Evolutionary genomics of host-microbe interactions

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Title

Evolutionary genomics of host-microbe interactions

Abstract

The microbes living inside hosts have highly important consequences for host health and fitness. From the host's perspective, some microbes exhibit mutualistic tendencies, others parasitic, and some commensal, but this is context-dependent and opportunistic lifestyles are widespread in nature. Our knowledge of how hosts interact molecularly with different microbes is, however, poor, and little research has been done on non-model organisms from a genomic and community-wide perspective. In this PhD thesis, I investigate host-microbe interactions from multiple angles, and utilize high-throughput sequencing techniques to paint a broad, overarching picture of the relationship between hosts and microbes.

My PhD comprised two related projects, 1) host-microbiome interactions and 2) host-parasite interactions. In the former, I have evaluated how to best sample and measure the gut microbiomes of avian hosts (Paper I and II). Different sections of the ostrich gastrointestinal tract were characterized and shown to harbour divergent microbial communities (Paper I, II, and IV). I have further demonstrated that the gut microbiome of juvenile ostriches is colonized in a successional manner and gradually develops over time (Paper III), and is strongly linked to growth and mortality (Paper III and IV). In the second project I described the avian transcriptome response to malaria infection over time and to parasites with different virulence (Paper V and VI). Birds with malaria infection experience a range of transcriptional changes that involves for example the immune system, stress response, cell death regulation, and regulatory genes. To evaluate the molecular response of the malaria parasite, I assembled the blood transcriptome of *Plasmodium ashfordi* and showed that parasite gene expression is host-specific (Paper VII). This transcriptome was subsequently used, together with a genome assembly of *Haemoproteus tartakovskyi*, to construct a phylogeny of haemosporidian parasites which showed strong support for a monophyletic clade of mammalian malaria parasites (Paper VIII). Finally, the assembled transcriptome and genome were utilized to identify thiamine biosynthesis enzymes in avian *Plasmodium* (Paper IX), and to demonstrate that the avian *Plasmodium* parasites exhibit the most AT-rich genes of eukaryotes (Paper X).

In summary, this work offers new insights into host-microbiome and host-parasite interactions, and enables a greater understanding of the multifaceted relationship between hosts and their microbes.

Key words

host, microbiota, avian malaria, ostrich, parasite, dual RNA-seq

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We have never been individuals – We are all lichens

Scott F. Gilbert

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I. **Elin Videvall**, Maria Strandh, Anel Engelbrecht, Schalk Cloete, Charlie K Cornwallis. (2017) Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources*. doi: 10.1111/1755-0998.12744.

II. **Elin Videvall**, Maria Strandh, Anel Engelbrecht, Schalk Cloete, Charlie K Cornwallis. (2017) Direct PCR offers a fast and reliable alternative to conventional DNA isolation methods for gut microbiomes. *mSystems*, 2: e00132-17.

III. **Elin Videvall**, Se Jin Song, Hanna M Bensch, Maria Strandh, Anel Engelbrecht, Naomi Serfontein, Olof Hellgren, Adriaan Olivier, Schalk Cloete, Rob Knight, Charlie K Cornwallis. (2018) The development of gut microbiota in ostriches and its association with juvenile growth. *bioRxiv*. 270017. doi: 10.1101/270017.

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V. **Elin Videvall**, Charlie K Cornwallis, Vaidas Palinauskas, Gediminas Valkiūnas, Olof Hellgren. (2015) The avian transcriptome response to malaria infection. *Molecular Biology and Evolution*, 32 (5): 1255–1267.

VI. **Elin Videvall**, Vaidas Palinauskas, Gediminas Valkiūnas, Olof Hellgren. (2018) Avian transcriptome responses to high- and low-virulent malaria strains over the course of infection. Manuscript.

VII. **Elin Videvall**, Charlie K Cornwallis, Dag Ahrén, Vaidas Palinauskas, Gediminas Valkiūnas, Olof Hellgren. (2017) The transcriptome of the avian malaria parasite *Plasmodium ashfordi* displays host-specific gene expression. *Molecular Ecology*, 26 (11): 2939–2958.

VIII. Staffan Bensch, Björn Canbäck, Jeremy D DeBarry, Tomas Johansson, Olof Hellgren, Jessica C Kissinger, Vaidas Palinauskas, **Elin Videvall**, Gediminas Valkiūnas. (2016) The genome of *Haemoproteus tartakovskyi* and its relationship with human malaria parasites. *Genome Biology and Evolution*, 8 (5): 1361-1373.

IX. Olof Hellgren, Staffan Bensch, **Elin Videvall**. (2017) *De novo* synthesis of thiamine (vitamin B_1) is the ancestral state in *Plasmodium* parasites – evidence from avian haemosporidians. *Parasitology*. doi: 10.1017/S0031182017002219.

X. **Elin Videvall**. (2018) *Plasmodium* parasites of birds have the most AT-rich genes of eukaryotes. *Microbial Genomics*, 4 (2): 1–9. doi: 10.1099/mgen.0.000150.

Paper I, VII, and IX have been reprinted with permission from the publishers.

Author contributions

I. E.V. and C.K.C. planned and designed the study. S.C. provided animal facilities and A.E. supervised the experiment. A.E., C.K.C., and E.V. collected the samples. M.S. planned and performed the laboratory work. E.V. performed the bioinformatic and data analyses. E.V. wrote the paper with assistance of C.K.C. All authors reviewed and approved of the final manuscript.

II. M.S., E.V., and C.K.C. planned the study. S.C. provided animal facilities, and A.E. supervised the experimental part of the study. M.S. planned and performed the laboratory work. E.V. performed the bioinformatic and data analyses. E.V., M.S., and C.K.C. wrote the paper with input from S.C. and A.E.

III. E.V. and C.K.C. planned and designed the study. S.C. provided animal facilities. A.E. supervised the experimental part of the study. N.S., A.E., C.K.C., and E.V. performed the sampling and cared for the animals. A.O. advised on sampling procedure. M.S. supervised the laboratorial part of the study, and together with H.M.B. prepared the samples for sequencing. E.V. performed the bioinformatic and data analyses. C.K.C., S.J.S., R.K., and O.H. provided advice on analyses and the interpretation of results. E.V. and C.K.C. wrote the paper with input from all authors.

IV. E.V. and C.K.C. planned and designed the study. S.C. provided animal facilities. A.E. supervised the experimental part of the study. N.S., A.E., C.K.C., and E.V. performed the sampling and cared for the animals. A.O. performed the euthanization procedure and advised on sampling. M.S. supervised the laboratorial part of the study, and together with H.M.B. prepared the samples for sequencing. E.V. performed the bioinformatic and data analyses. C.K.C., S.J.S., R.K., and O.H. provided advice on analyses and the interpretation of results. E.V. and C.K.C. wrote the paper with input from all authors.

V. O.H. and G.V. planned and designed the study. V.P. performed the experiment. O.H. performed the RNA extraction. E.V. performed the bioinformatic and data analyses. C.K.C. and O.H. provided advice on data analyses and the inter-

pretation of results. E.V., O.H., and C.K.C. wrote the paper with input from G.V. and V.P.

VI. O.H. planned and designed the study. G.V. and V.P. performed the experiment. E.V. performed the bioinformatic and data analyses. O.H. provided advice on analyses. E.V. wrote the paper with input from all authors.

VII. O.H. initially conceived the study design. O.H., V.P., and G.V. further developed the study. G.V. and V.P. planned the experiment. V.P. performed the experiment. O.H. performed the RNA extraction. E.V., O.H., and C.K.C. planned the analyses and interpreted the results. E.V. performed the assembly and the bioinformatic and data analyses. D.A. provided advice on the bioinformatic analyses. E.V. wrote the paper with extensive input from all authors.

VIII. S.B., O.H., V.P., and G.V. conceived the study. V.P. and G.V. collected the samples. T.J. sequenced the *H. tartakovskyi* genome. B.C. assembled the genome and performed the gene prediction. O.H. organized sequencing of *P. ashfordi* and E.V. performed the transcriptome assembly. E.V., B.C., and S.B. performed genome analyses. J.D.B. and J.C.K. performed the ortholog clustering, the multiple sequence alignments, and the synteny analyses. B.C. led the phylogenetic analyses with help from J.D.B. and S.B. The paper was written by S.B. with input from the other authors who approved the manuscript before submission.

IX. O.H. conceived the study, performed the analysis and wrote the first draft of the manuscript. E.V. performed the bioinformatic work on *P. ashfordi* and *P. relictum*. S.B. performed the bioinformatic work on *H. tartakovskyi*. All authors participated in interpreting the data, contributed to the writing and approved of the final manuscript.

X. E.V. planned the study, analysed the data, and wrote the paper.

Additional papers published during PhD

Additional papers to which I have devoted significant parts of my time during the PhD. They are not included in this thesis, but are available as open access at the corresponding journal web site.

XI. Martin N Andersson, **Elin Videvall**, Kimberly KO Walden, Marion O Harris, Hugh M Robertson, Christer Löfstedt. (2014) Sex- and tissue-specific profiles of chemosensory gene expression in a herbivorous gall-inducing fly (Diptera: Cecidomyiidae). *BMC Genomics*, 15 (1): 501.

XII. **Elin Videvall**, Nina Sletvold, Jenny Hagenblad, Jon Ågren, Bengt Hansson. (2016) Strong maternal effects on gene expression in *Arabidopsis lyrata* hybrids. *Molecular Biology and Evolution*, 33 (4): 984–994.

XIII. **Elin Videvall**, Erik Öckinger, Lars Pettersson. (2016) Butterfly monitoring using systematically placed transects in contrasting climatic regions – exploring an established spatial design for sampling. *Nature Conservation*, 14: 41–62.

XIV. Hannah Watson, **Elin Videvall**, Martin N Andersson, Caroline Isaksson. (2017) Transcriptome analysis of a wild bird reveals physiological responses to the urban environment. *Scientific Reports*, 7: 44180.

Abstract

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In summary, this work offers new insights into host-microbiome and host-parasite interactions, and enables a greater understanding of the multifaceted relationship between hosts and their microbes.

Svensk sammanfattning

Mikrober finns överallt, runt omkring oss i vår miljö och inuti våra kroppar. De som lever tillsammans med djur har mycket stor påverkan på värdens hälsa och evolutionära fitness. Från ett djurs perspektiv är vissa mikrober goda, andra onda, och vissa har ingen större påverkan på hälsan, men egentligen beror dessa egenskaper på sammanhanget, eftersom det är vanligt att mikrober byter strategi till det som passar stunden bäst. Vi har tyvärr väldigt lite kunskap om hur värdar interagerar molekylärt med olika mikrober, och få studier har gjorts ur ett genomiskt perspektiv på djur som inte tillhör de klassiska studieorganismerna. I denna doktorsavhandling undersöker jag interaktioner mellan mikrober och värdar från flera vinklar, och jag använder mig av nya DNA-sekvenseringstekniker för att illustrera en övergripande bild av förhållandet mellan djur och deras mikrober.

Mitt avhandlingsarbete omfattar två stora projekt, 1) värd-mikrobiom interaktioner och 2) värd-parasit interaktioner. I det första projektet har jag utvärderat hur man bäst kan provta och mäta mikrobiomet i magen hos fåglar (**Kapitel I** och **II**). Olika delar av mag-tarmkanalen analyserades på DNA och visade sig innehålla samhällen av olika mikrober (**Kapitel I**, **II** och **IV**). Jag har sedan kunnat visa att strutsungars mikrobiom koloniseras på ett successivt sätt och gradvis mognar över tid (**Kapitel III**), samt att mikrobiomet är starkt kopplat till både tillväxt och dödlighet (**Kapitel III** och **IV**). I det andra projektet beskrev jag fåglars transkriptom (totala genuttryck) i respons mot en malaria-infektion över tid och i respons mot parasiter med olika virulens (**Kapitel V** och **VI**). Fåglar med malariainfektion sätter igång en rad förändringar i sitt genuttryck som involverar till exempel immunsystemet, stressresponsen, regleringen av celldöd och regulatoriska gener. För att utvärdera den molekylära responsen hos malariaparasiter byggde jag ihop transkriptomet av *Plasmodium ashfordi* och kunde visa att genuttryck hos parasiten är specifik beroende på vilken värd den befinner sig i (**Kapitel VII**). Detta transkriptom användes därefter, tillsammans med ett genom av *Haemoproteus tartakovskyi*, för att konstruera ett fylogenetiskt träd av blodparasiter som resulterade i starka bevis för att malariaparasiter som infekterar människor är närmare släkt däggdjursparasiter än fågelparasiter (**Kapitel VIII**). Slutligen använde jag både parasit-transkriptomet samt genomet för att identifiera vitamin B_1-gener i fågelparasiter (**Kapitel IX**) och för att visa att malariaparasiterna som infekterar fåglar är de eukaryoter som uppvisar de mest AT-rika generna (**Kapitel X**).

Sammanfattningsvis innebär detta arbete nya insikter inom de molekylära interaktioner som äger rum mellan värdar och mikrobiom samt mellan värdar och parasiter. Denna nyvunna kunskap möjliggör en större förståelse för det mångfacetterade förhållandet mellan värdar och deras mikrober.

Glossary

Microbiota	The community of microorganisms within a confined host environment.
Microbiome	The community of microorganisms *and* their confined host environment.
Dysbiosis	A state of microbial imbalance resulting from a disturbed microbiota.
Commensal	An organism that lives in/on a host organism and benefits from the partnership, while the host is unaffected.
Mutualist	An organism that lives in/on a host organism and where both partners benefit from the association.
Parasite	An organism that lives in/on a host organism and benefits from the partnership, but at a fitness expense of the host.
Pathogen	An organism that causes disease in a host organism.
Pathobiont	A commensal or mutualist with opportunistic pathogenic potential.
Coevolution	Reciprocal evolution of interacting species.
Holobiont	A unit of biological organization composed of a host and all its associated microorganisms.
Symbiosis	A relationship between two organisms of dissimilar species that is constant and intimate. Can be mutualistic, commensalistic, or parasitic.
Phylosymbiosis	A strong coevolutionary trajectory between several host species and their microbiomes, resulting in a similar topology between the host phylogeny and the microbiome distance dendrogram.
Virulence	The degree of fitness reduction in a host by a given parasite.
Resistance	The capacity of a host to defend itself against parasites.
Tolerance	The capacity of a host to withstand parasites.
Parasitemia	The number of parasites in a host (e.g. proportion infected cells).
***in vivo* research**	Experiments using live whole organisms (as opposed to e.g. cell culture).
Plasmodium	Genus of protist parasites causing the disease malaria in vertebrates.
Haemoproteus	Genus of protist parasites, closely related to *Plasmodium*.
Haemosporidian	Protist parasites in the order Haemosporida, of which the majority belong to the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*.
Genome	The entire collection of DNA present in the cell nucleus of an organism.
Transcriptome	The collective transcripts from all expressed genes and their relative expression levels in a given tissue of an organism at a given time point.

Introduction

We live in a world dominated by microorganisms. All around us are miniscule microbes of various kinds, and inside our bodies we harbour entire ecosystems where diverse microbes coexist (**microbiomes**). The human body contains more microbes than human cells (Sender *et al.* 2016), and it has been estimated that as many as 37 million bacteria and 7 million fungal microbes are added to the indoor air when a person enters a room (Qian *et al.* 2012). Although we have, in modern times, gone to great lengths to exterminate as many microbes as possible from both our bodies and our environment, it has been discovered that we, together with other animals, cannot in fact survive without the many crucial functions performed by microbes.

Some microbes that are associated with hosts have been labelled as harmful (**parasites**), others beneficial (**mutualists**), and many do not affect hosts substantially (**commensalists**). However, as with most living things, it can quickly become difficult to categorize microbial species simply as good or bad, as their effects on hosts are largely contextual and environmentally-dependent. Certain microbes can be beneficial, but when opportunity strikes, they might change strategy to exploit their host (**pathobionts**). Then there are microbes which have been considered purely parasitic for a long time, but new research has illuminated some of their hidden mutualistic properties. A small amount of infectious microbes might even be advantageous, and in some cases necessary, to properly develop into a functional healthy adult (Gensollen *et al.* 2016; Ramanan *et al.* 2016). The famous 'hygiene hypothesis' (Strachan 1989) is based on this idea, and states that a lack of exposure to microbes and parasites during early childhood contributes to allergies such as asthma, and chronic inflammatory diseases.

The microbes hosts live with and are exposed to can have enormous consequences on their health and fitness. Several animal species have gone extinct because of the presence of parasitic microbes (van Riper *et al.* 1986; Atkinson & LaPointe 2009), while others may starve or become unable to mature into adulthood if they fail to acquire the correct microbial composition (Hirakawa 2002; Wahl *et al.* 2012). Microorganisms with parasitic tendencies often play major roles in their hosts' evolutionary trajectory. For example, it has been estimated that the greatest selection pressure exerted on humans in recent times is that from the parasitic microbe which cause malaria (Kwiatkowski 2005; McManus *et al.* 2017). In fact, the evolutionary influence of parasitic microorganism on hosts is so large, that one of the main hypotheses to the long-standing question of why

there are two sexes, implicates the presence of parasites as a causal factor by which hosts select for genetic heterogeneity (Hamilton *et al.* 1990). This **coevolutionary** arms race between hosts and parasites has been popularized as 'The Red Queen hypothesis' (van Valen 1973), derived from the Red Queen's comment to Alice in Lewis Carroll's book 'Through the Looking-Glass': "It takes all the running you can do, to keep in the same place". In a similar fashion, hosts need to constantly evolve to stay in the same place (survive), in response to parasitic microbes.

The genetic basis of the interactions that underlie the coevolution between hosts and their microorganisms was previously studied by examining single candidate genes and using targeted model organisms. For example, the host molecular response could be evaluated by characterizing parts of a specific immune gene, and a particular microbe could be identified by growing a culture. With the advent of high-throughput sequencing, we now have the opportunity to take an unbiased approach to examine the intricate host-microbe relationship. We now know that diverse communities of unculturable microorganisms reside within hosts and that interactions taking place between hosts and microbes are determined by a large and complex network of genes.

In this thesis, I use high-throughput sequencing techniques and genomic tools, to generate a broad but detailed view of the interactions taking place between hosts and their microbes.

Figure 1. The author, Elin Videvall (E.V.), at field work in South Africa. Photo: Charlie Cornwallis.

Background

Host-microbiome interactions

A world without microbes

Microbes are essential organisms in our world. Already in the 1800's, Louis Pasteur speculated that microbes are necessary for multicellular life (Pasteur 1885).

> "Life would not long remain possible in the absence of microbes."
> — Louis Pasteur

Although this statement is true, to some extent, all life would not suddenly cease to exist in the absence of microbes. Gilbert and Neufeld (2014) speculated that if all bacteria and archaea would suddenly disappear, humans would likely survive, initially, if we can artificially synthesize the essential vitamins and amino acids normally supplied by our gut microbiota. However, many other animals like ruminants and termites would starve to death, plants would rapidly deplete nitrogen levels and die, and the ocean would become virtually dead without bacteria supplying the essential nutrients to support phytoplankton. Most global biogeochemical cycling of nutrients would stop in a world without microbes and the authors predicted that complete human societal collapse would occur within a year (Gilbert & Neufeld 2014). The field of gnotobiology is the study of animals reared in germfree environments, and is built upon the concept of absent microbes. Gnotobiology altered the face of medical research, with a focus that lied within generating "pure units" of biology for sterile experimental research, however some misconceptions about the field has also contributed to a widespread cultural phenomenon revolving around an obsession with eliminating all microorganisms in our environment.

We now know that the microbiota of the gut is absolutely crucial for the development of the vertebrate immune system. Germ-free animals suffer from enlarged caecum, smaller lymph nodes, a poorly developed immune system, and reduced organ sizes, including heart, lungs, and liver (Gordon & Pesti 1971; Macpherson & Harris 2004).

Furthermore, animals without a developed gut microbiota are more susceptible to infection by **pathogenic** bacteria, viruses, and eukaryotes (Sprinz *et al.* 1961; Inagaki *et al.* 1996; Round & Mazmanian 2009), and eliminating bacteria in mice before an influenza infection initiates a reduced immune response, resulting in a higher viral load (Ichinohe *et al.* 2011). Besides modulating the host immune response, microbes in the gut perform a variety of important metabolic and biochemical functions, like metabolism of cholesterol to coprostanol (Sadzikowski *et al.* 1977), and the production of vitamin K (Hill 1997) and essential amino acids (Nicholson *et al.* 2012). Microbes are, as previously mentioned, not only important to vertebrates, but to many other animal hosts. They create biofilms which are essential for a large number of oceanic animals to complete their life cycles (Wahl *et al.* 2012), and a fifth of all insects are so dependent on having bacteria around that they have evolved unique cells, called bacteriocytes, or organs – bacteriomes, where they nurture tens of thousands of their important bacterial symbionts.

Microbiota in health and disease

Despite the many important roles gut microbiota have for human health, it has been largely understudied until recently, and is therefore sometimes nicknamed "the forgotten organ". Studies are now finding strong associations between the gut microbiome and multiple diseases such as autoimmune diseases (Macpherson & Harris 2004; Round & Mazmanian 2009), obesity (Turnbaugh *et al.* 2009), depression (Cryan & Dinan 2012; Maes *et al.* 2012), gut-inflammatory diseases, and cancer (Hope *et al.* 2005). Higher taxonomical diversity of the microbial composition in the gut has been associated with health both in humans and other animals (Spor *et al.* 2011). Low diversity of gut bacteria has been linked to disease-related imbalances of the microbial community, termed **dysbiosis** (Turnbaugh *et al.* 2009; Qin *et al.* 2010). However, that does not necessarily mean that a diverse microbiome is healthy and a less diverse is sick. The idea to directly associate a particular gut microbial composition with health has turned out to be very complicated task. The sheer variety of gut microbes, their interactions amongst each other, their interactions with the host, their interactions with macrobiota such as helminths, and the large inter-individual differences in host microbiomes complicate matter tremendously.

A healthy immune system is one that is able to differentiate between microbes, and allow commensal and mutualist microbes to flourish while keeping pathogens and pathobionts in check. To avoid infections by pathogenic strains via food sources, the intestinal immune system constantly monitors the gut microbiota. As many as 70% of all immune cells in the body are located in the gut-associated lymphoid tissue because of this reason (van der Heijden *et al.* 1987; Vighi *et al.* 2008). When pathogenic microbes are detected, specific antibodies called immunoglobulin A (IgA) are released and bind to the intruder, allowing B cells to target them (**Figure 2**). A highly developed

and complex communication system between the gut microbiota and the vertebrate mucosal immune system has evolved in response to the significance microbes have on host health. When parts of this communication fail, autoimmune or autoinflammatory diseases such as inflammatory bowel disease (IBD) may occur (Wu & Wu 2012).

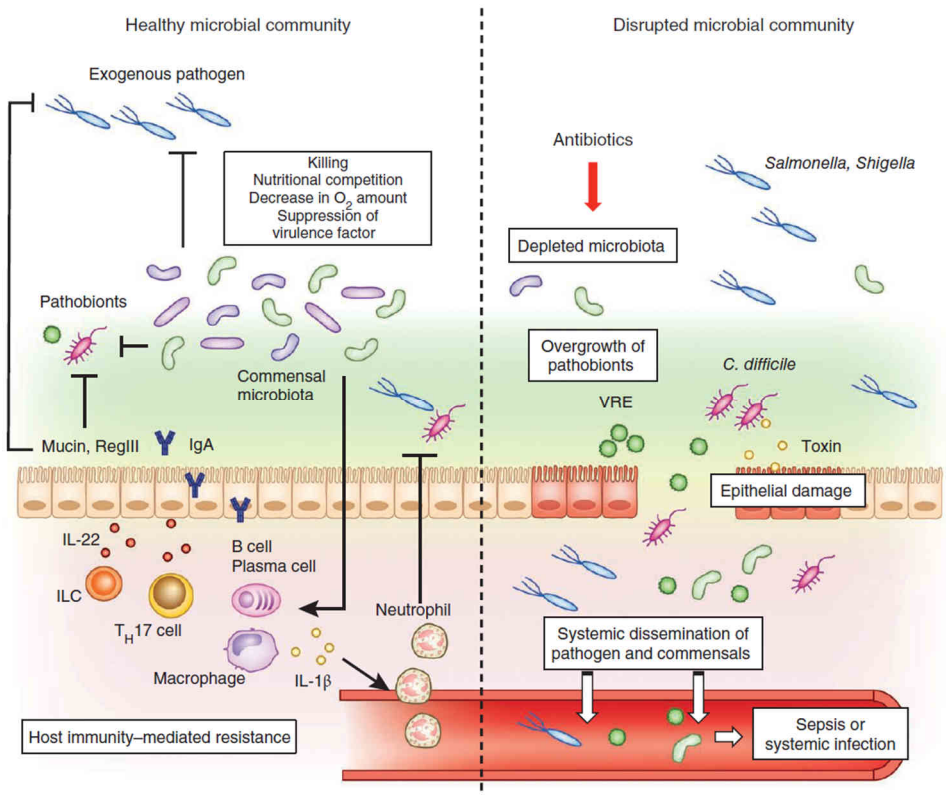

Figure 2. Healthy commensal gut microbiota prevents colonization by exogenous pathogens and pathobionts. Reprinted from Kamada *et al.* (2013) with permission from Springer Nature.

Recently, studies have increasingly been able to demonstrate some of the benefits hosts receive from their microbes. 'Defensive microbes' are those that provide their host with beneficial mechanisms against pathogens, and they can be divided into two main groups: those with direct effect on pathogens, and those that mediate host responses. Microbes that exhibit direct defensive mechanisms towards pathogens have been shown to produce toxic compounds that kill or reduce pathogen fitness, act as hyperparasites on the pathogens (Tollenaere *et al.* 2014), or directly compete with pathogens for host resources (Mideo 2009; Gerardo & Parker 2014). Microbes that have host-mediated

effects can influence either host resistance or host tolerance (Mideo 2009; Gerardo & Parker 2014; Yilmaz *et al.* 2014). For example, in *Aedes* mosquitoes, the **symbiotic** bacteria *Wolbachia* negatively impact infection by parasitic protozoans and viruses through resource competition and by positively influencing host immune responses (Moreira *et al.* 2009). Another example is the American chestnut tree (*Castanea dentata*), which was under the risk of extinction because of the parasitic fungi *Cryphonectria parasitica*, but was saved with the help of defensive fungal viruses in the family Hypoviridae (MacDonald 1991; Milgroom & Cortesi 2004). In humans, phage therapy using hyperparasitic phage viruses to kill targeted pathogenic bacteria, and faecal transplantation of healthy gut microbiota to treat *Clostridium difficile* infections, have shown great success and are two very promising methods of using defensive microbes to kill pathogens (Gough *et al.* 2011; Ford & King 2016). Mutualistic and defensive microbes will most likely become an important tool for future disease control in both humans and non-human animals, especially in the light of the increase in prevalence of drug-resistant pathogens. Probiotics-based vaccines containing defensive gut microbes are already being considered for certain human diseases, such as malaria (Ngwa & Pradel 2015).

Factors affecting host microbiota

The composition of the host microbiota can potentially be influenced by both genetic and environmental factors. When it comes to linking host genotype with gut microbiome composition, there has been some conflicting evidence. One of the largest microbiome studies to date investigated the gut microbiomes of 416 twin pairs and showed differentiated microbiota even in homozygotic twins, suggesting that genotype has a minor effect compared to the environment (Goodrich *et al.* 2014). However, the authors found that the effect of host genotype varied across different bacterial families with Christensenellaceae being the most heritable taxon in the human gut. Many other studies investigating the effects of host genotype on gut microbiota have failed, however, to find major contributions (Davenport 2016; Goodrich *et al.* 2016). The general view has therefore resulted in that individual gut microbiome composition in humans is mostly a product of environmental factors. Nevertheless, a few candidate immune genes, for example the major histocompatibility complex (MHC) (Toivanen *et al.* 2001; Bolnick *et al.* 2014; Kubinak *et al.* 2015), toll-like receptor 5 (TLR5) (Vijay-Kumar *et al.* 2010), myeloid differentiation primary response 88 (MYD88) (Turnbaugh *et al.* 2008; Wu & Wu 2012), and nucleotide-binding oligomerization domain containing 2 (NOD2) (Petnicki-Ocwieja *et al.* 2009; Frank *et al.* 2011), have been associated with effects on the gut microbial community. Vijay-Kumar *et al.* (2010) could show that, not only did mice with knocked-out TLR5 become obese and developed metabolic disease, but transplanting their gut microbiota to germ-free mice instilled the same physiological and metabolic changes as the donor mice, suggesting a strong causal effect of the gut

microbiota on host health. Although microbiome studies of laboratory mice and humans contribute much to our understanding, research on the effects of host genotype on microbiota in other animals is crucial for our understanding of this relationship. A handful of studies have evaluated the effects of host phylogenies on gut microbiota, called **phylosymbiosis** (Hird *et al.* 2015; Brooks *et al.* 2016; Groussin *et al.* 2017; Kohl *et al.* 2017). These studies have usually been able to show a minor to moderate effect of coevolution on the gut microbial composition (**Figure 3**), though they suffer from small and biased sampling efforts, with confounding effects such as physiological, ecological, and environmental differences between species. With an increasing number of animal gut microbiomes becoming sequenced, however, future phylosymbiosis research shows a lot of promise, and an increasing amount of data will allow for meta-analyses and more rigorous investigations across species. The outcome of these studies will be highly useful in further evaluating the effect of host genotype on the microbiota.

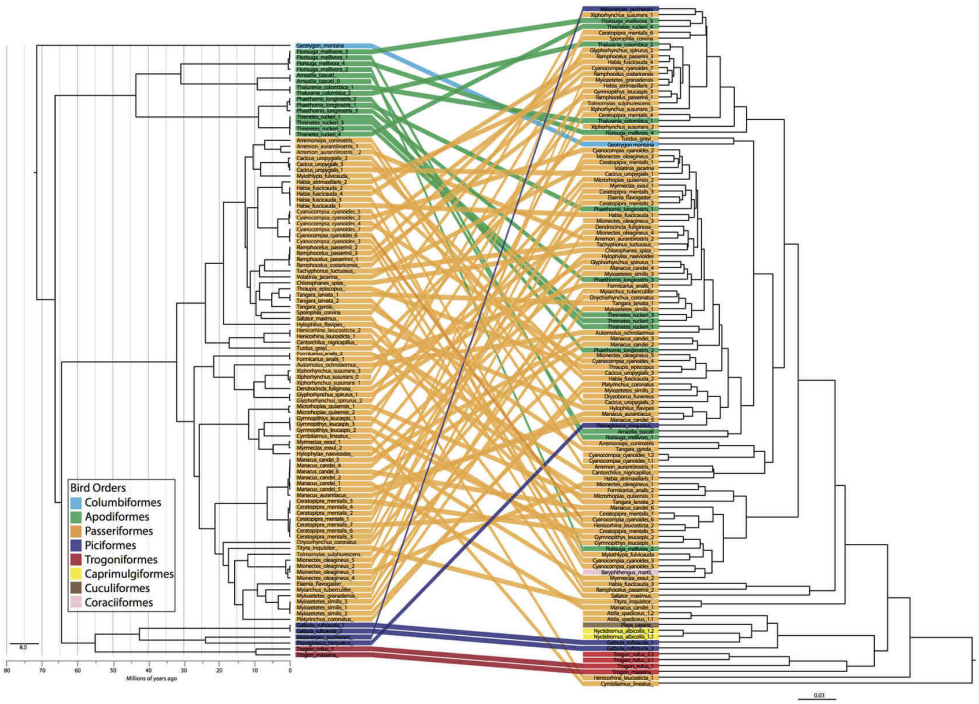

Figure 3. Bird phylogeny (left) compared to a dendrogram of gut microbiota distances (right). Individuals are tracked across the two topologies with lines. Reprinted from Hird *et al.* (2015) under a CC BY 4.0 license.

One of the most prominent drivers behind variation in gut microbiome of hosts is diet. Dramatic changes in the microbial composition due to a switch in diet may be seen as

early as within a day (David *et al.* 2014; Sonnenburg *et al.* 2016). An animal-based diet leads to an increase in the abundance of bile-tolerant microbes (Alistipes, Bilophila, Bacteroides), while Firmicutes bacteria primarily metabolize plant polysaccharides and are thus abundant in plant-based diets (David *et al.* 2014). Other factors that have been strongly associated with gut microbiota composition are age, antibiotics, drug usage, and climatic conditions. Chevalier *et al*. (2015) found that environmental temperature had a major influence on the gut microbiome and gut physiology. Cold exposure of mice markedly shifted the gut microbiota composition which caused energy homeostasis, leading to increases in gut size, insulin sensitivity, energy expenditure and absorptive gut surface (Chevalier *et al.* 2015). The development and maturation of gut microbiota in young animals are believed to be largely influenced by age. Human babies are born largely sterile and acquire their first gut microbes from their mother's vaginal microbiome. In babies born with caesarean section, however, the vaginal seeding of microbes is lost, and the gut microbiomes of these babies instead show similarities to the mother's skin microbiome (Dominguez-Bello *et al.* 2010). The mode of birth, together with early-life antibiotic exposure, is now believed to be an important factor explaining the higher levels of asthma and allergies in children born with caesarean section (Renz-Polster *et al.* 2005; Russell *et al.* 2012).

The importance of microbiota in evolutionary processes

In recent years, some people studying host microbiomes have argued that the field of evolutionary biology do not properly account for how organisms evolve together with their symbionts. The traditional view, to view species as separate entities evolving due to processes such as genetic drift, assortative mate selection, or local adaptation, has been challenged by some studies. For example, Brucker and Bordenstein (2013) found that when two species of *Nasonia* wasps mated with a more distant relative, the hybrid offspring would normally die. Treating the hybrids with antibiotics, however, made them viable and thriving. By simply eliminating the gut microbiota in the hosts, the authors could essentially tear down the ecological barrier separating the different species.

The importance of studying host microbiomes in light of evolution has recently been shown in the context of mate selection and its effect on speciation and trait evolution. Because many animals communicate by producing chemical signals, odour-realeasing microbes can have significant influence on their interactions. In humans, skin bacteria produce enzymes which interact with sterile apocrine sweat to produce axillary odour (Froebe *et al.* 1990). Without the bacteria, no pheromone signal is released. Symbiotic fermentative bacteria in the scent glands of striped and spotted hyenas produce odours that are species-specific, and further signal both sex and reproductive state (Theis *et al.* 2013). Similarly, the gut microbiota of termites produce colony-specific chemical cues which enable nestmate recognition. Manipulating the composition of gut bacteria with

antibiotics changes the termites' recognition behaviour toward nestmates (Matsuura 2001). Dodd (1989) discovered that rearing *Drosophila* fruit flies on two different diets drastically altered their mate choice. Flies reared on starch-based media preferred to mate with other flies that had been eating starch, and similarly, flies reared on maltose-based diet preferred maltose-eating flies, resulting in strong positive assortative mating. What was causing the changes in mate selection remained unknown until Sharon *et al*. (2013) successfully repeated the experiment and found assortative mating again after only one generation. This time, however, symbiotic microbes were the primary suspect, and Sharon *et al*. (2013) demonstrated that treating the flies with antibiotics suddenly removed their assortative mate preferences.

The idea that animals and plants are not separate entities that evolve discretely; but instead that individuals are a fusion of both host and microbe cells that evolve in symbiosis, called **holobionts**, was proposed already in 1994 by Jefferson. 'The hologenome theory of evolution' states that the fitness of a multicellular organism is derived from the entire collection of genomes (mostly microbial) representing the organism in an environment where selection occurs. Because microbes can be inherited both vertically and horizontally, the theory incorporates Lamarckian as well as Darwinian concepts. Holobionts and the hologenome theory of evolution have been widely discussed in the context of coral bleaching by infection of the bacteria *Vibrio shiloi* (Reshef *et al.* 2006; Rosenberg *et al.* 2007; Zilber-Rosenberg & Rosenberg 2008). After years of repeated bleaching events of the *Oculina patagonica* corals in the Mediterranean sea, the eastern population suddenly became resistant to *V. shiloi* infection, despite their decade-long lifespan and lack of adaptive immune system (Rosenberg *et al.* 2007). The phenomenon was termed 'experience-mediated tolerance' and attributed to the corals' symbiotic microbial communities, which can evolve and adapt much faster than the corals themselves in response to changing environmental conditions. However, the hologenome theory of evolution has encountered criticism, mainly because several microbes are not viewed as having high host fidelity, but rather environmentally acquired and context-dependent (Douglas & Werren 2016). Nevertheless, the currently rapid emerging importance of host microbiota expands our current view on how symbiotic microbes shape the evolution of hosts (Shapira 2016).

Unresolved questions in host-microbiome research

The research surrounding host microbiomes is relatively new, since it was only recently it became possible to evaluate entire communities of microbes simultaneously. In the last couple of years, a large part of the effort in microbiome research has been directed towards developing new methods and characterizing various microbial communities. The microbiome field is therefore still largely in its development phase with a large focus on evaluating methods that give reliable and repeatable results, and to describe the present variation in microbial communities within hosts. It is crucial to first

characterize and describe what constitutes a specific host microbiome, what makes it stable, and how it changes over time within and between individual hosts, in order to understand the processes and factors governing it. For microbiome research in model organisms, such as mice and humans, a lot of effort has been put into developing reliable and repeatable extraction and sequencing techniques, bioinformatic software, as well as statistical methods to analyse microbial communities (Lozupone & Knight 2005; Caporaso *et al.* 2010; Song *et al.* 2016; Callahan *et al.* 2016; Debelius *et al.* 2016; Morton *et al.* 2017). Microbiome studies using these model host organisms have recently shifted focus, from broad-scale characterisations and comparisons, to attempts at developing theoretical frameworks to understand the observed patterns.

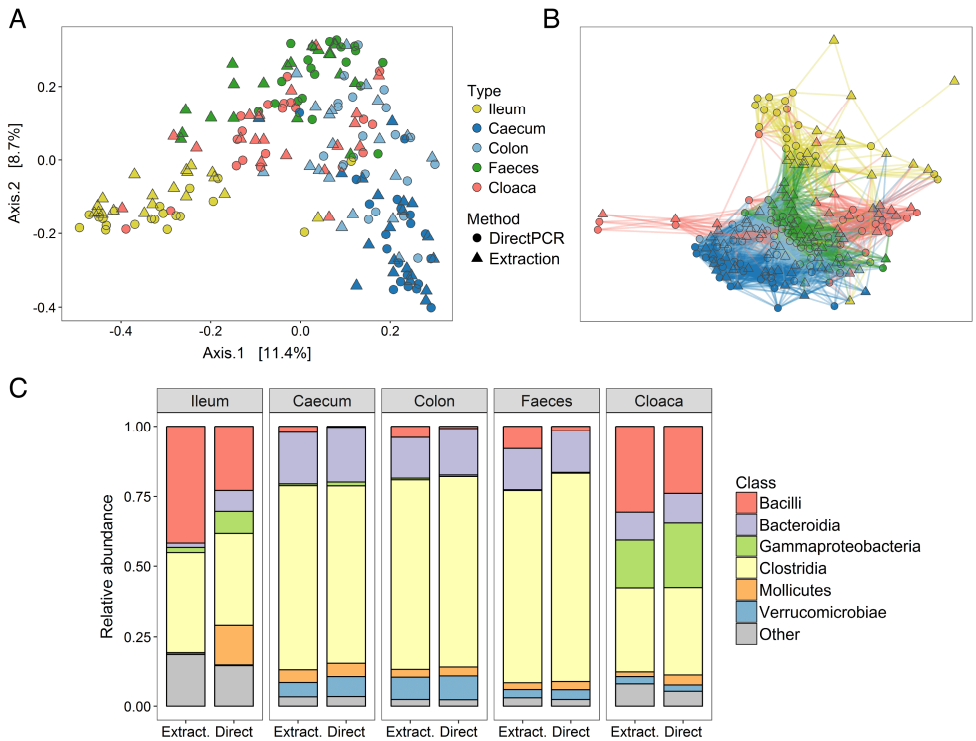

Figure 4. Evaluation of two different DNA isolation techniques (conventional DNA extraction versus direct PCR) for high-throughput amplicon sequencing of animal gut microbiomes. (**A**) PCoA, (**B**) distance network, and (**C**) taxonomic proportion in different sample types. Reprinted from **Paper II** (Videvall *et al.* 2017b).

However, the microbiome research of other animals are, as expected, lagging behind and is still in the development phase. As a result, there are both important research questions and methodological questions that are currently unresolved in the field of non-

model microbiomes. Almost no studies are available on how to best sample and measure the gut microbiota of animals (**Figure 4**). We also know extremely little of how the gut microbiota of different species look like and how it is colonized in offspring and subsequently matures over time. The adult microbiome seems to be heavily influenced by its earlier maturation process during juvenile stages, and this developmental window is therefore highly important to evaluate. One of the burning microbiome questions currently on several people's mind is how does the gut microbiome affect host fitness? Does the gut microbiota play a role in the growth and survival of hosts? And does the microbial community shift during disease? These are some of the questions I will address in this thesis. For an overview of host-microbiome related questions, please see 'Aim of thesis'.

Host-parasite interactions

Host coevolution with parasites

Though recent work on microbiomes has highlighted some of the beneficial effects microbes can have on hosts, several microbes ultimately pose a threat to host health and fitness. Being parasitic offers so many advantages, it is the most common way of life (Price 1977), and has evolved independently numerous times. The never-ending arms race between hosts and parasites has profound effects on their respective evolutionary path. Parasites continuously invent new invasion and avoidance strategies to circumvent the barriers raised by hosts. They usually have an evolutionary advantage relative their host because they tend to have short generation times, produce numerous offspring, and contain small genomes with high mutation rates. Particularly pathogenic parasites therefore place strong selective pressures on host genomes to evolve counter-adaptive measurements and defences to suppress the infection.

The extent to which parasitic organisms can influence the evolution of hosts was also highlighted by Dawkins (1999), who used parasites as one of his metaphors in 'The Extended Phenotype'. Dawkins expanded the gene-centric view, and reasons that the behaviour and physiology of hosts can be greatly modified by parasite genes, which ultimately determines the extended host phenotype. This means that the resulting host phenotype will depend, not only on the expression of genes in the host genome, but also on the expression of parasite genes, and the parasite's interactions with the host. A classic example illustrating this idea is trematode parasites which infect snails. Infected snails have thicker shells compared to uninfected snails, but are castrated by the parasite (Cheng 1973), presumably because the parasite has nothing to gain from the reproductive success of the snail, but are instead under strong selection pressure to keep

the snail alive. Hence, the parasite genes end up with a large influence on both host reproduction and host survival.

Although trematodes are not microbes, the same concept applies. There are many examples where parasitic microbes are known to significantly alter the physiology or behaviour of their host to their advantage. *Toxoplasma gondii* makes rats attracted to cat urine in order for the parasite to transfer to a feline host, the rabies virus induces e.g. hyperactivity, confusion, and hydrophobia in its host, and the malaria parasite makes vertebrates more attractive to blood-feeding mosquitoes. Although the concept of the extended phenotype is now commonly used to emphasize the importance of viewing both host and parasite genomes in light of their interactions, most studies still focus solely on the molecular aspect of one of the parties (Lambrechts *et al.* 2006).

Host defence

Vertebrate coevolution with parasites over hundreds of millions of years has created an intricate and complex defence system, known as the immune system. It consists of the innate immunity, acting as the first layer of defence, and the adaptive immunity, which adjusts to the presence of specific pathogens. The adaptive immune system can be further classified into humoral immunity and cell-mediated immunity, which primarily defend against extracellular and intracellular parasites, respectively, and utilize different cells and molecules. The memory lymphocytes protects the host against reinfection by pathogens with the same antigens, but not against similar pathogens that have different antigenic variants. Each host carries a specific memory profile based on its history of infections, which is why the immunological memory of older individuals are usually broader relative to that of younger ones. (Frank 2002; Abbas *et al.* 2014). Together, the strength and efficiency of different aspects of the host immune system can have major implications for the resulting disease progression and severity (**Figure 5**).

Host genomes continuously evolve **resistance** and **tolerance** mechanisms to minimize the negative effects of pathogenic microbes. For example, the resistance genes of the major histocompatibility complex (MHC) constitute the most polymorphic genes in the human genome, due to their role in recognizing and binding parasite antigens. Tolerance mechanisms allow hosts to cope with pathogens by expressing host genes involved in e.g. DNA repair. Where resistance is usually measured as the inverse of infection intensity (number of parasites per unit host tissue), tolerance can be defined as the rate of change in fitness as parasite burden increases, or more informally as ‘the ability to limit the damage of a given parasite burden’ (Simms & Triplett 1994; Råberg *et al.* 2009). In order to quantify tolerance, one needs to measure it across individuals of a given host species, since variation among hosts can be caused by factors other than tolerance, such as host condition or environmental factors.

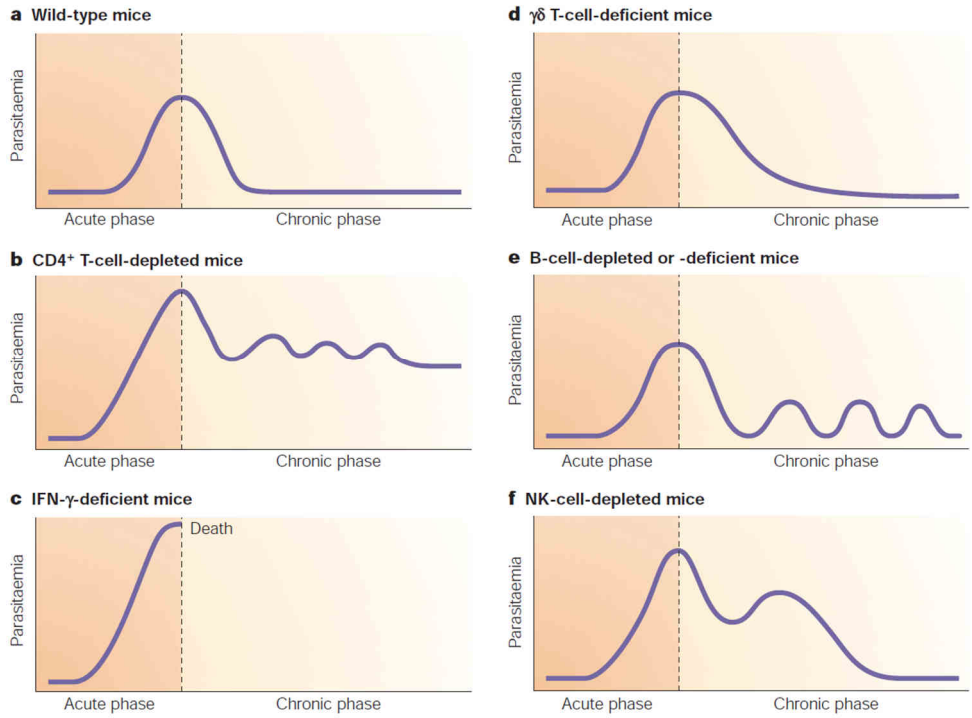

Figure 5. Different components of the immune system have implications for the course of infection. As an example are different mouse strains infected with the rodent malaria parasite *Plasmodium chabaudi*. Reprinted from Stevenson & Riley (2004) with permission from Springer Nature.

One of the best known examples of how hosts have evolved mechanisms to resist parasites is the human sickle cell trait, which confers resistance to malaria parasites (***Plasmodium*** species). Malaria is a dangerous disease which constitutes one of the leading causes of death worldwide in humans from an infectious agent. The haemoglobin allele that gives rise to the sickle red blood cell, HbS, has evolved independently several times in different areas, and is maintained at approximately 10% frequency in malaria-endemic regions (Flint *et al.* 1998). An individual homozygous for HbS suffers from sickle cell disease, but heterozygotes have a ten-fold reduction in the risk of acquiring severe malaria (Hill *et al.* 1991; Ackerman *et al.* 2005). Remarkably (as noted already by Haldane 1949), the selection pressure from *Plasmodium* parasites on the human genome has been so strong that, besides sickle cell disease, several other red blood cell disorders that increase resistance or tolerance to malaria have independently evolved in in humans. For example, alpha and beta thalassemia, ovalocytosis, glucose-6-phosphate dehydrogenase deficiency, and the Duffy-negative blood group (Flint *et al.* 1998; Kwiatkowski 2005).

Parasite virulence

Pathogens may cause disease in hosts because they possess structural, biochemical, or genetic traits that make them **virulent**. In an ecological context, virulence is usually defined as the reduction in fitness a host experiences by a particular parasite. Before the 1980's, conventional wisdom regarded virulence as a fixed artefact based on recent associations between a parasite and its host (Levin 1996), and assumed that selection always led parasites on a directional path to becoming commensals. Evolutionary biology was able to show, however, that selection processes could favour either an increase or a decrease in virulence. Work by Anderson and May (1979; 1981) improved our understanding of the natural selection pressures that act on parasite dynamics. A higher reproductive rate may allow a parasite to increase its likelihood of transmission to new hosts, thereby increasing its fitness, but this can result in higher virulence. If virulence increases too much, the parasite may risk killing the host before successful transmission has taken place. As such, the parasite faces important economic trade-offs between transmission and virulence.

Studying the evolution of virulence and its consequences on hosts is very important from both a medical and veterinary viewpoint, because a better understanding of parasite fitness optima may allow us to reduce the negative effects parasites have on hosts. Yet, very little is known about the distribution and effects of virulence in natural populations. Theory suggests that parasite virulence should increase with coinfection of multiple strains due to competition within hosts (Nowak & May 1994; Ebert 1998). It has also been hypothesized that the existence of multiple parasite strains of the same species are the outcome of host genotype-by-parasite genotype interactions. MHC has figured as one of the main genetic host components driving this view that some alleles function better toward certain parasite lineages (Westerdahl *et al.* 2005), but fare worse against other lineages due to binding properties. Few studies have been able to demonstrate specific genotype-by-genotype interactions, but some can clearly point to the fact that disease severity results from an interaction of both host and parasite genotype together (Lambrechts *et al.* 2005).

Immune evasion by parasites

A major part in the host-parasite relationship consists of parasites evolving variation in antigenic loci to escape host immunity. Having variable antigenic molecules can extend the time a parasite can persist in a particular host individual, and helps avoid the immunological memory of hosts. The large evolutionary pressure to generate antigenic variation in parasite genomes often cause antigenic loci to show signs of positive selection (Endo *et al.* 1996; Yang & Bielawski 2000). To cope with this strong selection pressure, some parasites have evolved genome-wide, or even localized, hypermutation mechanisms that allow them to generate more nucleotide variants via increased mutation

rates (Moxon *et al.* 1994; Caporale 1999; Ripley 1999). For example, mutator lines of the pathogenic bacterium *Pseudomonas aeruginosa*, has displayed non-random distribution of mutations along its chromosomes (Dettman *et al.* 2016), and pathogenic strains of *Escherichia coli* and *Salmonella enterica* have increased overall mutation rates (LeClerc *et al.* 1996; de Visser *et al.* 1999).

Other parasites store several genetic variants for an antigenic locus within their genome, effectively creating a vast library of antigenic variation to choose from. Parasites with this strategy express only one genetic variant at a time, called monoallelic expression, and is able to switch gene expression between the different variants. This is a strategy employed by some malaria parasites (Frank 2002; Ferreira *et al.* 2004; Recker *et al.* 2011; Bachmann *et al.* 2011; Guizetti & Scherf 2013). *Plasmodium falciparum* has a huge archive in its genome of up to 60 variants of the *var* genes coding for the surface-exposed PfEMP-1 protein. Switching gene expression between the different *var* genes can lead to some interesting population dynamics within a single host. When the host's immune system starts recognizing a particular type of antigen in a parasite, massive amounts of immune cells are proliferated that target this specific antigen. To escape this immune storm, the malaria parasite then switches expression from one *var* locus to another. If the parasite switches expression between variants too quickly, the host will develop immunity to all types early on and the infection will not be sustained. However, if the parasite is too slow switching between expression variants, it risks being cleared by the immune system before the switch has been made. This means that the timing of regulating expression between antigenic variants is crucial and finely tuned to the host's immune response. Which malaria parasites that utilize this immune evasion strategy, the rate of antigenic switching, and how the coordinated regulation of the switch works, are unanswered questions that future studies need to investigate.

More recent research has revealed the intricate immune evasion manoeuvres that parasites use to directly and effectively manipulate host gene expression to their advantage. This strategy have been detected in several organisms, but seems to be especially common in apicomplexan parasites. *Toxoplasma gondii* is well-known for inhibiting host gene expression of MHC (Leroux *et al.* 2015), *Plasmodium* parasites prevent host cell death expression pathways in liver cells (Hakimi & Cannella 2011), and *Theileria* parasites have a unique ability to completely transform host leukocytes and hijack their cellular machinery (Plattner & Soldati-Favre 2008). The challenge for the studies investigating these behaviours lies within proving that the altered host expression is indeed a direct manipulation by the parasite, and not a defence response induced by the host cell itself. It is clear, however, that parasites have evolved a wide range of sophisticated strategies to evade the immune defences of hosts; yet the molecular mechanisms underlying these activities are still largely unknown.

Controlled host-parasite experiments

Natural systems provide great opportunities to study the ongoing evolution and distribution of hosts and parasites. The drawback of using completely wild systems, however, is the inherent difficulties to disentangle the effects due to the large environmental variation and controlling previous host exposure to microbes. Vertebrates in nature are commonly infested with a plethora of various parasites; including (but not limited to) viruses, bacteria, fungi, intestinal nematodes, blood parasites, ticks, and mites (see e.g. Biard *et al.* 2015). The microbes inside natural hosts compete with and influence each other in various ways, and as such complicate any effort to evaluate their interactions with hosts. Taking control of the confounding environmental variables in the laboratory lets us investigate the dynamics and consequences of coevolution with fewer factors. This approach may allow for the distinction between cause and effect, and can reveal detailed molecular interactions between hosts and their microbes.

Though inbred laboratory mouse strains have been used as host models in studies of infectious disease for decades, they tend to give a highly specific picture of host-parasite interactions. The immune system of lab mice do not develop properly (Beura *et al.* 2016; Abolins *et al.* 2017), and because they have been heavily artificially selected, they are not very accurate at representing natural host responses in other vertebrate taxa (White *et al.* 2010). Investigating the molecular mechanisms of host-parasite interactions using natural hosts in controlled environments offers several potential advantages. The way natural selection shapes the evolution of host responses can be studied without interference by artificial selection, vaccinations, medicines, vector control, and other anthropogenic preventive measures. It is also possible to perform controlled infection experiments in wild hosts in order to follow host responses over the course of infections. Several non-model host organisms have now been successfully studied ***in vivo*** using endoparasites within controlled laboratory environments. These include for example *Daphnia magna* with microsporidia parasites (Ebert 1994; Ebert *et al.* 2000), the bumblebee *Bombus terrestris* with trypanosome gut parasites (Baer and Schmid-Hempel 1999; Barribeau *et al*. 2014), the freshwater snail *Potamopyrgus antipodarum* with trematode parasites (Koskella & Lively 2007, 2009), and passerine birds with malaria parasites (Atkinson *et al.* 2000; Zehtindjiev *et al.* 2008; Palinauskas *et al.* 2008, 2011; Cellier-Holzem *et al.* 2010; Ellis *et al.* 2015; Dimitrov *et al.* 2015).

Unresolved questions in host-parasite research

Understanding the mechanisms behind host-parasite interactions poses great challenges as the outcome depends both on the host, the pathogen, and the interaction between the two. Why do some host individuals get sick while others not? What are the mechanisms

causing these inter-individual differences in host resistance and tolerance? To start investigating this, it is highly important that we examine molecular responses to infection across different taxa (**Figure 6**). To date, there has been little integration between different host systems and between wild and model organisms. Despite extensive research conducted on a subset of parasitic microbes causing human disease, we know very little about the effects of parasitic infection on different hosts. Furthermore, we need to start investigating host molecular responses to parasites over the course of infection. Most studies investigating disease systems in wildlife are dependent on first catching the host in the wild in order to sample them. This approach can be useful to measure the prevalence of low, chronic infections in hosts that survived the acute infection. However, it will not enable an improved understanding of the full infection event, including initial disease stage with parasite proliferation, peak **parasitemia**, decreasing parasitemia, and the recovery period, as well as potential relapses of the infection.

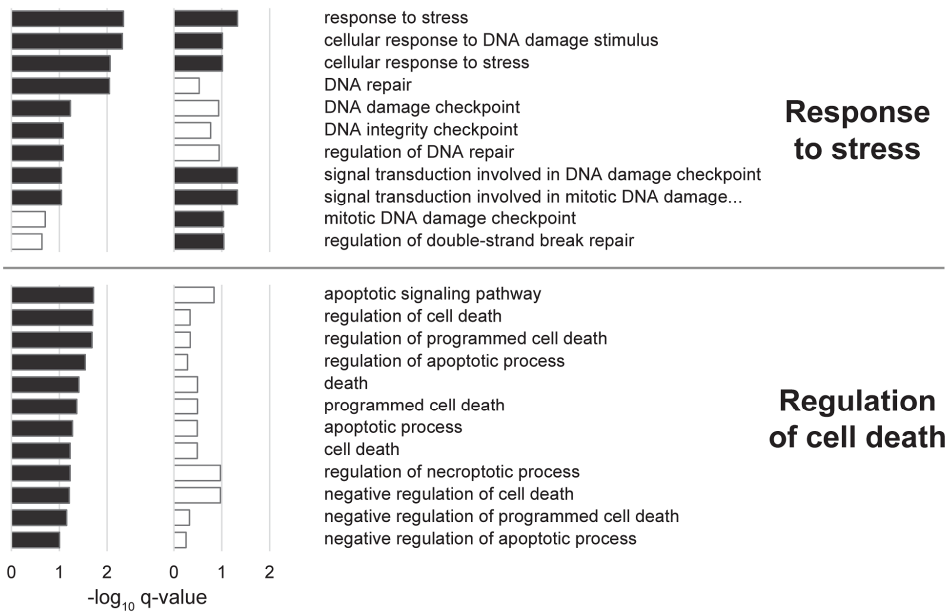

Figure 6. Similarities and differences in host processes related to stress response and regulation of cell death among genes differentially expressed in the avian transcriptome during peak parasitemia stage (left column) and decreasing parasitemia stage (right column). Black bars indicate significantly overrepresented processes and a higher $-\log_{10}$ q-value means higher statistical significance. Adapted from **Paper V** (Videvall *et al.* 2015) under a CC BY 4.0 license.

Finally, we have extremely little knowledge about the specific genes implicated in disease and infection, both from a host perspective and a parasite point of view. A

handful of candidate immune genes have been intensely studied in hosts, but there are thousands of genes with potentially critical roles in the host response to infection. Likewise, the complex network of interacting genes in the parasite genome has large implications for successful transmission and infection in hosts. If we want to better understand the molecular warfare between different parasites and their hosts, it will be important to look at their interactions from a **genome**-wide point of view and over the course of infection. These are some of the aspects I intend to take into account in this thesis. For an overview of research questions related to host-parasite interactions, please see 'Aim of thesis'.

Genomics

High-throughput sequencing of microbiomes

In 1977, Carl Woese and George Fox revolutionized the microbiology field by showing that the bacterial 16S ribosomal gene contains both highly conserved and hypervariable regions, and could be sequenced with universal primers to use in phylogenies and taxonomic identifications. Sanger sequencing, with its single-sequence approach, proved nonetheless highly impractical and time-consuming when it came to characterizing samples containing thousands of microbial species. Before the advent of high-throughput sequencing, most studies investigating microbial compositions had to first culture the organisms in the samples before sequencing them. Characterizing microbial communities with culture-based methods greatly underestimates the diversity, sometimes as much as 80% (Eckburg *et al.* 2005), because standard culturing medium is unsuitable for most microbes, of which many are anaerobes. This means that studies based on cultures become drastically biased towards aerobic microbes that are culturable and severely underestimate the true taxonomic diversity in a sample. However, even studies avoiding cultures drastically underestimated the microbial diversity previously. The reason was because PCR reactions with universal primers usually only result in single sequences derived from one or a few species that are common in the sample and/or bind well to the probes. As a result, when species composition was diverse, researchers had previously a lot of difficulties in recovering the full extent of the community.

With the new high-throughput sequencing techniques, the microbiome field has expanded dramatically (**Figure 7**). For the first time, we are now able to view the species making up entire bacterial communities, and we can accurately estimate their abundance and diversity. Excitingly, most of the species initially found have been completely novel (Venter *et al.* 2004), even inside the human body (Eckburg *et al.* 2005). The modern DNA sequencing techniques allow us to take a more holistic view of all the microbes in

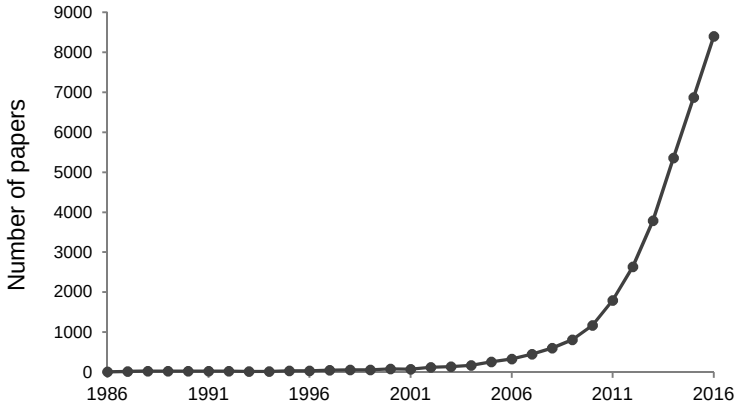

Figure 7. Number of published papers containing the term ”microbiome” indexed by PubMed each year.

a particular environment, and has led to a shift in the field of microbiology. Where previous studies mostly targeted single human disease-causing bacteria, such as *Escherichia coli* and *Salmonella* spp., today researchers have the possibility to evaluate the whole community of microbes in various parts of the body, and how they interact and change over time. This has led to the appreciation that the vast majority of microbes residing within our bodies are not pathogenic, but instead mutualistic or commensal, yet still have great implications for human and animal health.

High-throughput sequencing of host responses

Understanding the role of a specific gene, molecule, or pathway in the immune system of vertebrates is not an easy task. Specialized immune cells have been found to express at least 67% of all the genes in the genome, and less than 1% of genes in the genome are only expressed in one cell type (Hyatt *et al.* 2006). Given the size and complexity of the vertebrate immune defence, we need to consider all its components in the framework as a whole, in order to discern it. Moreover, any given immune molecule can have widely contrasting outcomes depending on the epistatic effects it has with other interacting gene products (Heng *et al.* 2008). The study of single classical immune-related genes is highly valuable, and contributes much to our understanding; however, such a narrow focus risks missing other genes with highly important functions, and does not advance our knowledge of the complex interacting network of responses that constitute the immune defence. Therefore, it is crucial that we decipher expression patterns of immune cells in the context of a network, and not limit our view to one type of cell, or molecule. By using high-throughput sequencing techniques, we can start investigating the roles of the various components of the immunity, their interactions

amongst each other, and how they vary in response to different diseases. Genomics provides us with the ability to view the immune system as a whole, and characterize it as the dynamic model it constitutes.

High-throughput sequencing of parasites

Before modern sequencing techniques, most research on parasite genetics targeted single candidate genes. It was not possible to study genome-wide expression of parasite genes. Later, microarray applications were developed and became useful at measuring gene expression, although they were inherently biased and required sequence information from a genome. With genomic tools, however, pathogens of humans quickly came into focus because of their importance in clinical studies, and their smaller, gene-dense genomes enabled easier assembly. The very first organism to have its genome sequenced, excluding viral phages, was indeed a pathogen, the bacterium *Haemophilus influenzae* (Fleischmann *et al.* 1995). After this pivotal moment, more pathogen and parasite species have had their genomes sequenced and characterized. Still lacking, however, are the many diverse parasite taxa infecting non-model organisms. Sequencing the genome of a new parasite does not only allow for the description of that particular species, but greatly enhances our understanding of parasite evolution through phylogenomic and comparative genomic studies. As a result, obtaining genomic sequences of non-model parasites have multiple benefits in the advancement of science.

In addition, with the development of RNA-sequencing methods, we now have the possibility to sequence the entire repertoire of expressed transcripts from a parasite. Some of the first studies using RNA-seq achieved this in eukaryotic pathogens such as *Schistosoma mansoni* (Almeida *et al.* 2012), *Giardia intestinalis* (Franzén *et al.* 2013), *Candida albicans* (Bruno *et al.* 2010), *Trypanosoma brucei* (Kolev *et al.* 2010), and *Plasmodium falciparum* (Otto *et al.* 2010). These **transcriptome** studies have been highly valuable as tools for improving parasite genome annotation; including information on splice sites, transcription start sites, UTR locations, novel ORFs, transcripts, and non-coding RNA. It is only recently, however, that studies have begun to evaluate parasite expression in several samples, over time, and compare different strains or genotypes (see e.g. Wurtzel *et al.* 2012). This approach allows for analyses of differential gene expression, coregulatory genes, alternative splicing events, and expression variation during infections. Furthermore, the new RNA-sequencing methods open up fantastic possibilities of evaluating simultaneous genome-wide gene expression of both host and parasite together, so-called dual RNA-seq. In conclusion, high-throughput sequencing techniques allow us for the first time to paint complete pictures of the complex molecular interactions taking place between hosts and microbes.

Aim of thesis

With this PhD thesis, I aim to provide a deeper understanding to the following broad research questions:

1. How do we reliably and accurately measure the gut microbiome of non-model animals? (**Paper I** and **II**)
2. How does the gut microbiome colonize juvenile hosts and develop over time? (**Paper III** and **IV**)
3. How does the gut microbiota affect host fitness? (**Paper III** and **IV**)
4. What constitutes a healthy and a diseased microbiome, and which microbes are specifically associated with health and disease? (**Paper IV**)
5. How does the transcriptome of a host respond to a parasitic infection over time and in relation to parasites with different virulence? (**Paper V** and **VI**)
6. How does the transcriptome of a parasite respond to different host individuals and which genes are being utilized at different stages of the infection? (**Paper VII**)
7. How do genomes of parasites evolve and what are their evolutionary relationships? (**Paper VIII**, **IX**, and **X**)

General methodology

Ostrich gut microbiomes

To study the gut microbiomes of hosts in relation to health and fitness (thesis aims 1–4), I have utilized a system of ostriches (*Struthio camelus*). The ostrich system consists of a research farm located in Oudtshoorn, Western Cape, South Africa (**Figure 8**). Ostriches are the world's largest bird species, and they are a valuable economic resource being farmed for feathers, eggs, meat, and leather, yet have only been kept in captivity for a short period of time relative to other agricultural animals (Cloete *et al.* 2012). Their chicks (**Figure 9**) are highly precocial, allowing them to be raised independently from parents. Studying ostriches at a research farm brings many benefits, including highly repeatable sampling, identification of individuals and their biological parents, simultaneous hatching, exclusion of predation, and reduced influence from environmental factors such as variation in diet. Both the genetic and growth variation among the ostrich chicks in the population is high, and I have had the privilege of working with a very large sample size of 234 individuals.

In the microbiome project, we collected faecal samples and weight measurements from all ostrich chicks every second week during the first three months of their lives. We also dissected all chicks that died naturally of disease (n = 68), as well as healthy controls

Figure 8. Study site in Oudtshoorn, South Africa with a group of ostrich chicks to the left and an adult male ostrich in his enclosure to the right. Photo: E.V.

(n = 60), in order to collect samples from three different sections of the gastrointestinal tract and the cloaca. All gut samples were collected in between rigorous cleaning routines, which included rinsing scalpels with hot water, soap, and 70% ethanol, followed by sterilization with a bunsen burner. Other samples we collected as part of this project were environmental samples (soil, food, water) and faecal samples from adults.

To analyse gut microbiota we have been using 16S rRNA gene sequencing. DNA from the samples was extracted using two different methods, standard DNA isolation according to the Earth Microbiome Project and Direct PCR where you circumvent the extraction steps. The results from these two DNA extraction methods were evaluated in **Paper II**. After 16S-amplicon library preparations, a total of 1152 samples were sequenced to produce 300 bp-long reads over three full sequencing runs on a Illumina MiSeq machine at the Department of Biology, Lund University. I analysed the sequence reads with bioinformatics and performed the statistical analyses in R. Further details of the study system, sample collection, laboratory procedures, and software used can be found within the methods section of each paper (**Paper I**, **II**, **III**, and **IV**).

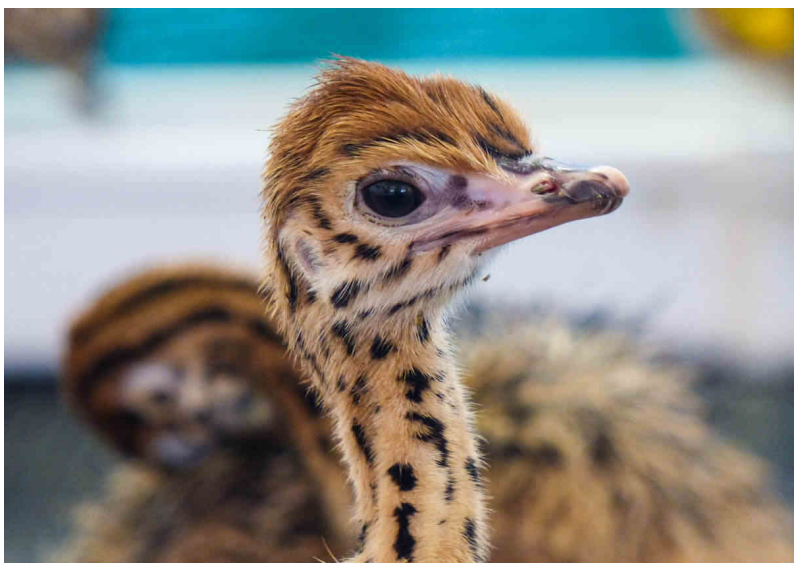

Figure 9. Ostrich chick, two weeks old. Photo: E.V.

Siskin malaria

To study host responses to parasites and parasite responses to hosts (thesis aims 5–7), I have been using a system of avian malaria. The eukaryotic protist parasites that cause the disease malaria are transmitted via dipteran vectors to a diverse range of vertebrate hosts, including primates, bats, rodents, ungulates, reptiles, and birds. Most of the research on host responses to malaria have been performed using primates and mice, with the effects of malaria on other vertebrate hosts remaining largely unknown. The definition of the term 'malaria parasite' varies greatly, but it is most commonly ascribed to all the species within the genus of *Plasmodium*. Species of *Plasmodium* should contain haemozoin pigment and use mosquitoes as their vector, however, exceptions to both of these rules exist. The term 'malaria parasite' is therefore sometimes also used for any haemosporidian parasites, including the genera ***Haemoproteus*** and *Leucocytozoon*. Malaria in birds is a highly suitable system for studying host-parasite interactions because the parasite replicates asexually in red blood cells of the host, which means we can perform repeated blood sampling of an individual to follow both the host response and the parasite over the course of an infection. The same parasite genotype

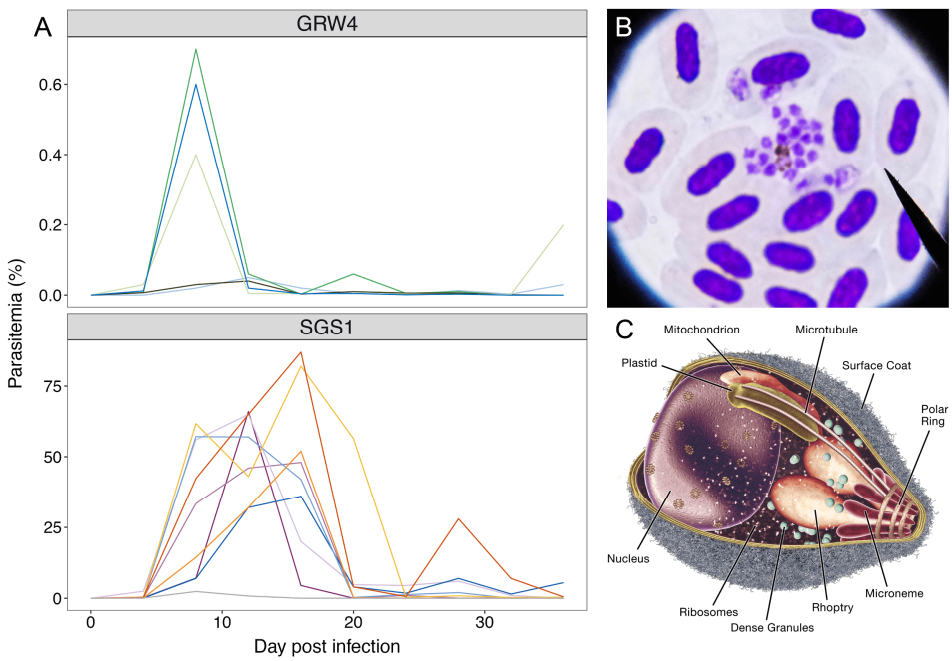

Figure 10. (**A**) Parasitemia levels over time in siskins infected with a low-virulent malaria parasite (GRW4) and a high-virulent parasite (SGS1). Reprinted from **Paper VI**. (**B**) Photograph of a microscope view of avian malaria parasites (in pink) on a blood smear. Photo: E.V. (**C**) Drawing of a malaria parasite merozoite highlighting major organelles. Reprinted from Cowman & Crabb (2006) with permission from Elsevier.

can reach different infection intensities (**parasitemia**) in different individuals (**Figure 10**), creating a great opportunity to investigate gene expression differences in hosts.

In collaboration with colleagues at the Nature Research Centre, in Vilnius, Lithuania who have the facilities to perform controlled infection experiments in birds, we have studied the molecular effects of avian malaria infection. Wild-caught juvenile Eurasian siskins (*Carduelis spinus*; **Figure 11**) were caught with mist nests and housed in aviaries at the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea. Siskins have previously proven to be excellent study organisms for avian malaria experiments (Palinauskas *et al.* 2008, 2011). They are susceptible to several *Plasmodium* lineages, yet juvenile siskins caught early in the summer are uninfected because the vectors have not yet emerged. Siskins are also abundant at the study site and suitable to house in captivity as opposed to several other small wild birds, which may not provide enough blood for high-throughput sequencing.

Figure 11. Eurasian siskin resting. Photo: Eva Mårtensson.

Three naturally infected siskins with high parasitemia intensities of *Haemoproteus tartakovskyi* lineage SISKIN1 were used to sequence the genome of this parasite (**Paper VIII**). Siskins used in the infection experiments were inoculated with blood from a single infected donor bird, and control birds were injected with blood from an uninfected

bird. The birds were then observed and blood samples for parasitemia calculations and for RNA-sequencing were collected at specific time points during the infection. We have used three different lineages of *Plasmodium* in three different infection experiments: *P. ashfordi* GRW2 (**Paper V** and **VII**), *P. relictum* GRW4 (**Paper VI**), and *P. relictum* SGS1 (**Paper VI**).

Collected blood samples were frozen in liquid nitrogen and subsequently extracted to retrieve RNA for transcriptome sequencing and DNA for genome sequencing. After library preparations, samples in the *H. tartakovskyi* genome project were sequenced on a 454 Life Sciences Genome Sequencer FLX+ machine at the Department of Biology, Lund University. The blood samples from the *Plasmodium* infection experiments were sequenced using paired-end Illumina HiSeq 2000 RNA-sequencing to generate transcriptome-wide gene expression data. The sequences were subsequently analysed with bioinformatic methods and software. For further details regarding the specific methods used in the avian malaria project, please see the individual papers (**Paper V**, **VI**, **VII**, **VIII**, **IX**, and **X**).

Results and discussion

Measuring the gut microbiome in birds

In order to investigate a new trait in vertebrates, it is crucial to first develop a methodological procedure that has been verified to give accurate and repeatable results. As an example, hormone levels in birds are often found to be non-repeatable, even

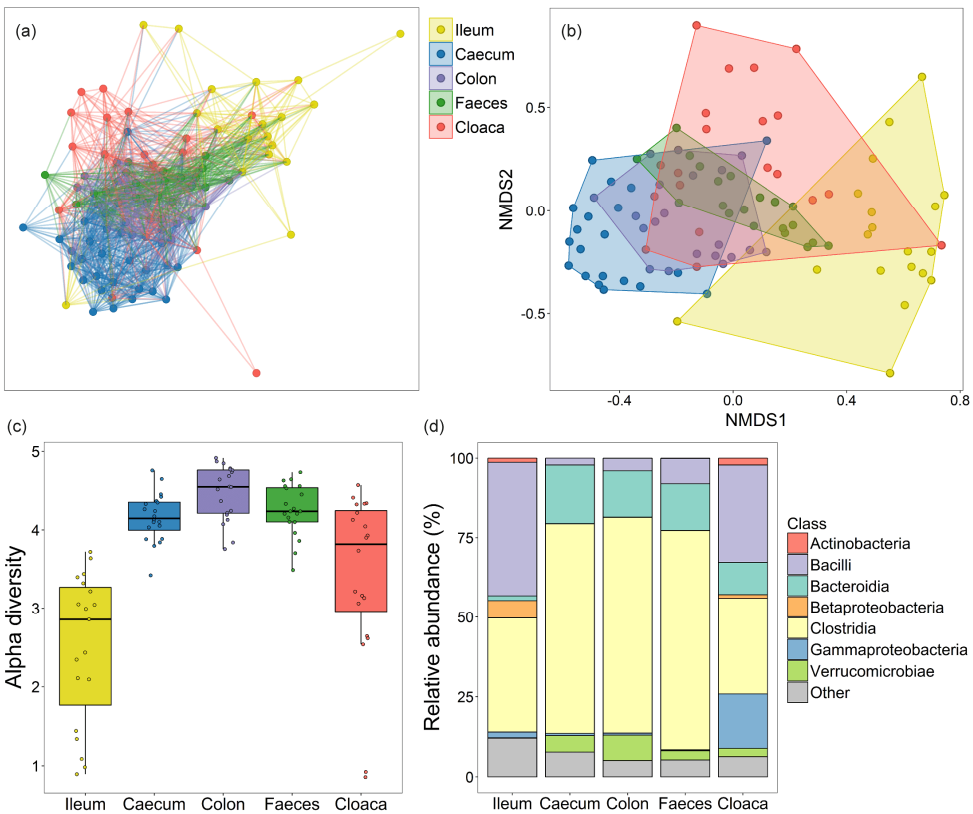

Figure 12. Microbiota differences between two sampling techniques (cloacal swabs and faeces) and three parts of the gastrointestinal tract (ileum, caecum, colon). (**a**) Network, (**b**) NMDS, (**c**) microbial diversity, (**d**) taxonomic composition. Reprinted from **Paper I** (Videvall *et al.* 2017c) with permission from John Wiley and Sons.

within the same study, as they can yield very high variation within individuals (Ouyang *et al.* 2011). Researchers must therefore be aware of what exactly they are measuring and how repeatable they can expect the results to be. Failure to do so may result in spurious relationships and false positives. Before we started large-scale investigations of ostrich gut microbiomes, we therefore set out to test the accuracy of two commonly used sampling techniques for bird microbiomes (**Paper I**), as well as the repeatability of two DNA isolation techniques (**Paper II**).

In gut microbiome research of birds and reptiles, a large amount of studies have sampled the animals by swabbing the cloacae with cotton swabs. By using ostrich juveniles, we tested in **Paper I** the ability of faecal and cloacal sampling at recovering the gut microbiota. We found that cloacal swabs yield a microbial community that is different from that of the ileum, caecum, and colon (**Figure 12**). Faecal samples were also poor representatives of the microbiota of the ileum and the caecum. However, faecal samples were significantly better than cloacal swabs at measuring the microbiota of the colon. We can therefore be confident that sampling faeces yields a microbial community that is not identical, but largely similar to that of the colon, and we recommend gut microbiome researchers to sample faeces, whenever possible, when studying birds.

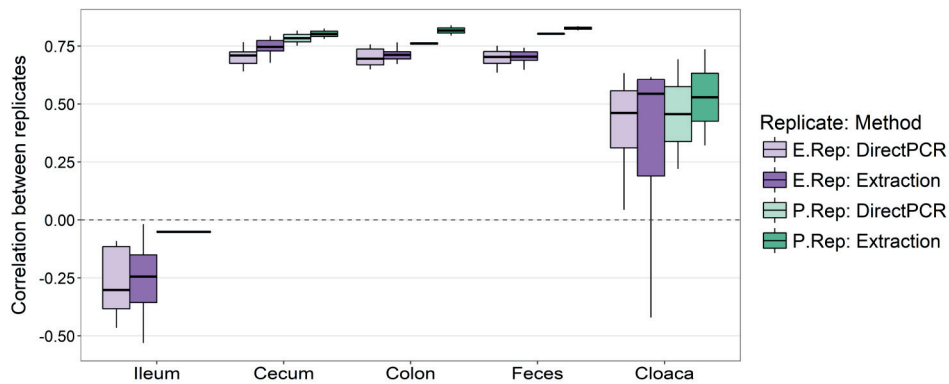

Figure 13. Repeatability of extraction replicates (purple) and PCR replicates (green) when using a direct PCR method (light colour) and a conventional DNA isolation method (dark colour). Adapted from **Paper II** (Videvall *et al.* 2017b) under a CC BY 4.0 license.

When preparing microbiome samples for 16 rRNA gene sequencing, the standard approach includes the isolation of DNA during a series of 32 cleaning and purification steps. In human microbiome research, a recent paper evaluated the possibility of using a potentially faster method with only 4 steps called 'Direct PCR', to circumvent the demanding laboratory procedure of DNA extraction (Flores *et al.* 2012). This method seemed promising, however, the study was performed on human samples only, and with

a small sample size and outdated sequencing technology. It was therefore unclear whether this potentially time-saving preparation technique would yield accurate results using different sample types from a bird species.

In **Paper II**, we set out to evaluate these two methods using five gut-related sample types from ostrich juveniles: gut content from the ileum, caecum, and colon, faecal samples, and cloacal swabs. In terms of costs, we found that the direct PCR method was faster and cheaper compared to the standard DNA extraction procedure. The direct PCR method produced highly repeatable and comparable microbial communities to the DNA extraction method in the caecal, colon, and faecal samples. However, the repeatability and accuracy of both library preparation techniques were much lower in the cloacal and ileal samples (**Figure 13**). In conclusion, both the direct PCR method and the DNA extraction method performed well and had high repeatability when using samples with high DNA concentration, but were equally poor at measuring the microbial community of low biomass samples. We can therefore recommend that researchers use whichever one of these methods they prefer.

Development of gut microbiota in juvenile ostriches

To evaluate how the gut microbiome develops and matures over time in juveniles of a non-model species, we studied in **Paper III** the faecal microbiota of ostrich chicks during their first three months of life. We found that age has a very strong effect on the microbial community (**Figure 14**). For example, the microbial diversity and richness progressively increased with age and the dissimilarities between the microbiota in individuals initially increased and later decreased with age. One week old individuals were highly dissimilar to all other ages, with a community largely dominated by *Akkermansia muciniphila*, likely a consequence of the internal yolk sac they still were

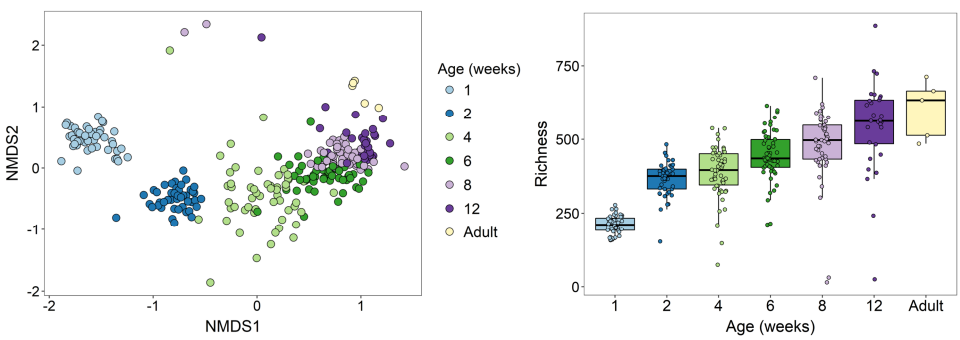

Figure 14. NMDS plot (left) and microbial richness (right) of ostrich gut microbiomes, with colours indicating age of individuals. Adapted from **Paper III** (Videvall *et al.* 2018).

absorbing during this early stage. There were striking differences in colonization and extinction patterns of different bacterial taxa. Some classes increased in relative abundances with age, for example Bacilli, Clostridia, and Planctomycetia, while others rapidly decreased with age, such as Verrucomicrobiae, Erysipelotrichi, and Gammaproteobacteria (**Figure 15**).

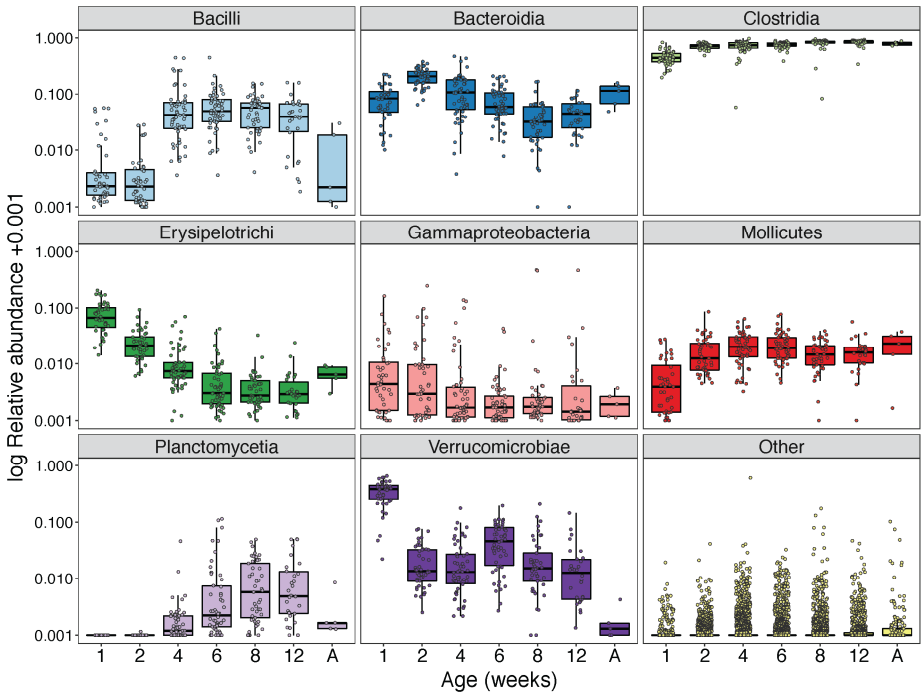

Figure 15. Relative abundances of microbial classes in the ostrich gut during development, with age in weeks on the x-axes and A = Adults. Reprinted from **Paper III** (Videvall *et al.* 2018).

The growth rates of the ostrich chicks were extremely variable, with some individuals weighing five times as much as their contemporaries at 12 weeks of age. We evaluated the effect of the microbiome on juvenile growth and found that individuals one week of age showed a strong positive correlation of microbial diversity with growth, while an overall negative association was found when evaluating all ages. Detailed analyses of specific taxa associated with growth showed that the family Bacteroidaceae was positively correlated with juvenile weight at week 1 of age, while Enterobacteriaceae, Enterococcaceae, and Lactobacillaceae were negatively correlated with weight at either week 2 or week 6.

Strong link between gut microbiota and mortality in ostriches

A large number of ostrich chicks died of suspected disease (n = 68) during the first three months of age (**Figure 16**). Despite attentive daily care and provisioning, high mortality rates of juveniles is common in ostrich rearing facilities, with some years up to 80% of the population dying within the first three months (Cloete *et al.* 2001). This high mortality is believed to be associated with some sort of gut-related disease, as many individuals that die first display characteristic disease behaviour such as lethargy and poor appetite, together with symptoms of diarrhea and an inflamed gastrointestinal tract. Several candidate bacterial pathogens have been appointed as potential culprits, but they differ between studies, and many of them are also present in a normal vertebrate gut microbiota as commensals or pathobionts.

In **Paper IV**, we found that weight was a good predictor of the ostrich chicks' probability of survival, but primarily during the first weeks, where low weight was associated with a low probability of surviving to the next week. Most of the chicks that died lost weight rapidly shortly before dying (**Figure 16**), likely an effect of disease symptoms and not eating. The gut microbiota showed major dissimilarities between diseased and control individuals, and this pattern was so strong, it explained a much larger part of the variation than that of the age of individuals, despite age being the most prominent explanatory variable of the faecal microbiota in healthy individuals (**Paper III**). These large differences between diseased and control individuals were highly significant in all three regions of the gastrointestinal tract. Interestingly, however, the beta diversity between individuals differed depending on gut region, with the diseased individuals being more similar to each other in the upper gastrointestinal tract (ileum) than the control individuals were to each other, but with a reversed pattern present in the lower part of the gut (caecum and colon).

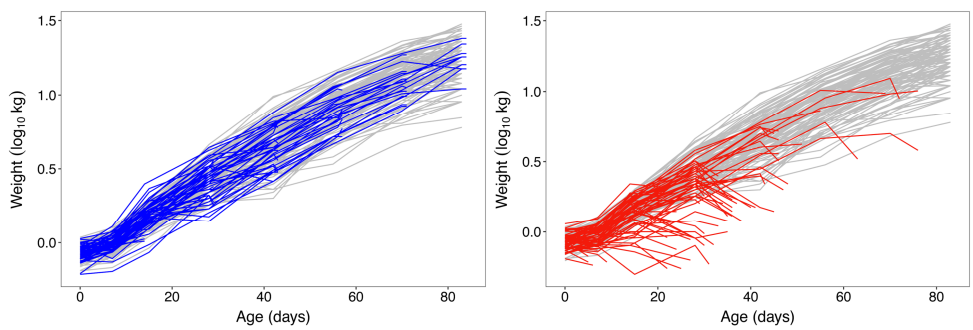

Figure 16. Log-transformed weight of control individuals (blue) and individuals that died from suspected disease (red) during the first three months of age. Grey lines represent all other individuals. Adapted from **Paper IV**.

The microbial diversity of diseased individuals was much lower than that of control individuals, in all three gut regions. Surprisingly, the ileum showed no sign of increasing in alpha diversity with age after controlling for disease, but the effects of age increased progressively along the gastrointestinal tract. We found several taxa associated with disease, i.e. enriched in the ileum, caecum, and colon of diseased individuals (for example Enterobacteriaceae, Peptostreptococcaceae, Porphyromonadaceae, *Clostridium*, *Paeniclostridium*), and several health-associated taxa depleted in diseased individuals (e.g. S24-7, Lachnospiraceae including *Roseburia*, *Coprococcus*, and *Blautia*, Ruminococcaceae, Erysipelotrichaceae, and *Turicibacter*) (**Figure 17**).

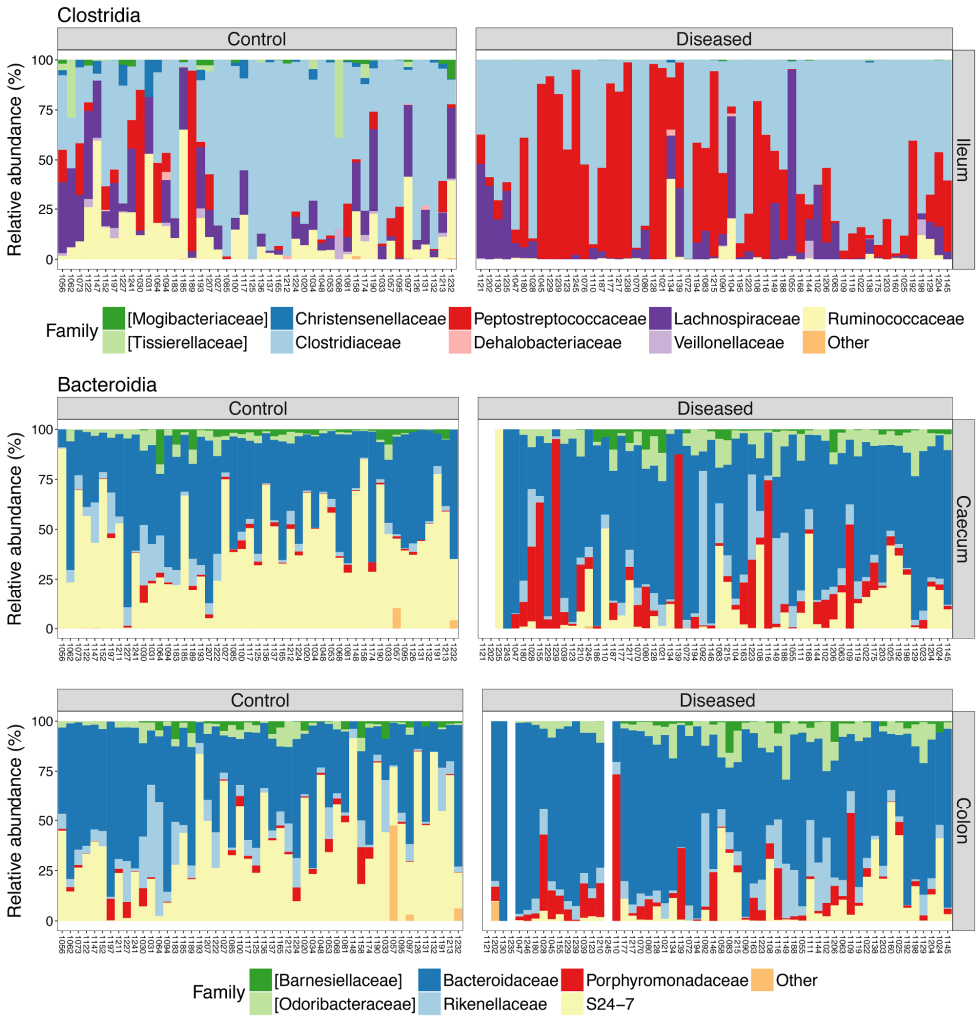

Figure 17. Taxonomic composition of Clostridia in the ileum (top) and of Bacteroidia in the caecum and colon (bottom). Left column = control individuals and right column = diseased individuals. Adapted from **Paper IV**.

We could also show that the food, water, and soil did not contribute significantly to the gut microbiota of diseased individuals, suggesting a small likelihood of bacterial contamination from these environmental sources. Taken together, all our results in **Paper IV** points to a pattern of extreme gut dysbiosis in the ostrich chicks that died from suspected disease.

Avian transcriptome responses to malaria parasites

In **Paper V** and **VI** we evaluated transcriptome responses of siskins to malaria parasites over the course of infection. A large number of genes was found differentially expressed in birds during infection with the high-virulent lineages *P. ashfordi* GRW2 (**Paper V**) and *P. relictum* SGS1 (**Paper VI**), but not during infection with the low-virulent malaria lineage *P. relictum* GRW4 (**Paper VI**). The differentially expressed genes in the birds infected with GRW2 were largely similar during the peak and decreasing parasitemia stages (**Figure 18**). However, a high number of genes involved in the immune system was upregulated during peak parasitemia compared to decreasing parasitemia. The decreasing parasitemia stage had instead genes overrepresented within functions related to 'mature B cell differentiation'. The most highly expressed genes in the blood were those related to haemoglobin and betaglobin (**Paper V**).

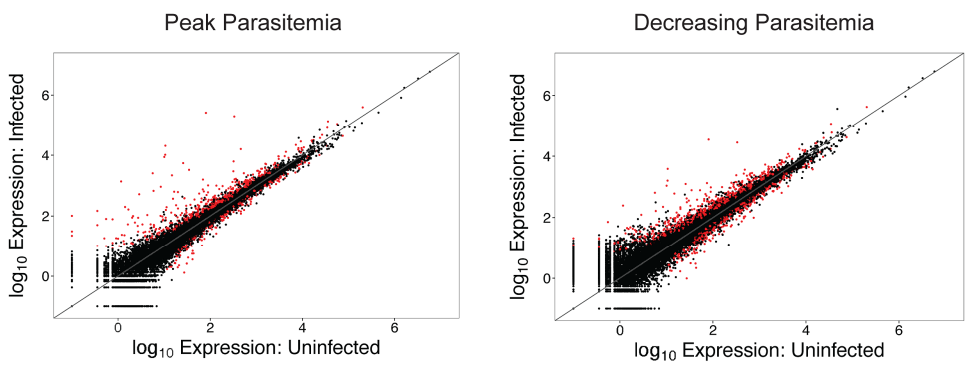

Figure 18. Transcriptome expression in birds with malaria infection during peak parasitemia (left) and decreasing parasitemia (right) compared to control birds. Each point illustrates the mean normalized log-transformed expression levels for one gene (n = 18,618 genes) and significant differentially expressed genes are coloured in red. Adapted from **Paper V** (Videvall *et al.* 2015) under a CC BY 4.0 license.

In the transcriptome responses to the high-virulent parasites GRW2 and SGS1, we found that up- and down-regulated genes in infected birds were overrepresented within processes related to oxidative stress, negative regulation of cell death, metabolic and

catabolic processes, and regulation of gene expression. In fact, the regulatory miRNA genes belonged to the most significantly upregulated genes during both malaria infection experiments, and some of them were also highly correlated with parasitemia intensity. In **Paper VI** we further evaluated the expression of protein-coding genes that have essential roles in producing mature miRNA molecules and in the miRNA gene silencing pathway, and could demonstrate that these miRNA-related genes were also significantly upregulated during infection.

In **Paper VI**, we had the possibility of sequencing a much larger number of individuals and more time points, resulting in a total of 76 bird transcriptomes. Interestingly, the host molecular response to the high-virulent parasite SGS1 resulted in a clear circular trajectory of individuals over time in the principal component analysis (**Figure 19**), with the size of the trajectory being highly correlated with the number of parasites in the blood. That the magnitude of the host response was highly associated with the quantity

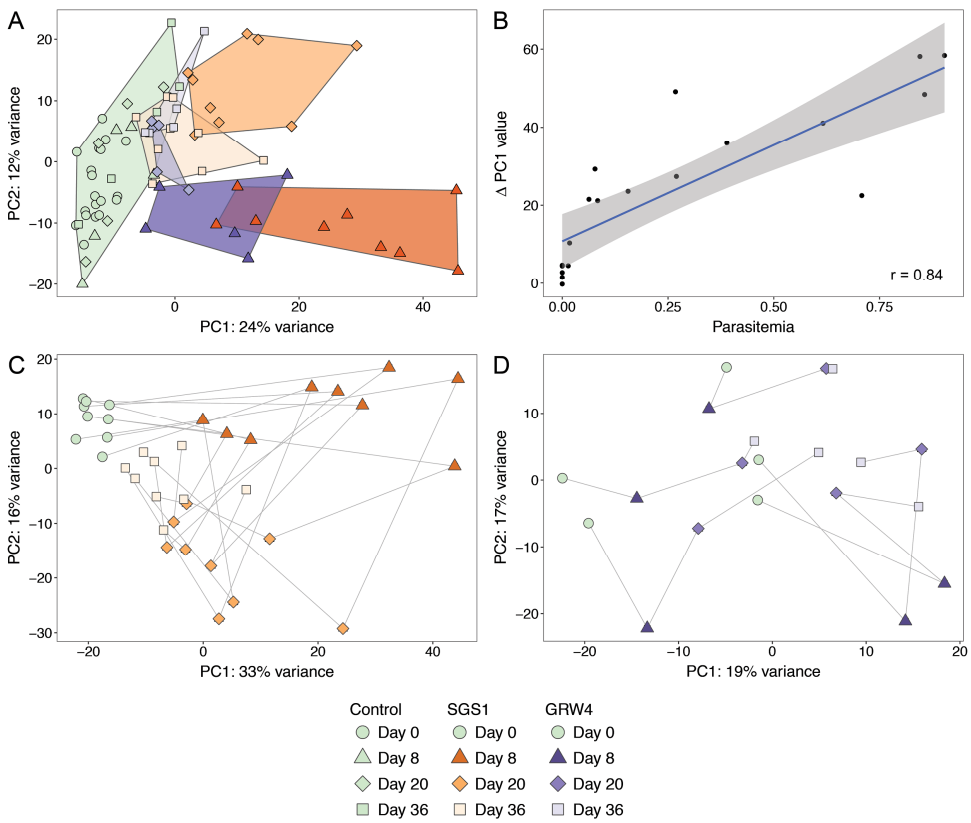

Figure 19. (**A**) PCA of all 76 host transcriptomes. (**B**) Correlation between host individual movement on the PC1 dimension and parasitemia levels. (**C**) The high-virulent parasite SGS1 causes a circular trajectory of host transcriptomes in the PCA, whereas (**D**) the low-virulent parasite GRW4 does not. Reprinted from **Paper VI**.

of parasites during infection was also demonstrated for ~800 genes which showed significant positive or negative associations with parasitemia intensity.

Malaria parasite transcriptome responses to hosts

Because of the largely unbiased nature of high-throughput sequencing, as compared to e.g. targeted microarrays, we retrieve sequence data from all the material in our sample, regardless of origin. This means that dual RNA-sequencing can be utilized to collect reads in samples that contain transcripts from more than one organism, for example in the case of a host and a parasite. At the time I started my PhD, the field of dual RNA-seq was in its infancy and the only relevant study that had used this method was one evaluating the fungal model pathogen *Candida albicans* in mice (Tierney *et al.* 2012).

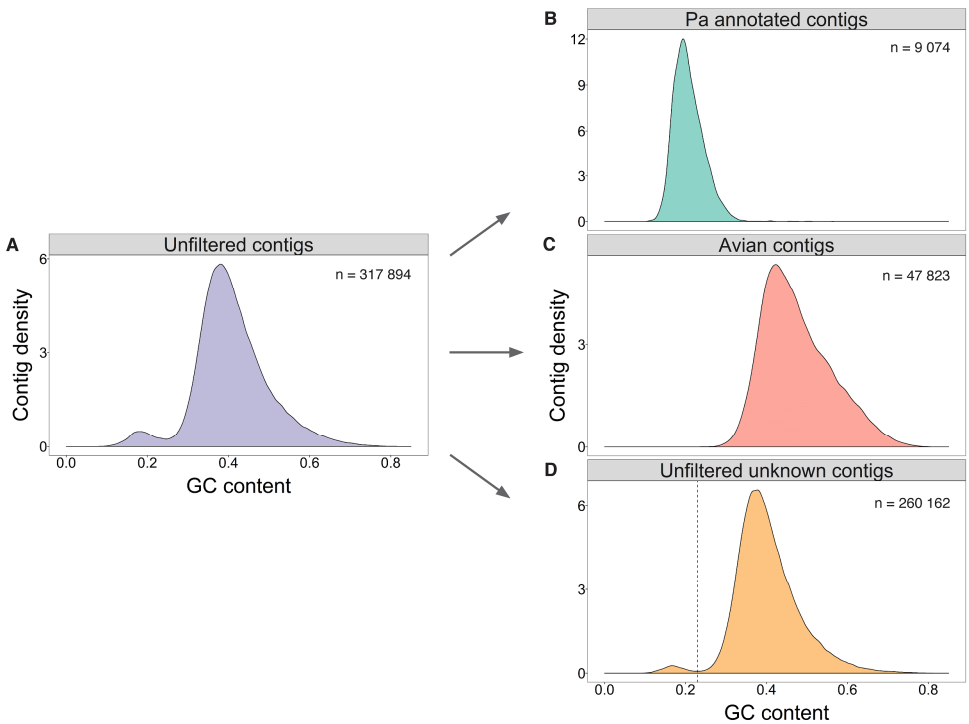

Figure 20. Density graphs of transcript GC content in (**A**) the initial unfiltered transcriptome assembly. After rigorous bioinformatic filtering steps, I constructed (**B**) the filtered *Plasmodium ashfordi* assembly, (**C**) transcripts matching bird sequences, and (**D**) unknown contigs containing both bird and parasite transcripts which were subsequently utilized. Reprinted from **Paper VII** (Videvall *et al.* 2017a) with permission from John Wiley and Sons.

In several aspects, my journey into this field of dual RNA-seq data was novel. Not only was I working with a relatively understudied wildlife disease system in terms of molecular studies, the dual RNA-seq data was derived from an infection experiment involving a non-model host species without any genome sequence *and* a non-model parasite species without any genome sequence. The aim of building a clean parasite transcriptome assembly from scratch without any genomic references was therefore monumental.

In **Paper VII**, I show that this endeavour was possible through multiple bioinformatic filtering steps, because we present the first transcriptome assembly of a wildlife bird malaria parasite, *Plasmodium ashfordi* lineage GRW2 (**Figure 20**). Expression of *P. ashfordi* transcripts was analysed in three different host individuals and during two time points of the infection, peak parasitemia and decreasing parasitemia. We could demonstrate that *P. ashfordi* exhibits host-specific gene expression as there were several differentially expressed parasite transcripts between individual hosts, but no differences between time points. We further identified single nucleotide polymorphisms in the *P. ashfordi* transcriptome, that could potentially be a result of real variants in the parasite population, or rare gene duplication events where the transcribed transcripts remain near-identical to each other. When evaluating sequence identity with other apicomplexans, *P. ashfordi* was most similar to the human parasite *P. falciparum*, and we directly compared annotated gene functions of these two parasites (**Figure 21**). Finally, we identified a long list of *P. ashfordi* transcripts derived from genes documented to be involved in the *Plasmodium* invasion of red blood cells.

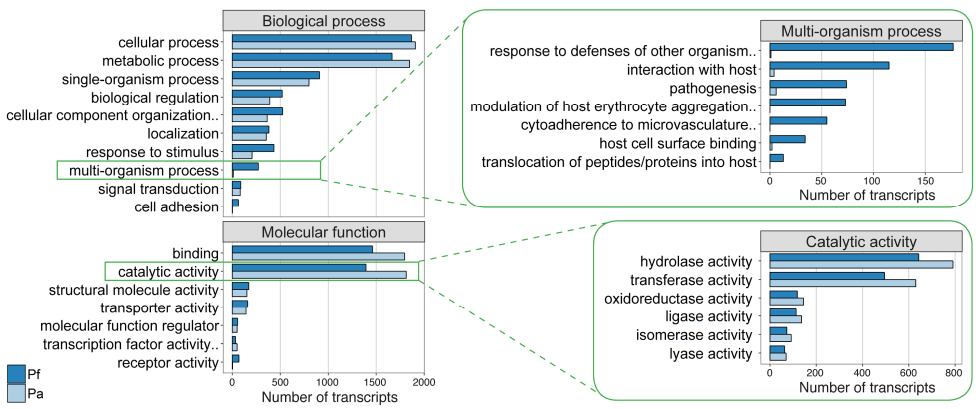

Figure 21. Differences between the transcriptomes of the avian malaria parasite *Plasmodium ashfordi* (light blue) and the human malaria parasite *Plasmodium falciparum* (dark blue) in terms of transcripts annotated with specific gene ontology terms. *P. ashfordi* has fewer transcripts annotated as processes involved in host interactions because these sequences are likely evolving faster compared to other genes, and thus have become too differentiated in avian malaria parasites for sequence similarity searches. Reprinted from **Paper VII** (Videvall *et al.* 2017a) with permission from John Wiley and Sons.

Evolution of malaria parasite genomes

Because the recently assembled *P. ashfordi* transcriptome represented the only available genomic resource of a non-mammalian malaria parasite (**Paper VII)**, it became incredibly valuable in our phylogenomic analyses of **haemosporidians**. Previous studies have found sequence similarities between human malaria parasites and avian malaria parasites. It has therefore been repeatedly suggested that mammalian *Plasmodium* are not monophyletic, and that instead host switches of parasites from birds to primates are responsible for these similarities (Waters *et al.* 1991; Pick *et al.* 2011).

We assembled the genome of *Haemoproteus tartakovskyi*, a bird blood parasite in the sister genus to *Plasmodium*, to use as an outgroup in a new phylogeny based on genomic data. In **Paper VIII**, we described the genome characteristics of *H. tartakovskyi*, and use it together with *P. ashfordi* to create a well-supported phylogeny of *Plasmodium* parasites. The *H. tartakovskyi* genome is small and AT-rich (25.4%), similar to many *Plasmodium* parasites. It also contains expansion clusters of gene families that are slightly higher in GC%, a possible sign of species-specific invasion-related genes similar to *rif*, *var*, and *stevor* in *P. falciparum*.

Phylogenetic analyses were conducted in two steps. First, an initial phylum-wide phylogeny of 17 apicomplexan species was constructed, which confirmed the placement of *H. tartakovskyi* as an outgroup to *Plasmodium*. Second, we constructed two phylogenetic trees with only Haemosporidian sequence data, which resulted in a supported monophyletic clade of mammalian-infecting *Plasmodium* parasites, with the only available non-mammalian *Plasmodium* (*P. ashfordi*) as a sister taxon (**Figure 22**).

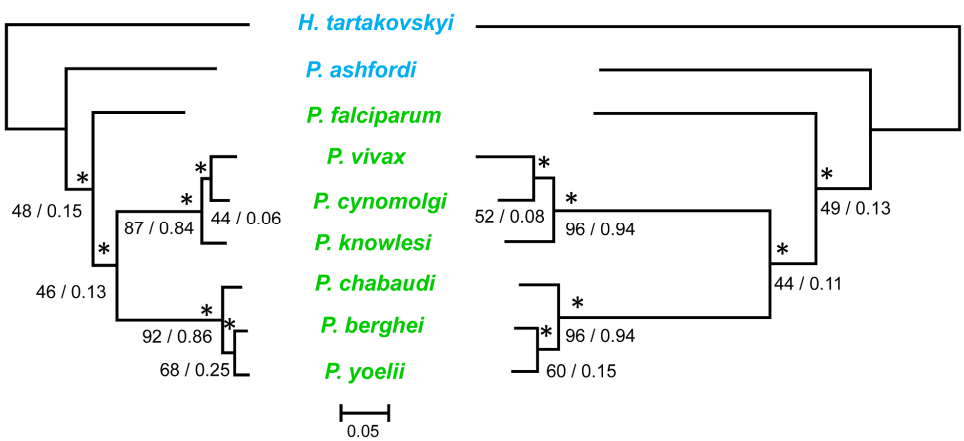

Figure 22. Two *Plasmodium* phylogenetic trees based on genomic sequence data with *H. tartakovskyi* as outgroup, supporting a monophyletic clade of mammalian malaria parasites (green) with avian haemosporidians (blue) as sister taxa. Reprinted from **Paper VIII** (Bensch *et al.* 2016) under a CC BY-NC 4.0 license.

In **Paper IX**, we utilized both of these newly assembled genomic resources of *P. ashfordi* and *H. tartakovskyi* to search for three key genes involved in the thiamine (vitamin B_1) biosynthesis pathway. Interestingly, the genes coding for this essential vitamin are present in the genomes of primate malaria parasites, but absent in the genomes of rodent malaria parasites and other closely related apicomplexans. It has been suggested that these genes in primate malaria parasites are a result of horizontal gene transfer from bacteria (Frech & Chen 2011). However, our analyses show that these genes are not only present in the genomes of all three avian *Plasmodium* parasites evaluated, but are also actively expressed as transcripts. Furthermore, we located these genes in the genome of a species in the sister genus, *Haemoproteus*, which suggests that the thiamine genes have been present in the common ancestor but subsequently lost in the rodent malaria species (Hellgren *et al.* 2017).

Finally, in **Paper X**, I again utilized our assembled *H. tartakovskyi* genome and *P. ashfordi* transcriptome, together with the genomes of two newly sequenced avian malaria parasites, *P. relictum* and *P. gallinaceum* (Böhme *et al.* 2016). I conducted comparative genomic analyses of a large number of eukaryotes with AT-rich genomes to identify the species with the most extreme AT-bias. The human malaria parasite *P. falciparum*, has repeatedly been described as the most extreme eukaryotic organism when it comes to nucleotide composition. However, my results show that the sequenced avian malaria parasites (all three of them) have a much lower GC content, by a large margin (**Figure 23**).

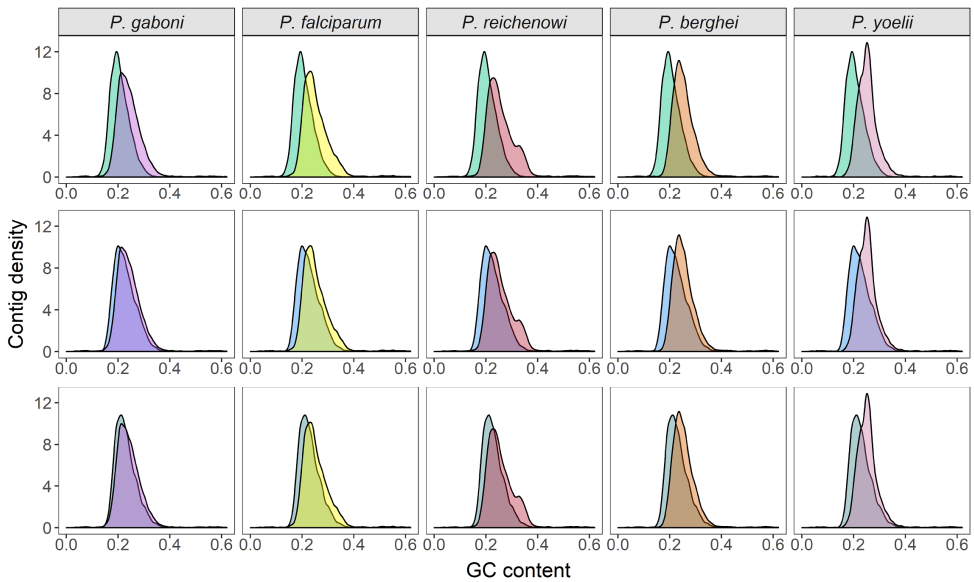

Figure 23. Density of transcript GC content in three avian malaria parasites (green–blue) compared to five AT-rich mammalian parasites (columns). Adapted from **Paper X** (Videvall 2018) under a CC BY-NC 4.0 license.

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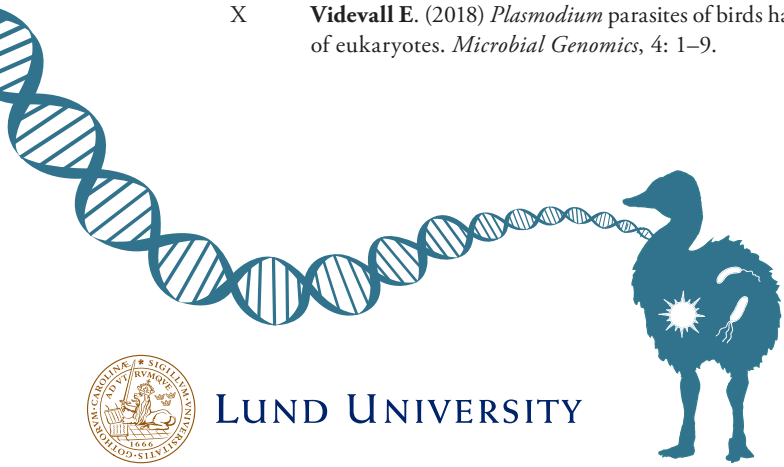

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