicks were characterised by having more Firmicutes whereas adults had a higher abundance of Bacteroidetes and Proteobacteria in their gut (Barbosa et al. 2016). Additionally, adults' gut microbiotas were more diverse compared to chicks (Barbosa et al. 2016).

Wild living rodent populations, such as the field vole (*Microtus agrestis*) are also nest forming, primarily as a result of extended maternal families living together (Madison, FitzGerald, and McShea 1984). It was found that on average 2-5 individuals will share and sleep in the same nest, though numbers would fluctuate depending on season as more voles were recruited into nest "clusters" in the winter months (Madison, FitzGerald, and McShea 1984). As there is a thermoregulatory benefit to huddling during colder months non-related individuals would be accepted into nest clusters during late December and early January (Madison, FitzGerald, and McShea 1984) which could potentially introduce new microbes to the nest and its occupants. The overall roaming ranges of wild living field voles were studied for 2 years as part of a mark and

recapture study (Erlinge et al. 1990). The home ranges of voles were shown to depend on population density as well as breeding or non-breeding season as ranges of voles were seen to expand during breeding season as well as during low density periods (Erlinge et al. 1990). Males were shown to have greater home ranges than females and to be overlapping with other males during breeding season (Erlinge et al. 1990).

2. Aims and hypotheses

The aim of the present study is to identify the role of relatedness on the composition of the faecal microbiota in wild living voles. We hypothesise that animals that are closely related to each other will exhibit a significantly more similar microbiota composition than compared to non-related animals. We expect that this similarity will be most noticeable between the offspring and their respective dam and not the sire due to longer contact to the dams during suckling and therefore longer nest sharing with the dams. Sharing a nest and living in close proximity to each other leads to individuals sharing significant similarities in their microbiota. We expect to see that individuals trapped in close proximity to each other will be more similar in their microbiota composition than animals trapped further apart from one another as this might suggest close proximity of their nests to one another.

For a detailed description of the study site and species see Chapter 2. For the present study we utilised faecal pellets collected from the voles at each capture time-point. Faeces, which are made up of 40% bacterial cells, are a representative way of sampling the microbiota of the colonic lumen though samples will most likely also contain microbes from other sites of the gastrointestinal tract due to mucus excretion and epithelial turnover (Moore et al. 1975; Eckburg et al. 2005).

3. Methods

The methods for trapping of voles, collecting and storage of samples as well as sequencing and metagenomics analysis of microbiota samples are described in detail in Chapter 2. The central trapping grid used was approximately 0.375 hectare in size with Ugglan small animal traps spaced 3-5m apart within the same trapping grid.

3.1 SNP identification

The methods for genotyping and identifying of SNPs are described in greater detail in Wanleik et al. (2018). In short, DNA was extracted from ear and tail bleeds using DNeasy Blood and Tissue Kit (Qiagen) and genotyping was then performed by KBiosciences (Hoddesdon, UK; http://www.kbioscience.co.uk) using the KASPar SNP genotyping system. This included negative controls (water) and duplicate samples to validate reproducibility.

The generation of pedigree data from the SNPs was done using Sequoia which assumes Hardy-Weinberg equilibrium in samples and removed any SNPs which violated this assumption. In total 114 SNPs were identified and 2667 samples obtained. A pedigree was assigned for each trapping site separately; a dam and sire were assigned to the offspring and a log10 likelihood ratio (LLR) was calculated to confirm the dam, sire and the parent pair as the most suitable match. For further information on the generating of SNP and relatedness data please see Appendix 5.

The pedigree data was generated by Dr. Klara Wanelik (University of Liverpool).

3.2 Statistical analysis

Analysis of the pedigree data in relation to the microbiota data was carried out using R (R version 3.5.0; 2018-04-23).

Pedigree data and faecal microbiota data were merged by the individuals' PIT tag numbers and the earliest recorded faecal sample was used in the analysis to ensure sampling at the "youngest" point in the individual's life. Bray-Curtis dissimilarity index (Bray and Curtis, 1957) was calculated for all individuals and the median Bray-Curtis index score for offspring animals was compared to Bray-Curtis index values of their respective assigned dams using a Wilcoxon's paired T-test (Wilcoxon 1946).

A Mantel test (Mantel 1967) was performed comparing generated matrices from the geographical distance of the individual traps voles were caught in and the microbiota sequence data from the same individuals. Similarly, a comparison was made between a matrix generated from temporal distance, measuring the days between capture of the dam and capture of the respective offspring, and the microbiota sequence data, using the Bray-Curtis distance index.

4. Results

A total of 1585 OTUs were identified in the longitudinal faecal samples used for the present analyses with a mean of 17103 reads per OTU and a median read value of 4972. A total of 74 offspring animals were identified as having both faecal microbiota data and pedigree data available as well as 20 sires and 21 dams.

4.1 Beta diversity of the faeces is not significantly more similar between closely related individuals

Using a paired Wilcoxon's t-test we investigated the relationship between the beta diversity of the faecal microbiota at one point in time and the relatedness of the hosts to one another. The median Bray-Curtis distance index of offspring animals was compared to the Bray-Curtis distance index of their relative mother animals (dams) but no significant association (V = 1147, p = 0.63) was found suggesting that closely related animals do not have a more similar microbiota to one another than any other two individuals (Figure 1).

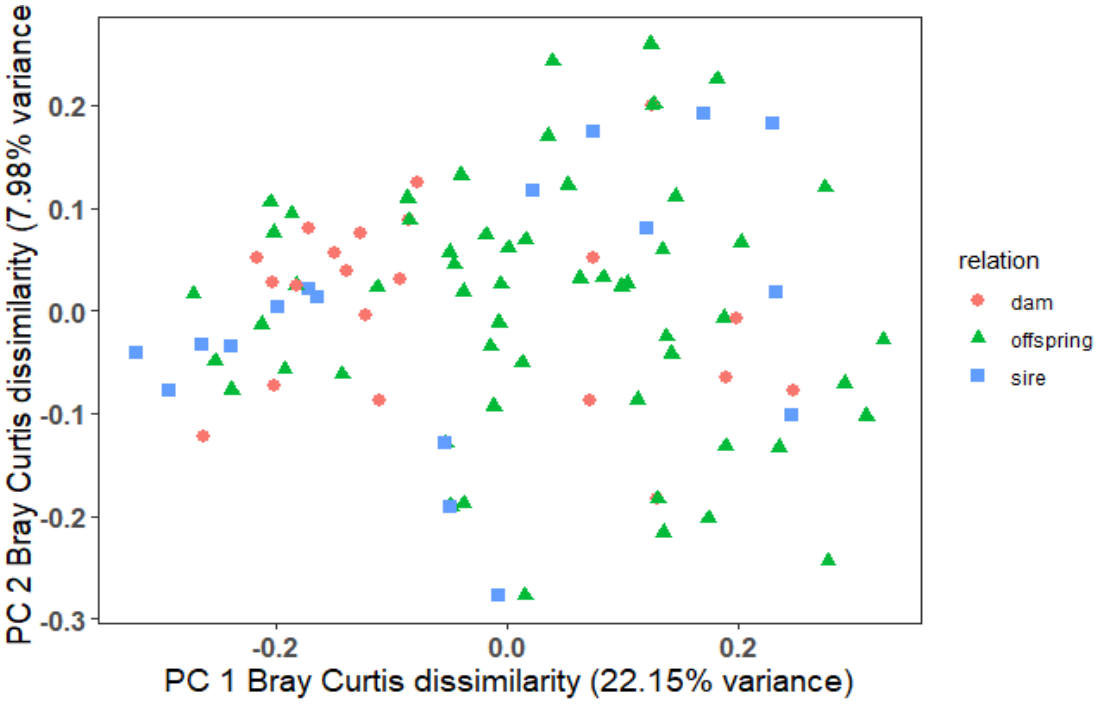


Figure 1. Principal coordinate plot shows the Bray Curtis dissimilarity between dams (red circles), sires (blue squares) and offspring (green triangle) animals. No clear-cut pattern or clustering can be detected showing that there is no major difference in the diversity of the microbiota based on relatedness.

4.2 Individual trap location does not significantly influence the beta diversity of the faecal microbiota

A Mantel test was performed between faecal microbiota compositions (using Bray Curtis dissimilarity index as distance measure) and geographical distance measures of individual traps to identify any associations between microbiota composition and geographical proximity of the offspring to its dam. Individual trap location and therefore the relative proximity of animals to one another did not show a significant association with the faecal microbiota composition (Mantel statistic r = -0.021, p = 0.7).

4.3 The temporal distance in sampling offspring and dam is not significantly associated with the variation in beta diversity

The temporal distance between trapping the offspring animal and the dam was compared to faecal microbiota composition. The days between capture of the offspring animal and its corresponding dam were calculated from the date of capture. Bray Curtis distance was used as a measure of faecal microbiota composition. The two distance variables were then compared using a mantel test. The association between temporal distance and microbiota composition was found to be non-significant (Mantel statistic R = 0.084, p = 0.08).

5. Discussion

The present study found no significant association between relatedness and the composition of the faecal microbiota in wild living field voles. Dams and their offspring did not have a more similar beta diversity of their faecal microbiota compared to random non-related individuals. Whilst other studies have identified a significant association between microbiota composition, measured using

UniFrac distance, and relatedness in humans these studies compared individuals known to live within the same household (Song et al. 2013; Yatsunenko et al. 2012). This might suggest that close physical proximity rather than genetic relatedness is more important for the association between relatedness and microbiota composition. The present study further supports this theory as it did not find an association between genetic relatedness and microbiota composition in voles. Comparing the microbiota composition of nest-sharing individuals would be an interesting future comparison but would require data gathering on live vole movement to track which animals share their nest.

The geographical distance between individual traps, and therefore the potential location of individual nests, did not have a significant effect on microbiota similarities; neither did the time of sample collection. As traps were spaced between 3-5m apart it is possible that voles were overlapping across these ranges and that no real association between trap location and microbiota could be established as traps were too close together.

Mothers and their offspring have been shown to have very similar microbiota compositions after birth and during breast feeding (Song et al. 2013; McVeagh and Miller 1997). A study on wild living barn swallows (*Hirundo rustica*) found a maternal but not paternal effect on the composition of the faecal microbiota of newly hatched offspring; however, this effect is unlikely to persist into adulthood (Kreisinger et al. 2017). Once nest sharing ceases and offspring became fully fledged horizontal transmission of bacteria between offspring and parent no longer occurs and allows the offspring's microbiota to be colonised by new and different bacteria to their parent animal (Bäckhed et al. 2015; van Veelen, Falcao Salles, and Tieleman 2017).

Differences both in the taxonomic and functional composition were significant between adult and juvenile faecal microbiota (Kreisinger et al. 2017). The faecal microbiota observed in adult animals was found to be two-times higher in terms of observed OTUs and alpha diversity compared to juveniles suggesting that the juvenile microbiota increases significantly until adulthood (Kreisinger et al. 2017). Within juveniles the faecal microbiota was found to have a lower microbiota variation compared to adults (Palmer, 2007; Dominguez-Bello et al., 2011; Kreisinger et al. 2017). Social offspring showed a significant similarity in faecal microbiota composition to their mother animals when using a weighted UniFrac analysis. Offspring did not show such similarity in faecal composition with sires (Kreisinger et al. 2017). The present study similarly did not find any association between sires' and their offspring's' microbiota composition. However, neither did it find an association between offspring and dams. This may be due to sampling occurring weeks after the birth process, once offspring have reached a level of maturity at which maternal impact on the initial microbiota is no longer detectable. In humans, after 11 to 12 months once solid foods have been introduced to the infants diet and the offspring becomes more independent from the mother, the functional microbiota was shown to become more similar to that of an adult and the composition between maternal and infant microbiota became more distinctly different (Bäckhed et al. 2015; Vaishampayan et al. 2010).

Microbiota may be transferred from the parent animal to the offspring during the physical contact of food provisioning, as is the case in some mammals (Lucas and Heeb 2005). In mammals, initial inoculation of newborn young by vaginal microbiota during the delivery has a long- term effect on their gut microbiota composition (Salminen et al. 2004), while pre-biotic compounds included in breast milk enable the colonisation by beneficial microbes in the gut during the first few

months of life (Bode 2012). Once the offspring is weaned and an adult diet is introduced the microbiota composition changes and becomes more dependent on the environment the individual lives in (Yatsunenko et al. 2012; Kreisinger et al. 2017).

When comparing multiple samples from the same individuals within one breeding season and across two breading season juvenile barn swallows showed a higher similarity within the same individual as would be expected by chance but only within the first breeding season (Kreisinger et al., 2017). Adults, on the other hand, retained a highly similar microbiota composition within one individual across multiple breeding seasons (Kreisinger et al. 2017). The stability of the adult microbiota opposed to the variability of the juvenile microbial composition supports the idea that as the juvenile reached adulthood its microbiota significantly changes and may no longer resemble the initially transferred microbiota of the maternal bird, and that external sources during the nestling period introduce more bacteria over time. In humans and other mammalian taxa, shifts in microbiota composition occur during the transition between the pre- and post-weaning period due to associated nutritional changes (Schloss et al. 2012; Bäckhed et al. 2015; Mach et al. 2015). Other studies on free-living vertebrates have also revealed significant population-wide changes in the faecal microbiota in response to switches in response to physiological state or health and infection status as well as diet composition changes (Amato et al. 2014; Kreisinger et al. 2015; Maurice et al. 2015b; Kreisinger et al. 2017). These findings are also supported by a study on captive mice where juveniles exhibited a rapid change in faecal microbiota composition compared to adult animals whose microbiota remained relatively stable over time (Schloss et al. 2012; Kreisinger et al. 2017).

A twin study in newly born humans suggests that incidental environmental exposures play a major role in determining the distinctive characteristics of the microbial community in each baby (Palmer et al. 2007). Whilst investigating the changes of the microbiota over the first year of life each baby showed a distinct microbial ecosystem but all twins' microbiotas had converged toward a profile characteristic of the adult gastrointestinal tract (Palmer et al. 2007). Human adults have been shown by multiple studies to have a unique gut microbiota which remains stable on the time scale of months (Ley et al. 2006; Eckburg et al., 2005; Zoetendal et al. 1998). The alterations in human gut microbiota have been shown to be significantly associated with changes in diet. In young children the changes from breast milk to solid foods, and even in later life the switch from a mainly animal based diet to a purely plant based diet (David et al. 2014). Short-term consumption of diets composed entirely of animal or plant products alters microbial community structure and the inter-individual differences in microbial gene expression (David et al. 2014). The animal-based diet increased the abundance of bile-tolerant microorganisms (*Alistipes, Bilophila*, and *Bacteroides*) and decreased the levels of Firmicutes that which are responsible for the breaking down of dietary plant polysaccharides (*Roseburia, Eubacterium rectale*, and *Ruminococcus bromii*) (David et al. 2014). Diet and the changes in seasonal availability of foods was also shown to be strongly correlated with changes in gut microbiota in wild living black howler monkeys (*Alouatta pigra*) over a ten-month period (Amato et al. 2014). Seasonal changes, most likely associated with changes in diet, were also found to be significantly associated with gut microbiota composition changes in wild living mice (*Apodemus sylvaticus*) (Maurice et al. 2015b). Across a study period of two years strong seasonal shift in gut microbiota composition could be observed, due to the transitions from insect- to a seed-

based diet (Maurice et al. 2015b). This involved decreased levels of Lactobacillus, and increased levels of Alistipes (Bacteroidetes phylum) and Helicobacter (Maurice et al. 2015b).

The trans-generational transfer of microbiota from parent generations to their offspring could have a long-standing effect on microbiota composition in the progeny of parental generations (Salminen et al. 2004; Kreisinger et al. 2017). A very small number of studies have focused on the stability and heritability of host-associated microbiota with the majority of studies focusing on human subjects or laboratory reared animals (Sporr et al., 2011; Yatsunenko et al., 2012). As individuals within captivity and laboratory breeding facilities will experience very little environmental variation or exposure to changes in condition (such as seasonality or food availability) transfer of host-associated microbiota from parent to offspring may be exaggerated and of greater importance when studying these systems (Kreisinger et al. 2017).

The present study made the assumption that by utilising the earliest capture record available chances that offspring would still have been young enough to resemble their mothers might be higher. However, no significant relationship between offspring and their mothers' microbiota composition was found, potentially due to the animals sampled already being weaned and independent and no longer in close enough contact with their mother animal to share a significant portion of their microbes.

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Chapter 4: Haemoparasites identified in a wild living house mouse (*Mus musculus domesticus*) population on the Isle of May, Forth of Firth (Scotland) and their associations with host biology

Abstract

Both macroparasites, such as helminths, and haemoparasites will often co-occur within their wild hosts and compete for resources and space. The present study investigates which haemoparasites are present in a wild living population of house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. Macroparasites present in this mouse population have previously been investigated and reported. However, no information about any existing haemoparasites was available. *Bartonella taylorii* and *Sarcocystis spp.* were found to be present at 47% and 6.9% respectively in the mice. Neither haemoparasite species showed any significant association with each other or the presence of helminths. *Bartonella taylorii* was, however, found to be significantly associated with the weight of the host as well as the year samples were collected in. *Sarcocystis* was significantly associated with the age of the host, occurring more frequently in older individuals. Finally, *Bartonella taylorii* was found to be significantly associated with the cytokine gene expression in the spleen of the host and was associated with IL10 and IL13 expression.

1. Introduction

Wild animals are often simultaneously infected by a multitude of parasites, bacteria and viruses (Cox et al., 2001; Telfer et al. 2010; Fenton 2008). Co-infections have been shown to significantly impact each other and even increase or decrease the likelihood of other infections (Telfer et al. 2010). The interactions by which parasites may increase or decrease the presence of other parasites in the host will be either direct competition, for example for resources, or indirectly by interacting with and changing the host's immune response (Lello et al., 2004; Graham et al., 2005; Behnke et al., 2008). If the interactions within the host are antagonistic then the presence of one parasite will limit or decrease the presence of others, for example as a result of limiting food resources (Lello et al. 2004; Graham, 2008; Telfer et al. 2010). If the effects of the parasite infection are mutualistic, for example by inducing immunosuppression within the host, then multiple parasites may benefit and co-exist with one another (Telfer et al. 2010; Fenton 2008). Parasites which induce generalised immunosuppression in their host, however, will not only allow for other non-pathogenic parasite infections to take hold but also potentially increase the risk of pathogenic infections (Fenton 2008). Evidence suggests that parasite infections may be driving susceptibility to the bacterial infection causing Tuberculosis in human populations living in Africa (Liu et al., 2010; Li et al., 2013). It is therefore of great importance to understand the dynamics between multiple infections within the host and to indentify all potential pathogens present within a population. It is also important to consider environmental and host variables which may drive the observed infection and susceptibility patterns. Certain parasite species may be more abundant in summer months compared to spring or autumn or occur more frequently in older individuals than younger in ones (Telfer et al. 2010; Fenton 2008).

Wild rodents are often found to be infected with helminths which are associated with a decreased expression of the inflammatory cytokine IFNγ which facilitates increased susceptibility to haemoparasites (Graham 2008). These haemoparasites often require red blood cells to successfully infect their host and will compete with each other for resources as well as compete with helminths which induce anaemia in the host (Hung et al., 2005; Graham 2008; Kuo et al., 2010). Macroparasites, such as helminths, compared to haemoparasites will often induce a different immune response in the host (Fenton 2008). Haemoparasites will often induce T helper type 1 (Th1) responses whereas helminths are usually the catalyst for T helper 2 (Th2) responses in their host (Abbas, Murphy, and Sher 1996; Mosmann and Sad, 1996; Romagnani, 1996; Pritchard et al. 1997; Stewart et al. 1999). Th1 responses will usually promote phagocytosis of infected cells, such as infected red blood cells, by macrophages, whilst Th2 responses often lead to an increased mucus production to help purge the gut of helminths (Fenton 2008). These responses are typically antagonistic to each other and will limit the presence and abundance of co-infecting parasites (Fenton 2008).

A common haemoparasite in wild rodent populations are *Bartonella* species - a bacterium found in the blood of its host - which are often species specific in their selection of host. *B. doshiae* is associated with *Microtus* species, for example, whilst *B. birtlesii* is commonly found in *Apodemus* species. Other *Bartonella* species that are common in western European rodents are *B. grahamii* and *B. taylorii* .Typically, *Bartonella* is transferred by fleas via faecal inoculation and then entering the blood stream. Infection with *Bartonella* is detectable for approximately four to eight weeks and will predominantly invade erythrocytes (Schülein et al. 2001). Fleas have commonly been observed in the fur of captured mice (*Mus musculus domesticus*) on the Isle of May, Scotland; however, to my

knowledge, no studies have been carried out on either the fleas or the mice to establish the presence of *Bartonella* infection on the island.

Other common haemoparasites in rodents include *Anaplasma spp*., *Borrelia spp*., *Trypanosoma spp.* and *Babesia spp*. (Bajer et al. 2014). The protozoan *Babesia microti* is transferred to rodents by ticks and will infect the erythrocytes of its host and eventually transfer and be detectable in the spleen (Telfer et al. 2010). Infection with *B. microti* is chronic and will usually be detectable in the blood throughout the host's life once infected (Persing et al. 1992; Siński et al. 2006). This means that the older the individual the more likely it will be to test positive for *B. microti* infection. The bacterium *Anaplasma phagocytophilum* is also transmitted by tick bites but unlike *B. microti* it is a limited infection, usually detectable for four to eight weeks (Telfer et al., 2010). It predominantly infects neutrophils of its host (Shaw et al., 2001; Springer et al., 2015).

The spirochete *Borrelia burgdorferi sensu lato* is commonly transmitted in Europe by the bite of *Ixodes ricinus* ticks (Humair and Gern 1998). *B. burgdorferi* can be acquired by the tick whilst feeding on the blood of an infected host and, once infected, ticks remain infective for life (Walker 2009; Gassner et al. 2013). Hosts which can harbour *B. burgdorferi* include wood mice and voles and but the parasite can also found be among medium-sized mammals such as hedgehogs, rats, squirrels, hares (Matuschka et al. 1994; Humair and Gern 1998). Wild wood mice, once infected with *B. burgdorferi,* will remain infective to ticks feeding on their blood over winter until the next spring (Humair and Gern 1998). Even though *Apodemus* mice have been shown to successfully transmit *B. burgdorferi* to ticks, *Borrelia* could not be detected in the rodents' blood itself using PCR. Ticks fed on

the rodents did however test positive for the infection suggesting that blood PCR analysis of rodents may not always be indicative of the presence of the spirochetes (Humair and Gern 1998)

Chronic exposure to certain haemoparasites can either increase or decrease the susceptibility to other parasites. Infection with *Babesia microti*, for example, will increase the risk of acquiring *Anaplasma phagocytophilum* which in turn increases susceptibility to *B. microti* (Telfer et al. 2010).

An individual infected with *Bartonella spp*. will have decreased susceptibility to *B. microti* as they both negatively correspond to one another. Both infections utilise red blood cells and therefore compete for resources in their host (Telfer et al. 2010; Taylor et al. 2018).

A recent study in the field vole *M agrestis* found a strong negative correlation between the presence of *B. microti* infection and *Bartonella* infection (Taylor et al. 2018). Once individuals were infected with *B. microti* a secondary infection with *Bartonella* became significantly less likely. However, the reverse was not true as the rate of infection with *B. microti* remained stable even with *Bartonella* present. A noticeable physiological difference in the field voles was the size of the spleen once infected with *B. microti*. Spleen size increased significantly in infected individuals but show no detrimental signs for the overall fitness of the animal (Taylor et al. 2018).

The composition of haemoparasites on the Isle of May has not yet been described and could be of particular interest due to the distinctly separate nature of the rodent population on the island from mainland mice.

2. Aims and Hypotheses

The aim of this study is to identify the haemoparasites present in *Mus musculus domesticus* on the Isle of May and investigate the effect of environmental variables, such as season and habitat, as well as life history traits, such as age and sex, on the presence or absence of haemoparasites.

Bartonella spp. and *Babesia spp*. are hypothesised to be present in mice on the Isle of May as these are common pathogens found in mice and other rodent species on the mainland. As lyme disease has not previously been reported on the island the presence of *Borrelia* species in the mice would be less likely but of great interest as the infection can spread to humans.

Transmission of the pathogens to the mice may be possible through ticks, carriers of *Babesia* spp., and fleas, transmitters of *Bartonella.* We expect that *Bartonella* spp. will be most prevalent among the mouse population, as fleas have been detected directly on the mice whereas ticks have not been directly observed on the island; however, ticks may not be entirely absent due to high number of migratory birds which may carry ticks and transfer them onto the mice.

The effects of *Bartonella spp.* infections on the individual host will also be investigated as infection with the microparasite has been reported to influence the body mass in other rodents (Taylor et al. 2018). An individual infected with *Bartonella* is, therefore, expected to exhibit a lower weight than an uninfected individual.

As well as exhibiting physiological associations with microparasite infection it is expected that the immune response will show significant associations with microparasite presence. Specifically infection with *Bartonella* is hypothesised to lead to an increased expression of IL10 in the mice as this association has been previously observed in field voles (Taylor et al. 2018).

2.1 Study site and species

Please see Chapter 1 for detailed description of our study site, the Isle of May in the Forth of Firth, and our study species the house mouse (*Mus musculus domesticus*).

3. Methods

3.1 Field work Isle of May

Please see Chapter 1 for field sampling methods. Mice collected in 2015 and 2016 were used in the present study, amounting to a total of 261 samples. In addition to the methods described in Chapter 1 blood samples were taken by collecting drops of blood from the tail and stored at room temperature for an hour. Samples were then placed in the fridge at 4°C for another hour before being spun at 7.000rpm for 10 minutes to separate serum and blood clots. Blood smears were collected on glass slides and fixed several days later and stained using giemsa stain.

Cytokine gene expression was obtained from spleen tissue following the same extraction and sequencing methods as used for mesenteric lymph node cytokine gene expression. Please refer to Chapter 1 for more details.

3.2 Identification of haemoparasites

Microparasite analysis for the Isle of May mice was established by PCR using DNA obtained from blood clots on culled animals. The samples were stored at -80°C, the Quantana Extracta tissue kit for DNA extraction for PCR is used for DNA extractions following the instructions outlined for tissue samples. A number of different PCRs were carried out for the detection of *Bartonella*, *Babesia, Trypanosoma, Borrelia* and *Anaplasma* species (see Table 1). All PCRs, with the exception of the *Borrelia spp.* detection, were carried out using the AccuStart II GelTrack PCR Suoermix (Quanta BioSciences) which is a two times concentrated ready mix for the use in PCR amplification of DNA fragments up to 4kb. PCR products were run on 2% agarose gels to detect presence or absence of the microparasite. A real-time PCR protocol was used for the detection of *Borrelia* as detailed in Courtney et al. (2004).

PCRs returned positive results, of which two random positive samples for each parasite PCR were sent off for sequencing to confirm the species. The samples from the *Bartonella spp*. PCR product returned a sequence which matched *B. taylorii* strains (>98% similarity value, >95% similarity with other *Bartonella spp*. strains) when analysing the sequences using BLAST. The samples sent off from the *Babesia* PCR did not return a positive match for any *Babesia* species, instead the closest match was *Sarcocystis spp*. strain at >97% similarity value using BLAST.

In addition to PCR, blood slides were also used to identify blood parasites by randomly selecting 200 fields per sample slide and recording any parasites seen by microscopy (magnification x150). A total of 261 mice were screened for haemoparasites and included in the analysis.

3.3 Statistical Analysis

All data analysis was carried out using R (R version 3.5.0; 2018-04-23). Body mass residuals were calculated using the raw body weight measures as the dependent variable in a linear model and including snout to vent length and sex as effector variables. The residual values obtained would either be negative or positive indentifying animals which were lower than average weight for their length and sex or heavier than average respectively.

Three separate generalised linear models were used to investigate the associations between microparasite presence and host variables, using a binomial approach as the dependent variables were grouped as a factor (present/absent). Each model was first run as a "full model" including all listed variables (see details for each model below) and was then reduced to the minimum adequate model by using chi-squared back-step testing in R. The results of the minimum adequate models are displayed in the results.

The first generalised linear model (Table 3) investigated the associations between *Sarcocystis* presence and sex of the host, age (based on eye lens weight and converted into age in days), trapping year, *Trichuris muris* intensity (log transformed), *Syphacia oblevata* intensity (log transformed) and weight residuals (as a proxy of condition).

A similar generalised linear model, using the same host variables, was used to test the associations between *B. taylorii* presence and host variables (Table 4). The final generalised linear model investigated the associations between *B. taylorii* presence and cytokine gene expression of the host's spleen cells. Cytokine

expressions from the spleen were investigated and included in the model, specifically expression of FoxP3, GATA3, IFNγ, IL2, IL5, IL6, IL10, IL13, and TBx21. In addition to these variables, sex, age and weight residuals of the host were included in the model. A PCA was used to summarise these cytokine expression variables into one variable (PCAcyto) to be used in the generalised linear model.

Table 1. PCR primer pairs, annealing temperature in cycling conditions, and end product length for the detection of different haemoparasites from blood clots.

Microparasite species	Forward Primer	Reverse Primer	Reference
Bartonella	CS140f 5'- TTACTTATGAT CC(GT)GG(CT)TTTA	CS1137r 5'- AATGCAAAAA GAACAGTAAACA	Birtles and Raoult, 1996 Norman et al., 1995
Babesia	Bab1F 5'- CTTAGTATAAGC TTTTATACAGC	Bab1R 5'- ATAGGTCAGAA ACTTGAATGATACA	Persing *et al*. 1992
Anaplasma	Ana3F 5'- GGATTATTCTTT ATAGCTTGCT	Ana3R 5'- TTCCGTTAAGA AGGATCTAATCTC	Chen et al., 1994 Chu et al., 1998
Trypanosoma	Nested 1st round Tryp1F 5' - GAAACAAGAA ACACGGGAG 2nd Round Tryp2F 5'-TGGGATAACAA AGGAGCA	1st round Tryp1R 5'-CTACTGGGCAG CTTGGA 2nd round Tryp2R 5'-CTGAGACTGTAA CCTCAAAGC	Noyes et al., 1998
Borrelia	Bb23Sf 5'-CGA GTC TTA AAA GGG CGA TTT AGT	Bb23Sr 5'-GCTTCAGCCTGG CCATAAATAG	Courtney et al., 2004 ***

*** Borrelia detection was carried out as a real-time PCR using the Taqman probe 5'- FAM-AGATGTGGTAGACCCGAAGCCGAGTG-TAMRA

4. Results

4.1 Microparasite species detected in mouse population on the Isle of May

Of the five haemoparasites that were tested for only two species were found in the Isle of May mouse population, testing a total of 261 animals. *Bartonella spp*. and the *Babesia spp*. All individuals that were tested were negative for *Anaplasma spp., Trypanosoma spp*. and *Borrelia spp*.. *B. taylorii* occurred most frequently, infecting 47% of all tested animals, whereas *Sarcocystis spp.* infected only 6.9% of animals. Animals testing positive for *Sarcocystis spp*. were only captured in spring sessions whereas *B. taylorii* was detected in all but one sampling session (Table 2).

Table 2. Total number of mice caught in each season/sampling session as well as number of individuals testing positive for *Bartonella taylorii* or *Sarcocystis spp.*

Season	Total number of mice caught	No. of mice infected with *Bartonella taylorii*	No. of mice infected with *Sarcocystis spp.*
Autumn 2015	100	76	0
Autumn 2016	54	0	0
Spring 2015	49	27	9
Spring 2016	42	9	9
Summer 2015	16	10	0

4.2 *Sarcocystis* infection is significantly associated with age of host and presence of *Syphacia oblevata*

Sarcocystis spp. infections were only detected in spring across two separate trapping years. The minimum adequate model, to test the associations between *Sarcocystis* and biology of the host as well as helminth infections, showed that there was a significant positive association of host age on the probability of *Sarcocystis* being present ($p < 0.005$; Figure 1). The presence of *Syphacia oblevata*

was also significantly negatively associated with *Sarcocystis* presence (p = 0.0003; Table 3).

All other testes variables included in the original generalised linear model did not show significant associations with *Sarcocystis* presence or absence (see Table 3).

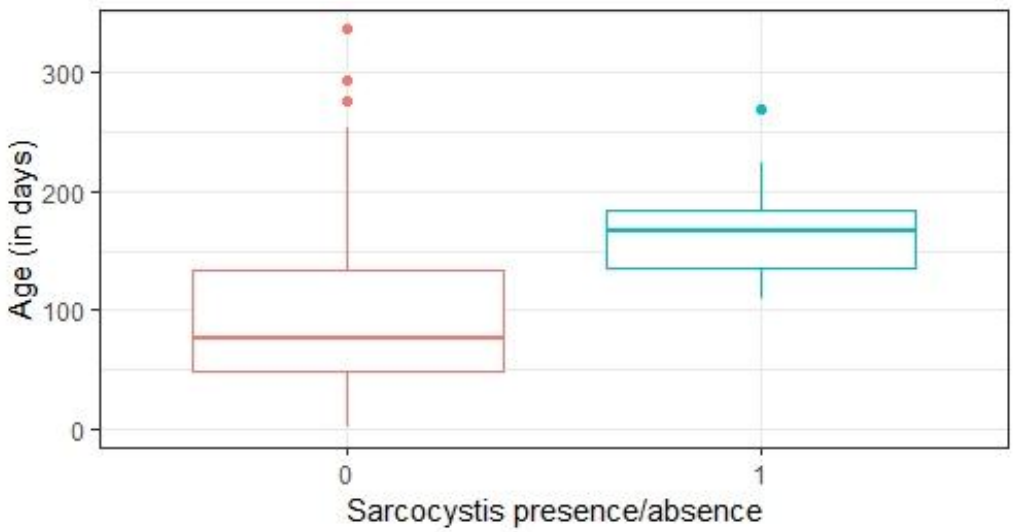


Figure 1. Association between age (in days) and presence or absence of *Sarcocystis*. Presence of Sarcocystis is denoted by "1" and represented in blue on the right hand side.

Table 3. The minimum adequate model results of the generalised linear model for the association between *Sarcocystis* presence and host variables. Likelihood ratio (LR) test were used to obtain p-values. Null Deviance: 131; Residual Deviance: 97.36; AIC: 103.4 .

Variable	Coefficient	Standard error	Residual Deviance	Degrees of freedom	LR	p-value Chi-squared
Age (in days)	2.176e-02	5.799e-03	110.2	1	20.1	0.00033 ***
Syphacia oblevata (log+1)	-1.997e+01	2.038e+03	117.5	1	12.86	7.398e-06 ***
Trichuris muris (log +1)	-2.884e-01	2.804e-01	97.36	1	1.87	0.17
Bartonella	1.379e-01	6.684e-01	92.11	1		0.83
Weight residual	3.059e-01	1.217e-01	95.49	1	1.51	0.22
Year	4.227e-01	6.753e-01	92.5	1	0.39	0.53
Sex	-6.466e-01	6.098e-01	93.98	1	1.48	0.22
Age (in days) : Weight residual	-9.836e-04	1.796e-03	92.1	1	0.33	0.57

4.3 *Bartonella taylorii* infection is associated with condition of the host, age of the host and year of trapping

The minimum adequate model for the association between *Bartonella taylorii* infection and host biology (Table 4) showed a strong effect of weight residuals on the probability of the host being infected with *B. taylorii*. Age of the host (in days) was still included in the minimum adequate model and showed a potential negative association with *B. taylorii* presence (Table 4). In addition, the year of capture was significantly negatively associated with *Bartonella taylorii* infection ($p < 0.005$; Figure 2).

Infection with either *T. muris* or *S. oblevata* did not show any significant association with the presence of absence of *B. taylorii* and was removed by the minimum adequate model chi-squared analysis.

Sex of the host was also not significantly associated with presence or absence of *B. taylorii* (p-value Chi-squared = 0.2). An interaction between sex and weight residuals was tested for, however, no significance was detected and the interaction was removed from the minimum adequate model (Table 4).

Table 4. The minimum adequate model results for the generalized linear model to investigate associations between *Bartonella taylorii* presence and host variables. Likelihood ratio (LR) tests were used to obtain p-values. Null Deviance: 361; Residual Deviance: 256.6; AIC: 264.6.

Variable	Coefficient	Standard error	Residual Deviance	Degrees of freedom	LR	p-value (Chi-squared)
Age (in days)	-0.005	0.003	260.1	1	3.44	0.063 .
Year (2016)	-3.076	0.39	262.8	1	93.54	0.013*
Weight Residual	0.167	0.068	350.1	1	6.24	< 2e-16 ***
Sex (male)	-0.44	0.372	256.56	1	1.4	0.24
Sex: Weight residuals	-0.03	0.149	255.16	1	0.05	0.815
T. muris (+1 log)	0.039	0.165	255.11	1	0.05	0.82
S. oblevata (+1 log)	0.009	0.09	255.1	1	0.009	0.92
Sarcocystis	0.022	0.677	255.1	1	0.001	0.97

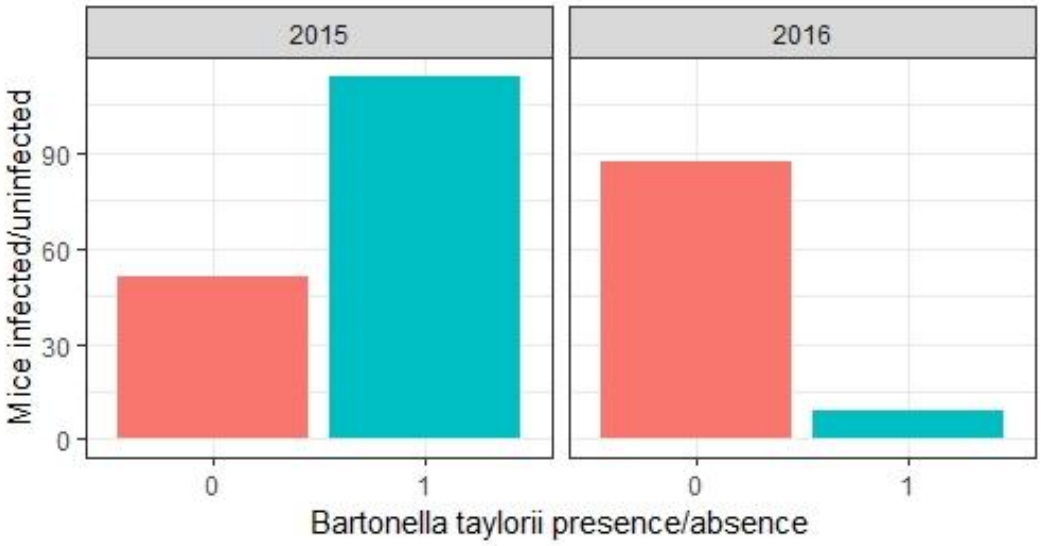


Figure 2. *Bartonella taylorii* presence and absence in samples collected in 2015 and samples from 2016. Presence of *B. taylorii* is shown in blue, absence in red.

4.4 No significant association between *Bartonella taylorii* infection and expression cytokines in the spleen was detected

A PCA was used to summarise the multiple cytokine expression variables to be used in a generalized linear model. PC1 and PC2 were selected as summarising variables for cytokine expression, based on their Eigenvalues being above 1 (PC1: Eigenvalue = 2.69; PC2: Eigenvalue = 1.22). The loadings of PC1 and PC2 can be found in Table 5. From the loadings of PC1 and PC2 it becomes evident that PC1 has no individual components strongly correlating to the variables as none of the values are above 0.5/ -0.5. In comparison, PC2 has two important components, namely IFNγ and IL1β, which are negatively correlated at -0.64 and -0.61 respectively. PC1 was negatively correlated with all raw variables and should therefore be considered as a general index for all cytokine activity.

A generalised linear model was used to investigate the associations between *Bartonella taylorii* infection and cytokine gene expression of the spleen. A two-

way interaction between age (in days) and weight residuals of the host was included in the full model but removed by back-step chi-square testing to find the minimum adequate model. Age of the host (in days) was found to be significantly associated with *Bartonella taylorii* (Table 6). PC2 of cytokine expression was also found to be significantly associated with the probability of presence of *B. taylorii* suggesting that important associations are mainly driven by the two important components of PC2, namely IFNγ and IL1β.

Table 5. The loadings for all individuals terms included in the PCA for PC1 and PC2.

Cytokine (log)	PC1 (Loading)	PC2 (Loading)
FoxP3	-0.344	0.02
Gata3	-0.34	-0.13
IFNγ	-0.19	**-0.64**
IL1β	-0.21	**-0.61**
IL2	-0.33	-0.04
IL5	-0.34	0.22
IL6	-0.33	0.27
IL10	-0.34	0.06
IL12b	-0.36	0.09
IL13	-0.34	0.24

Table 6. The minimum adequate model results of the generalized linear model to investigate the associations between Bartonella taylorii presence and spleen cytokine gene expression. Likelihood ratio (LR) tests were used to obtain p-values. Null Deviance: 136.1; Residual Deviance: 127.7; AIC: 131.7.

Variables	Coefficient	Standard error	Residual Deviance	Degrees of freedom	LR	p-value (Chi-squared)
Age (in days)	-0.011	0.004	130.94	1	8.13	0.004 **
PC2 Cytokines	0.39	0.20	127.69	1	4.87	0.027 *
Sex	-0.29	0.50	122.82	1	0.41	0.52
Weight residuals	0.078	0.21	122.41	1	0.0039	0.95
Age (in days) : Weight residuals	-0.0007	0.002	122.41	1	0.148	0.70
PC1 Cytokines	0.005	0.09	122.26	1	0.004	0.94

5. Discussion

The presence of haemoparasites on the Isle of May in a freely roaming population of house mice was investigated in the present study. *B. taylorii* was found to be present in mice throughout the trapping seasons and across both trapping years. Sequencing of PCR products revealed that some mice were also infected with an unidentified *Sarcocystis* species. *Anaplasma*, *Trypanosoma* or *Borrelia* species were not found in the mouse population.

B. taylorii was found to be most commonly present parasite, potentially due to the high number of fleas recorded on the mice whilst ticks were not observed. Fleas are an important insect vector for transmitting *Bartonella* species (Breitschwerdt and Kordick 2000). An experimental study transferred fleas collected from infected cats to specific pathogen free cats which tested positive

for *Bartonella* after exposure to the infected fleas (Chomel et al. 2009). Fleas will become infected and support replication of *Bartonella* species after ingesting blood from an infected host (Higgins et al. 1996). The fleas on the Isle of May are therefore the perfect insect vector to infect and spread the parasite to the mice.

The present study only detected *Sarcocystis* in the spring sampling session across two separate sampling years. Parasite infections on the island may not only occur through horizontal acquisition, such as sharing of infected ticks and fleas between nest mates, but could potentially be introduced by migratory birds, such as robins (*Erithacus rubecula*) and puffins (*Fratercula arctica*), to the island during spring. The first full life cycle of sarcocysts was described in 1977 by Munday et al.(1977) and involved the Red junglefowl (*Gallus gallus*) as intermediate host and a dog (*Canis familiaris*) as definite host. Since then birds such as the Northern goshawk (*Accipiter gentilis*) have been identified as potential definite hosts as well as intermediate hosts (Cerna and Kvasnovska 2013). Raptors, such as peregrines (*Falco peregrinus*) and short-eared owl (*Asio flammeus*), have repeatedly been observed on the Isle of May (personal communication David Steel, Isle of May Warden) and could be potential carriers of sarcocysts onto the island.

Sarcocystis is the largest genus within the family Sarcocystidae and consists of species which infect a range of animals including mammals, birds and reptiles. *Frenkelia*, another genus within this family, consists of parasites that use rodents as intermediate hosts and birds of prey as definitive hosts. The parasite reproduces sexually in the gut of the definitive host, is passed with the faeces and ingested by the intermediate host. Once the intermediate host is consumed by

the definitive host, the cycle is completed (Markus, Killick-Kendrick, and Garnham 1974).

Presence of *B. taylorii* in Isle of May mice was significantly associated with trapping year and a higher number of mice were infected in 2015 compared to 2016. This observation could be a true representation of infection with *B taylorii* in the wild but might also be due to chance sampling of more infected individuals in 2015 compared to the following year or variability in the detection of *Bartonella* in the laboratory. However, studies have reported difficulties in isolating and documenting low levels of *Bartonella spp*. infection which may lead to under-detection of the parasite in the wild (Breitschwerdt and Kordick 2000). A possible explanation for the findings of the present study could therefore be lower success in detection of *Bartonella spp.* infection in mice sampled in 2016, potentially due to lower levels of parasites present in the blood samples collected.

B. taylorii was found to be significantly associated with the host's weight residuals. The present study hypothesised that infected individuals will have a lower overall weight; however, the opposite seems to be the case as animals with a higher weight appear to be more likely to be infected with *B. taylorii* whilst individuals with a low weight residuals are highly unlikely to be infected. Though these observations could be driven by age difference, as older individuals are more likely to have been exposed to the parasite in their lifetime compared to young mice, age was considered in the overall model and was not significantly associated with *B. taylorii* infection. Larger and heavier individuals are often thought to be in better condition and it is possible that only individuals in good condition exhibit *Bartonella taylorii* infection as lighter individuals may have died

from the infection or become susceptible to other pathogens which may have killed them.

Sarcocystis infection did not show an association with weight of the host but was associated with age of the host (in days). Older individuals were found to be more likely to be infected with *Sarcocystis*. A study involving several different rodent species found that heavier individuals were significantly more likely to be infected with *Sarcocystis* (Svobodová et al. 2004) which may explain the findings of the present study as older individuals will often also be the heavier individuals.

Longitudinal sampling enabled data collection on when the parasite was first recorded within a host and whether or not the parasite was later cleared or not (Taylor et al. 2018). To improve the findings of the present study adding longitudinal data in future sample collections would yield more information about the nature of the parasite infections and the immune response within the host.

The present study did not find any significant associations between microparasite infections and helminths' presence. Nor did the present study identify a significant association between presence and absence of the haemoparasites on each other. These results are unexpected as other studies have found significant impact of macroparasites on the presence of haemoparasites as well as competition among haemoparasites themselves influencing susceptibility of the host to other parasite infections (Telfer et al. 2010; Fenton 2008). Differences in the findings of the present study in comparison to other studies may be due to differences in methodological approach.

A significant association could be detected between *B. taylorii* presence or absence and cytokine expression in the spleen, specifically the cytokines IFNγ and IL1β. A relationship between *B. taylorii* infection and the immune response in the spleen was expected as *Bartonella*-infected red blood cells would be in direct contact with the spleen (Dehio, 1999; Dehio, 2001). Infection with haemoparasites has been found to affect the spleen size in rodents (Taylor et al., 2017), which in turn was shown to be associated with inflammatory responses in the immune system (Jackson et al., 2011). IFNγ is expressed during Th1 inflammatory responses against intracellular parasites (Mosmann et al., 1989; O'Garra et al., 2000; Theofilopoulos et al., 2001). IFNγ produced by Th1 cells has the dual effect of both stimulating Th1 development, and inhibiting Th2 development (Abbas et al., 1996). Interleukin (IL)-1 is also part of the inflammatory response in the context of infections, and refers to both IL1α and IL1β. Monocytes and macrophages are the primary source of IL1β. (Jacques et al., 2006). The findings of the present study are supported by another study conducted on laboratory reared mice which showed an increase in IFNγ secretion in spleen cells following infection with *Bartonella henselae* (Kabeya et al., 2007). The production of IFNγ was induced by antigen-specific lymphocytes as well as nonspecific cell-mediated immune responses, including activated macrophages (Kabeya et al., 2007). Another laboratory based murine model showed that nitric oxide derived from macrophages activated by IFNγ play a significant role in eliminating *B. henselae* from infected cells (Musso et al., 2001). Therefore, the association between *B. taylorii* infection and expression of IFNγ in the spleen is most likely due to the immune response trying to eliminate the intracellular infection.

Measuring the responses to experimentally removing or adding other parasite species within a wild living host population would be the most reliable way to investigate the interactions between parasite species and the effects they have on the host (Fenton et al. 2014). However, these types of experiments, especially in wild populations, are rare. Instead observational studies try to investigate parasite interactions and will often report significant correlations in co-occurrence of parasite species (Lello et al. 2004; Telfer et al. 2010), which might not be found by other studies investigating the same natural populations (Fenton et al. 2014). Correlation-based approaches have been found to be unreliable for investigating parasite interactions and the most reliable way to study these associations would be through longitudinal studies sampling the same individuals over several months (Fenton et al. 2014). To improve the output of the present study longitudinal sampling would be of great benefit and would offer the opportunity to investigate exactly when and how long for individuals are infected with each parasite.

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General Discussion

The present thesis aimed to investigate the variability and composition of the gut microbiota in two wild rodent populations. The effects of host and environmental variables were explored as well as any associations with the hosts' immunological responses.

The two rodent populations investigated in this thesis showed great similarities in their microbiota compositions as well as the responses and associations of the microbiota with host, environmental and immunological variables. Both voles and mice showed a significant association between the seasons in which samples were collected and the gut microbiota composition. Explanations for this have been given in the respective chapters but essentially involve potential differences in diet of the host.

To further investigate this point a detailed study of the rodents diets would need to be carried out, using both vegetation surveys of the sampling locations and isotope data taken from the rodents to confirm what they have been eating.

Tissue sampling type was found to be impacting on microbiota composition as different sample tissues taken from the same individual showed significant differences in microbiota composition. Human studies have similarly found that the microbiota composition varies within one individual depending on sampling location within the body (Costello et al., 2009). These differences are believed to be due to the specialised functions that the microbes perform in each location. An interesting next step for the present thesis would be to generate functional data

of the investigated microbiota and add the functionality of microbes to the analysis to further understand the emerging patterns. One such method would be to add functionality data by analysing microbiota sequences using “Greengenes” (McDonald et al., 2012) and “BugBase” (Ward et al., 2017).

Another finding of the present thesis was the variation between proximal colon samples sampled at 24 hours after capture and colon samples taken from individuals after 48 hours of captivity. This difference could be explained by exposure to standard laboratory foods instead of the voles’ original wild diet or potentially elevated stress levels which impact microbiota composition. A way to further investigate this would be to monitor changes in stress levels, for example by measuring cortisol, an important hormone involved during stress responses (Cryan and Dinan, 2012). Potential changes in these stress levels should then be compared to the microbiota composition as well as the changes in functional genes within the microbiota. By so doing potential associations between stress and microbiota could be determined.

No significant association could be found between the role of relatedness on the similarity or dissimilarity of the gut microbiota composition. In human studies new-born infants show significant similarities in their microbiota composition to their mothers, suggesting that the sampling of wild voles occurred at an age at which offspring had outgrown such similarities to their dams. By sampling younger animals, still in their suckling stage, it could be possible to detect a similar association between dams, their offspring and the shared microbiota. Sampling animals directly in their nests would also allow to be certain of parentage. Observations around nest-sharing into adulthood among voles would also allow

further investigation into the role of close physical proximity among the animals and the microbiota composition of the individuals.

Finally, the haemoparasite present on the Isle of May within its wild house mouse population were identified as these parasites had never been investigated on the island before. *Bartonella taylorii* and *Sarcocstis spp*. were found to be present within the mouse population. The haemoparasites showed no significant association with each other or the presence of other parasites, namely helminths. Whilst *B. taylorii* was found across multiple sampling sessions and in both autumn and spring, *Sarcocystis spp*. were only indentified in a small number of mice in the spring months. Further investigation would be necessary to determine a cause for this observation. Other mammals and birds inhabiting the island ought to be screened for the microparasites as a potential explanation for transmission to the mice.

Overall, wild rodent populations were found to have stable alpha diversity of their gut microbiota; however, environmental variables, especially location, host variables, such as sex, and immunological variables showed significant impact on the beta diversity of the microbiota.

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Appendix 1

Primerdesign *Precision nanoScript™ 2* Reverse Transcription Kit

Kit Contents

Sufficient reagents are supplied to complete 50 Reverse Transcription reactions
 Oligo-dT primer (YELLOW)

 Random nonamer primer (RED)

 dNTP mix (10mM of each) (ORANGE)

 nanoScript 2 enzyme (WHITE)

 nanoScript 2 4X reaction buffer (BLACK)

 RNAse/DNAse free water (WHITE)

 (Optional) Gene specific primer, lyophilised (GREEN) re-suspend in 990ul Water

Reagents and Equipment to Be Supplied by User

 Thermostatically controlled “hot block” or water bath

 Pipettors and Tips

 Ice

 Vortex and centrifuge

 RNA Template

We recommend RNA be DNAse treated prior to use to reduce the potential for real-time PCR detection of contaminating genomic DNA.
 Thin walled 0.5ml tubes with close fitting lids

 Thin walled 0.2ml PCR tubes

Bench-side Protocol

Annealing Step

1. For each RNA sample add the following reagents to a thin walled 0.2 ml PCR tube or equivalent.

Component	1 reaction
RNA template (up to 2µg)	X µl
RT primer (YELLOW) and/or (RED)* or (GREEN)	1.0 µl
RNAse/DNAse free water (WHITE))	X µl
Final volume	10 µl

2. Apply a lid to each sample and then heat to 65°C for 5 minutes. This can be achieved using a thermostatically controlled “hot block” or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.
3. Immediately cool the tubes in an ice water bath.

For optimal results, samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

Extension step

1. Make up a mix according to the protocol below:

You will need 10µl for each RT reaction.

When working with 1ng - 2µg of RNA: Component	1 reaction
nanoScript2 4X Buffer (BLACK)	5.0 µl
dNTP mix 10mM (ORANGE)	1.0 µl
RNAse/DNAse free water (WHITE))	3.0 µl
nanoScript2 enzyme (WHITE)*	1.0 µl
Final volume	10 µl

Extension step

1. Make up a mix according to the protocol below:

You will need 10µl for each RT reaction.

When working with 1ng - 2µg of RNA: Component	1 reaction
nanoScript2 4X Buffer (BLACK)	5.0 µl
dNTP mix 10mM	1.0 µl

(ORANGE)	
RNAse/DNAse free water (WHITE))	3.0 µl
nanoScript2 enzyme (WHITE)*	1.0 µl
Final volume	10 µl

2. Add 10 µl of this mix to each of the samples on ice.

3a. For oligo-dT or gene specific priming alone (YELLOW or GREEN)
Apply a lid to each sample, mix tubes by briefly vortexing followed by a pulse spin and incubate at 42°C for 20 minutes.

3b. When using random primers alone (RED) or in combination with oligo dT or gene specific primers
Apply a lid to each sample, mix tubes by briefly vortexing followed by a pulse spin and incubate at 25°C (room temperature) for 5 minutes and then at 42□C for 20 minutes.

4. Heat inactivate the reaction by incubation at 75□C for 10 minutes.

5. Store cDNA samples at -20ºC until use.

Appendix 2

Project Report
LIMS15632
Mouse and Vole Metagenetics Analysis

Laboratory analysis was performed by Lucille Rainbow (library production) and Anita Lucaci
(sequencing).
Bioinformatics analysis was performed by Luca Lenzi.
For queries regarding the laboratory analysis, please contact:
Lucille Rainbow, library preparation (lrainbow@liverpool.ac.uk)
Anita Lucaci, sequencing production (anita@liverpool.ac.uk)
John Kenny (Jkenny@liverpool.ac.uk)
For queries regarding the bioinformatics analysis, please contact:
Luca Lenzi (llenzi@liverpool.ac.uk)
Sam Haldenby (s.haldenby@liverpool.ac.uk)

1. BACKGROUND

The aim of this project is to identify bacterial communities in mice from the Isle of May and
vole from the Kielder forest. Faecal samples as well as colon samples from voles were
collected to perform longitudinal comparative studies. The mice were used to collect faecal
samples only. Online Table 1 (Table1.ExperimentalDesign.xlsx) summarises the samples that
underwent sequencing.
The project requires the following steps:
1. Amplicon production and sequencing.
2. Quality trimming of the reads.
3. Selecting the best quality reads for each amplicon targets.
4. Filtering for PhiX phage sequences used in the sequencing process.
5. Defining cluster of amplicons for each locus.

2. LABORATORY ANALYSIS

Primers described by Caporaso et al (2011 PNAS 108, 4516-4522) were used to amplify the
V4 region of 16s. A total of 658 samples were submitted and 5 µl of each DNA sample at
1ng/µl was entered into the first-round PCR with conditions 98oC for 2 mins, 20sec at 95oC,
15secs at 65oC, 30sec at 70oC for 10 cycles then a 5min extension at 72oC.
F:
5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA3'
R:
5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT3'
The primer design incorporates a recognition sequence to allow a secondary nested PCR
process. Samples are first purified with AMPure SPRI Beads before entering the second PCR
performed to incorporate Illumina sequencing adapter sequences containing indexes (i5 and
i7) for sample identification. 15 cycles of PCR were performed using the same conditions as
above for a total of 25 cycles in all. Samples were purified using AMPure SPRI Beads before
being quantified using Qubit and assessed using the Fragment Analyzer. Successfully
generated amplicon libraries were taken forward.
Those samples that failed to amplify were not taken forward for sequencing. Collaborator
positive and negative controls were included, as were CGR positive and negative controls.
Final amplified libraries and controls were pooled in equimolar amounts into 8 pools using
the Qubit and Fragment Analyzer data, and the Mosquito X1, followed by size selection on a
1.5% Pippin prep gel using a size range of 300-600bp.
Quantity of the size selected pools of amplicon libraries was completed using a Qubit® dsDNA
HS Assay Kit, while the quality and average size was assessed using the High Sensitivity DNA
Kit on either the Agilent Bioanalyzer or the Advanced Analytical Fragment Analyzer.
Subsequently, a quantitative real-time PCR (qPCR) assay, designed to specifically detect
adapter sequences flanking the Illumina libraries, was performed using an Illumina® KAPA
Library Quantification Kit (Kapa Biosystems, Wilmington, USA). Briefly, a 20 µl PCR reaction

(performed in triplicate for each pooled library) was prepared on ice with 12 µl SYBR Green I
Master Mix and 4 µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial
concentration determined by the Qubit® dsDNA HS Assay Kit). PCR thermal cycling conditions
consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds
(denaturation) and 60°C for 45 seconds (annealing and extension), melt curve analysis to 95°C
(continuous) and cooling at 37°C (LightCycler® LC48011, Roche Diagnostics Ltd, Burgess Hill,
UK).
The template DNA was denatured for 5 minutes at room temperature using freshly diluted
0.1 N sodium hydroxide (NaOH) and the reaction was subsequently terminated by the
addition of hybridization buffer. Following calculation of the molarity using qPCR data,
template DNA was diluted to a loading concentration of 8 pM using the hybridization buffer.
The amplicon libraries were sequenced on an Illumina® HiSeq 2500 platform with version 2
chemistry using sequencing by synthesis (SBS) technology to generate 2 x 300 bp paired-end
reads.

3. BIOINFORMATIC ANALYSIS

3.1. INITIAL PROCESSING AND QUALITY ASSESSMENT OF THE SEQUENCE DATA

Initial processing and quality assessment of the sequence data was performed using an inhouse pipeline (developed by Richard Gregory). Briefly, base-calling and de-multiplexing of indexed reads was performed by CASAVA version
1.8.2 (Illumina) to produce 658 samples across the two runs, in FASTQ format. The raw FASTQ files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011).
The option “-O 3” was set, so the 3' end of any reads which matched the adapter sequence over at least 3 bp was trimmed off. The reads were further trimmed to
remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20 bp were removed. If both reads from a pair passed this filter, each was included in

the R1 (forward reads) or R2 (reverse reads) file. If only one of a read pair passed this filter, it was included in the R0 (unpaired reads) file.
Online Table 2 (Table2.SequenceTrimming.xlsx) summarises the read counts before and after adapter and quality trimming for the dataset. All raw and trimmed read data for the dataset as well as detailed statistics on the read trimming are available from the following URLs:
http://cgr.liv.ac.uk/illum/LIMS15632_fec3feda10cd25a5
This analysis was performed to assess the quality of the reads, later analyses utilise R1 and R2 raw reads.

3.2. READS QUALITY FILTERING

The raw reads were subjected to a Cutadapt trimming step to remove PCR primer sequences
that could potentially introduce an artificial level of complexity in the samples. Due to the
design of the Illumina sequencing adapters, the PCR primer sequence is located downstream
of the sequencing adapter sequence, therefore with this step both Illumina adapters and
sequencing primers are removed.
To improve base quality in both read pairs, sequencing errors were corrected in both forward and reverse reads using the error-correct module within SPAdes assembler, version 3.1.0 (Bankevich et al. 2012). Read pairs were aligned to produce a single sequence for each pair that would entirely span
the amplicon using PEAR (version 0.9.10; Zhang et al., 2014). This applies an optimal quality
trimming to each sequence mate to obtain the best possible alignment. Additionally,
sequences with uncalled bases (Ns) were removed. To remove sequences originating from
potential PCR primer dimers or from any spurious amplification events, a size selection was
applied to select sequences between 200 bp and 600 bp within merged sequence set.
Fragmented PhiX phage genome was added to the sequence library to increase the sequence complexity (which can be low for amplicon sequencing and prevents the Illumina technology from functioning correctly). PhiX sequences are not indexed, so should not be associated with indexed reads after de-multiplexing. However, a very small proportion of PhiX reads can become associated with an index (likely as a result of 'cross-talk' between adjacent clusters on the sequencing flowcell). To remove these sequences, each sample was compared with the complete PhiX sequence (GenBank gi9626372) using BLASTN (Altschul et al., 1990). Sequences matching PhiX (E-value < 10^{-5}) were filtered out of the dataset.
Online Table 3 (Table3.SequencePreprocessing.xlsx) summarizes the metrics for these

filtering steps for the data. Overall, for most of the samples more than 99.61% of the initial
sequences pass the filtering steps.

3.3. METAGENETICS ANALYSIS

Any sequences passing the filters for each sample were merged into a single file. This final
sequence file, plus its own metadata file describing each sample, was used for the analysis
by using a custom pipeline based on QIIME1.9.1 (Caporaso et al., 2010).
GreenGenes database (version 13.8) was used throughout the analysis.
The following analysis steps were performed keeping separate 'vole' from 'mice' samples, to
achieve the best possible resolution of the microbial communities within each species.

3.3.1. Defining clusters of amplicons, OTUs and their taxonomy identifications

To identify the sequence variability in each sample, the obtained amplicon sequences were
sorted and assigned to groups (clusters) according to their sequence similarity. Ideally, a
group of sequences (cluster) should contain all, and only, the sequences derived from the
same original amplicon. This step has been performed using SWARM (version 2.2.1, Mahé et
al., 2014) with the strictest parameters. This parameter indicates the maximum number of
differences that a sequence must have from another sequence of the same cluster, default
equal to 1. This will ensure that for each sequence aggregate to a cluster, there is at least one sequence that is only 1 base distant from it.
To calculate the abundance of each cluster, sequences were then aligned on the identified
centroid clusters sequences, using a minimum similarity threshold of 97% for the entire length
of the sequence. The '`--usearch_global`' function implemented in VSEARCH2.6.2 was
used for this step. VSEARCH (a free tool implementing the UCLUST algorithm in USEARCH,
Edgar, 2010) tool is designed to retain and output the best possible alignment after screening
for all the possible alignments passing the defined similarity thresholds (97% in this case).
Taxonomic assignment of each cluster (now referred to as operational taxonomic unit, OTU)
was carried out using the QIIME script **assign_taxonomy.py**, using the RDP classifier (Wang

et al., 2007) to match the centroid sequence of each cluster obtained by swarm, to a sequence from the database.
Number of OTUs associated to each kingdom are indicative, reflecting the taxonomy in the database used. 16S derived from chloroplast and mitochondria may be
assigned to either bacteria or eukaryotes taxonomy according to the convention used by the
database curators, and these specific cases are not taken into account when computing such
statistics.

The analysis resulted in a phylogeny of the OTUs and an OTU table file (in "biom" format, for
details see http://biom-format.org/), which contains the OTU information for each sample as
well as taxonomic information on the OTUs and sample metadata. Given that the de-novo
clustering approach performed is subjected to produce several low abundant clusters, the
abundance table is post-processed to remove any OTU below 0.005% of the total sequence
count of sequences (Bokulich, et al., 2013). The final number of sequences clustered to any
of the newly identified OTUs for each sample are summarised in online Tables 5 and 6,
respectively for 'Vole' and 'Mice' (Table5.OTUsCount.Vole.xlsx, Table6.OTUsCount.Mice.xlsx).
They show the number of identified clusters in each sample and the percentage of sequences
aligned to any of them.

4. DESCRIPTION OF THE RESULTS FILES

All of the raw data files can be downloaded from the following URL:
http://cgr.liv.ac.uk/illum/LIMS15632_fec3feda10cd25a5
All of the results files can be downloaded from the following URL:
http://www.cgr.liv.ac.uk/illum/LIMS15632Results_165978892ecc8211/
All the analysis results are included into the 'Vole' and 'Mice', each including the following
files:

'**qiime.seq.fasta.gz**'
All the sequences passing the filtering steps.

'**otu_table.biom**'
The main QIIME output, containing the OTUs abundance table in binary format

‘**otu_table.classic.taxa.txt**'
OTUs table in human readable format (loadable in Excel).

‘**rep_set.fna**'
List of representative sequence for each OTU, used in the assign taxonomy step with
RDP taxonomy assigner.

‘**rep_set_tax_assignment.txt**'
The RDP main output including the taxonomy identification for each OTU.

‘**rep_set_aligned_filtered.tree**'
Phylogenetic tree (Netwick format) for the identified taxa.

‘summary.even.abs’ ‘summary.even.rel’
Files obtained after summarising abundances for each identified taxon, at multiple possible taxonomic levels.
Absolute and relative abundances were used according to the folder name (‘abs’ and ‘rel’, respectively).
The folder also includes all the tables cited in this documents:

‘**Table1.ExperimentalDesign.xls**’, summarising the metadata information associated
to the samples in the dataset.

‘**Table2.SequenceTrimming.xlsx’**, summarising the quality trimming information
associated to the sequenced samples (although not used in the analysis).

‘**Table3.SequencePreprocessing.xlsx**, summarising the pre-processing steps and the
QC of the sequences utilised in the analysis, for each sample.

‘**Table5.OTUsCount.Vole.xlsx**, summarising the number of OTUs identified and the
number of aligned sequences onto them for each vole sample.

‘**Table6.OTUsCount.Mice.xlsx’**, summarising the number of OTUs identified and the
number of aligned sequences onto them for each mice sample.

Although not discussed in this document because they are now obsolete, the results of the
previous analysis obtained clustering together vole and mice samples then separating the
OTUs accordingly to the species of the samples were also provided in the following folders

‘**Vole.splitted**’ ‘**Mice.splitted**’.

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Appendix 3

PowerSoil® DNA Isolation Kit

Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.

2. Gently vortex to mix.

3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.

7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note

Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).

14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds. 11

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Appendix 4

NuceloSpin® RNA extraction kit Bench-side Protocol

1. Add 10X Precision DNase reaction buffer directly to your RNA

The amount added will vary depending on the volume of your RNA. Add 5µl of buffer for every 50µl of RNA.
2. Add 1µl of Precision DNase enzyme to each reaction.

1µl of enzyme will successfully eliminate DNA from up to 100µl of RNA solution.
3. Incubate for 10 minutes at 30°C. (DNase Treatment)

Where necessary this can be extended to 30 minutes without any degradation of RNA.
4. Incubate for 5 minutes at 55°C. (DNase inactivation)

5. Proceed to Reverse Transcription step or store DNase treated RNA at < -20°C for later use

Appendix 5

Genotyping data preparation

Sequoia assumes HW equilibrium in samples so removed SNPs which violated this assumption.

Sequoia also requires low levels of LD (high levels can lead to spurious results):

1. 1) Calculated LDs

2. 2) Found all pairs with D' > 0.7 (n = 240) - mostly within genes

3. 3) So, for genes appearing in these pairs, if multiple SNPs, choosing SNP with highest minor allele frequency (MAF) and removing rest

4. 4) Recalculating LDs -> a few pairs of SNPs still with D' > 0.7 (n = 8) but this time, between genes

5. 6) For these remaining pairs choosing SNP/gene with highest MAF and removing other.

Sequoia favours SNPs with higher MAF (e.g. > 0.3) for large datasets but since we have c. 100 SNPs only being less stringent:

1. 1) MAF > 0.1

2. 2) Call rate > 0.70

3. 3) Removing samples for which > 50% of SNPs missing

4.

Final numbers

n = 114 SNPs

n = 2667 samples

Life history data preparation

Sequoia required Life history (LH) information re. individual's sex and an indication of their birth date (e.g. birth month – BM): "The age differences calculated from the birth years provided in Life history data are used as a

secondary source of information, amongst others to help distinguish between half-siblings, grandparent–grand-offspring and full avuncular pairs".

Within our (original) genotype dataset there are 5 classes of individuals. Each of these require looking into different datasets for this LH information

1. 1) Tail sample only -> longitudinal field data (sex and BM)

2. 2) Multiple tail samples -> longitudinal field data (sex and BM)

3. 3) Tail sample(s) and ear sample -> longitudinal field data (BM), cross-sectional lab data (sex as more reliable)

4. 4) Ear sample, tagged -> longitudinal field data (BM), cross-sectional lab data (sex as more reliable)

5. 5) Ear sample, not tagged -> cross-sectional lab data (sex and BM)

Sex

In longitudinal dataset, sometimes get mismatch between sex classifications for same individual. To get around this, identified the most common sex classification.

Birth date

This is the trickiest variable to estimate. I have used the following criteria:

Only individuals that juvenile or already breeding at first capture given a birth date.

Birth date then assumed to be:

1. 1) 2 weeks prior to first capture if juvenile and 12g or less.

2. 2) 4 weeks prior to first capture if juvenile and between 12-15g (Burthe et al. 2010; Begon et al. 2009) - if bigger possible that wrongly assigned juvenile status so leave blank.

3. 3) 6 weeks prior to first capture if breeding (minimum age at first breeding) - if non-breeding at capture don't know if previously bred or not i.e. could be juvenile or adult (with very different birth dates), but if breeding know that individual is at least 6 weeks.

Birth month (BM) then assigned from this birth date.

Pedigree inference: re. "Site_spec_pedigrees"

A pedigree was inferred for each site separately. Excel files, each including following information can be found in folder "Site_spec_pedigrees":

1) id: PIT TAG number of individual (if available), otherwise tail tube number or vole ID. In order to build the pedigree sequoia infers ‘dummy’ individuals (id beginning with F for hypothetical female and M for hypothetical male), this allows e.g. half-sibling relationships to be established without the need for shared parent to be genotyped. NB/ Dummy individuals in different sites share the same ids but they are not the same individuals

2) dam: assigned mother

3) sire: assigned father

4) LLRdam/LLRsire/LLRpair: The log10 likelihood ratio (LLR) of the dam, sire and the parent pair; this is the ratio between the likelihood of the assigned parent being the parent, versus the most likely alternative type of being related to the focal individual

Deducing reproductive success from the inferred pedigrees: re. “RS_pedigree_indivs.xlsx”

I have counted up the number of times a female/male appears as a dam/sire which gives you the reproductive success as inferred from the pedigree. This is for individuals with a reproductive success of 1 or more.

In doing this, you miss two types of individuals with ‘zero’ reproductive success. Those which don’t appear in the pedigree at all (for which we have no information) and those which appear as e.g. sibling, parent or grandparent match. I am assuming here that we are more confident about the latter class of ‘zeros’ i.e. those which never appear as a dam or a sire but do appear in another capacity in the pedigree. Only these zeros as included in the spreadsheet for now.