Evolution of Diversity in Avian Innate Immunity

Michal Vinkler

HABILITATION THESIS

Zoology

Charles University, Faculty of Science

December 2018

CONTENTS

Concepts and methods of ecological immunology

Avian inflammation

Traits associated with inflammatory responsiveness and general health in birds

PREFACE

Much faster than any time in the past, methodology in life sciences develops to allow detailed understanding to all sorts of the biological systems represented on this planet. The massive increase in our research possibilities brings both euphory of thoughts regarding our future plans, as much as the depression from our steadily growing, yet limited capacity to master all the diverse high-tech and high-throughput instrumentation that is becoming reasonably cheap and accessible. This general pattern is becoming clearly visible especially in interdisciplinary research that is forming larger and larger part of the total global scientific output. Many interesting and relevant questions cannot be answered using conceptual framework and methodology of a single scientific discipline, driving interdisciplinarity among basic qualities of the contemporary research. In biological sciences it has been recognised since the times of Charles Darwin and his followers that answering basically any question requires evolutionary insight to bring ultimate understanding of the system. Hence, having evolutionary biology as a platform for all other research, the boundaries between individual disciplines dissolve making harder and harder to categorise the research.

However, this trend is unfortunately not always reflected by the main-stream journals, funding agencies and higher education. Less than a half century ago different biological disciplines were not so much remoted, specialised and self-absorbed as they are now. In the Charles Janeway's times evolution was perceived as an integral part of immunology which was used to explain the immunological function, which was then used to explain the very basic concepts of the evolution of life on Earth (Janeway 1989). Yet, with our advance in the understanding of the very details of the problematic on both sides the individual biological disciplines became more isolated and now we are facing a situation when many students in immunology lack even the basic understanding of evolutionary principles while most zoologists and ecologists never passed any immunological course during their studies. In my work I have attempted to promote the principle of interdisciplinarity and melt again the borders between the disciplines presently considered as distant as the "green" zoology and "white" immunology. This was driven by the hope to contribute to re-unification of the "rainbow" biology in general and development of a relatively young branch of research named evolutionary immunology in particular. Starting with a zoological background of the molecular-ecological research performed by the team of my former doctoral thesis supervisor prof. Tomáš Albrecht, I adopted immunology as my second specialisation to avoid the so called "scientific inbreeding", while allowing progress of the investigation towards deeper understanding of specific phenomena within the broad process of evolution. My particular scientific questions have been:

1. What are the genetic differences between individuals and species that contribute to variation in the resistance to infectious diseases? How did they evolve and what is the mechanism of their function?

2. What is the effect of this genetic diversity on the general physiology of the organisms in a given ecological context?

These two categories of questions determined both my scientific career as well as the structure of this habilitation thesis. As a model taxon for my investigation I selected birds, a vertebrate group with clear multi-fold convergence to mammals that, by comparison, can be used to determine general principles involved in evolution of the immune defence in homoiothermic animals. Regrettably, I must admit that despite my continuous effort to answer the two categories of selected questions, huge gaps are still

present in my understanding of the investigated systems that precluded combining the content of this thesis into a single logical and comprehensive source of information. Instead, the work is still logically divided into two broad parts – one focusing on the evolution of the innate immune genes and the other one describing the immunological state and responses, with the bridge between the two easily conceivable, yet after more than ten years of research still missing. Be it a motivation for us for further research.

Prague, 1st November 2018

ACKNOWLEDGEMENTS

Although habilitation thesis is submitted as a contribution of a single author, vast majority of the research work being done in contemporary biological sciences results from a team collaboration, accomplishing tasks which could not be performed by an individual. I have been very lucky in my career with the teams I belonged to and which I led. They all have been rich with stimulating ideas and multidisciplinary skills. To mention everyone who helped me approach the goals of this thesis is a difficult job. Therefore, I wish to make a more general acknowledgement to all my excellent co-workers (every and each one of them) and here mention only several most prominent names from my long list. Namely I would like to thank my former scientific mentors: prof. Tomáš Albrecht and assoc. prof. Pavel Munclinger from Charles University, assoc. prof. Josef Bryja, Anna Bryjová and Dr. Jaroslav Piálek from the Institute of Vertebrate Biology, Czech Academy of Sciences, Vladimír Holáň from the Institute of Experimental Medicine, Czech Academy of Sciences, and Dr. Lisa Rothwell and late prof. Pete Kaiser from the Roslin Institute, University of Edinburgh. Without their help my ultimate goal to combine immunology and zoology as two distinct biological disciplines would come certainly in vain. My next expression of thankfulness belongs to all of my students who, over the years, became my bottomless well of inspiration and more common than not also my prolonged hands to handle the science. Here I would like to name especially the doctoral students Hana Velová, Zuzana Świderská, Barbora Bílková, Petra Bauerová, Tereza Králová and Martin Těšický. Similarly, I wish to appreciate the help from my senior collaborators, Dr. Marian Novotný, Dr. Jana Svobodová, Dr. Jakub Kreisinger, Dr. Oldřich Tomášek, prof. Dave Burt, assoc. prof. Dana M. Hawley, prof. Rami A. Dalloul, prof. Arild Johnsen, prof. Jan T. Lifjeld, assoc. prof. Pavel Hyršl and Dr. Jakub Hraníček. Finally, the research would not be possible without appropriate funding provided mainly by the Czech Science Foundation (projects Nos. 206/06/0851, 206/08/1281, P505/10/1871, P502/12/P179, P506/15-11782S) and Charles University, (projects Nos. PRIMUS/17/SCI/12, GAUK/127507, GAUK/44809, GAUK/540214, GAUK/504214, GAUK/275715). I am also highly obliged to the Fulbright Commission in the Czech Republic for providing me the possibility to do my research in USA during my stay at Virginia Tech (2015-2016; grant No. 2015-21-04). And last but certainly not least, I would like to express my uttermost gratitude to my beloved family for their non-accusing understanding and support.

INTRODUCTION

While presently viewed by the general public as a mostly resolved field of medicine, infection biology still represents an ever surprising and relevant research branch. For more than a half of a century modern medicine fights the pathogens with rapidly developing drugs and yet the pathogen-borne infectious diseases remain globally the second leading cause of human death (Fauci 2001) and a source of dramatic economic losses in domestic animals (Wilkinson et al. 2011; Dehove et al. 2012) as well as a severe threat to wildlife (Wiethoelter et al. 2015). Even in the highly developed countries such as the EU or the USA, the mortality rates due to pathogens decrease only slowly over time representing an important hazard and most infections were not successfully eradicated (Dye 2014). In the light of these facts, it is fascinating to see how well the pathogens have adapted to the human effort to eliminate them as much as to realise how perfectly our own (animal) immune system protected us against them during the earlier millions of years of our history when no medical science existed. Furthermore, microbes do not represent only pathogens with the capacity to kill the host, but, probably on the first place, symbionts that continually modulate animal physiology for good and bad. Therefore, investigation of the host interaction with microbes remains highly important and the research bringing understanding of the evolutionary changes in this relationship provides a key to resolve even merely practical challenges of the current society, such as microbial drug resistance issues or replacement of antibiotics with more sustainable treatments.

Observed from the evolutionary-ecological perspective, pathogens (and parasites in general) represent one of the chief factors driving organic evolution. Since pathogenesis results from the activity of many different microbial taxa, the diversity of selection pressures acting on animal immune system is enormous, being the generator of extensive diversity of the immune defence strategies. This can be documented by the fact that immune genes belong among the most rapidly evolving genes in animal genomes (Hillier et al. 2004; Bustamante et al. 2005; Fumagalli et al. 2011) and also genes showing the highest recombination rates (Frazer et al. 2007). Not only in the Major Histocompatibility Complex (*MHC*) that is involved in adaptive immunity, but also in animal innate immune genes we can track numerous footprints of the past or ongoing positive selection, which can be studied based on different types of molecular traits, including population differentiation in certain genes (Ferwerda et al. 2007; Carnero-Montoro et al. 2012; Nédélec et al. 2016), their allelic frequencies (Hellgren 2015; Kloch et al. 2018), dN/dS substitution rates (Smirnova et al. 2000; Vinkler et al. 2009; Grueber et al. 2014) or trans-species polymorphism occurrence (Tesicky and Vinkler 2015). Shaped by the contact with pathogen structures, the immune-related genes adapt with their expression or shapes and other physicochemical features of the resultant protein molecules to optimally respond to pathogen incursion (Walsh et al. 2008; Guivier et al. 2010; Wang et al. 2012). This selection can be either linear (selective sweeps) or cyclic (fluctuating or balancing selection; Woolhouse et al. 2002) and can target a rich variety of different genes and genomic regions to achieve functionally comparable results (evolutionary convergence). This fascinating wealth of strategies the immune system may take to combat the pathogens and achieve homeostasis is in the focus of evolutionary immunology. The diversity-based approach adopted by this discipline can serve as a rich source of understanding especially for the developing field of evolutionary medicine. Evolutionary medicine considers individual adaptations in different patients, being a fore step to personalised medicine, which is a modern medical model treating patients as heterogeneous units to be cured based on the insight into their individual needs (Henneberg and Saniotis 2012). Similarly, application of evolutionary thinking can (and should) be adopted in agriculture to the protection of different species and breeds of the domestic animals against diseases or in the wildlife protection where vulnerable populations of endangered species may be threatened by pathogenic challenges.

Regardless of the possible practical application of the information gained, biodiversity-based investigation of the immune function within the evolutionary and ecological context brings us essential and philosophically plausible basic understanding of the constrains in animal defence against diseases (see, e.g., Janeway 1989). The parasite-driven selective forces modulate various host traits, starting with the population dynamics and

territoriality, and ending with the mating systems and investments into reproduction (Clayton and Moore 1997). The study of evolution of the host-parasite relationships as well as the research of relationships between individual immunological and physiological pathways enables identification of the general trade-offs that limit the evolution of life-history traits in animals (Lochmiller and Deerenberg 2000). This information is needed to answer so much simple and in the same time basal questions as why there are fifteen nestlings in a clutch of some species while others have a single one or why some birds are bright and not cryptic.

For several reasons, birds represent a suitable taxon for evolutionary investigation of the host-pathogen interactions (Clayton and Moore 1997). For example, the domestic chicken and to lesser extent also the duck, turkey and the quail belong among traditional model species for the immunological research (Schat et al. 2014), a wide range of avian genomes is now available (Zhang et al. 2014; though this was not true a few years ago), avian models play a central role in evolutionary studies of parasite-mediated sexual selection (Hamilton & Zuk 1982) and avian unique physiology is being extensively investigated mainly with respect to their capability of flight (Videler 2005) and longevity (Vleck et al. 2007). Furthermore, in certain aspects of their physiology (e.g. day-time activity, visual orientation, advanced cognition, longevity) some birds are more similar to humans than are the rodents typically used in immunological research. Yet, so far only insufficient attention has been paid to avian evolutionary immunology, which precludes the comparative approach and full exploitation of the avian models. Therefore, in my work I oriented almost exclusively to the research in avian evolutionary immunology. Among my favourite model species belong passerine birds (avian highly derived crown taxon) on one side and the galliform birds (representing ancient avian lineage) on the other (Jarvis et al. 2014). Apart from thoroughly comparative studies where a high number of species were investigated, my research in passerines focused both at man-kept zebra finches (*Taeniopygia guttata*) and free-living populations of great tits (*Parus major*), scarlet rosefinches (*Carpodacus erythrinus ~ Erythrina erythrina*) and North American housefinches (*Carpodacus mexicanus ~ Haemorhous mexicanus*). In galliforms, my research oriented to wild as well as captive grey partridge (*Perdix perdix*) and namely to the traditional domestic fowl (*Gallus gallus* f. *domestica*) breeds that mostly escape the attention of both immunologists and evolutionary biologists. Thus, all contributions included in this thesis represent studies in birds, a taxon in which immunological investigation is highly needed to provide a comparable insight into the research topics studied presently in wild and laboratory mammals.

GENERAL DISCUSSION

Relevance of the innate immune gene diversity investigation

For more than two decades the evolutionary research aimed at understanding the diversity maintenance in immunity was dominated by the focus on the Major histocompatibility complex (*MHC*; Piertney and Oliver 2006). This results from the fact that in earlier days of the immunological research (until 1990s) much attention was attracted by the mechanism of clonal diversification of T cells, which lead to the idea of paramount importance of the acquired immunity for disease resistance (Janeway 1989). The enormous and by all means fascinating MHC diversity has evolved to reflect the T-cell receptor (TCR) somatic variation to allow the vertebrate immune system to achieve virtually unlimited capacity for pathogen recognition. Thus, numerous evolutionary studies both in model and non-model species assessed the interspecific and intraspecific levels of *MHC* variation to show their ecological and evolutionary consequences (see e.g. the review Adelman et al. 2014). However, the enormous variability of *MHC* may also represent its main drawback for an attempt to understand the molecular principles of host-pathogen evolutionary co-adaptations. The tens to hundreds of alleles encoded at a variable number of loci (Piertney and Oliver 2006), common pseudogenisation (Sepil et al. 2012), together with conformational plasticity responsible for structurally flexible ligand binding in some MHC variants (Koch et al. 2007) may simply bring too much complexity that can (at the current stage of the research) prevent us from describing some of the basic principles of molecular co-evolution between hosts and microbes.

While over the years of the research it became clear that the complexity of the immune function and its variation in the ecological context cannot be fully captured by the MHC-oriented research only (Acevedo-Whitehouse and Cunningham 2006), no clear alternative focus was indicated. To contribute to the advance in the wildlife evolutionary and ecological immunology research, Tomáš Albrecht and I (PAPER I) suggested the research focus towards the Pattern-recognition receptors (PRRs). The discovery of PRRs is probably one of the greatest stories of current immunology (I humbly remark a story that started its life approximately at the same time as myself, so I am pleased to be somehow, many years later, part of it). Although yet undiscovered by that time, their existence was correctly foreseen already in 1980s by Charles A. Janeway (Janeway 1989). Since their discovery in insects (Rosetto et al. 1995) and later in vertebrates (Medzhitov et al. 1997) PRRs and namely Toll-like receptors (TLRs) as their most intensively studied representatives raised great attention among immunologists and medical scientists. Their scientific beauty is multifold. First, by classical categorisation of immune mechanisms, the PRRs belong to innate immunity which was originally thought to be invariantly boring (and, therefore, remained understudied), yet they turned out to be essential for triggering any successful immune response (Medzhitov and Janeway 1998) – PRRs are the very first host receptors to detect the pathogen, i.e. they are far more important than had been assumed (Kawai and Akira 2010). Second, PRRs form a functional bridge between the so called innate and acquired immunity – a combination of signals provided by PRRs direct and govern the form, timing and intensity of a developing adaptive immune response (Palm and Medzhitov 2009). Third, the effector response commonly triggered by PRRs is inflammation (Uematsu and Akira 2006; Figure 1), an immune mechanism which is efficient in pathogen clearance, but also costly in terms of resource allocation and damage of host tissues (Ashley et al. 2012). Fourth and last, the enormous scientific interest in the biology of PRRs in general and TLRs in particular brought great wealth of knowledge that can be used to interpret new data (presently ca. 50 000 articles on WOS earned during the twenty years since their discovery, only small fraction, however, investigating their evolution). TLRs are definitely not the only PRRs (Palsson-McDermott and O'Neill 2007). Nevertheless, the facts that TLRs are generally highly expressed in many different tissues and their genes typically consist of only few exons, which simplifies the molecular work, undoubtedly contribute to their popularity. In the PAPER I we encouraged the (by then limited) investigation of PRR variation, namely in wild-living animals and its interpretation within the evolutionary framework of the host-pathogen interactions. We selected TLRs, the most extensively studied group of the innate immunity receptors as an

example and a model gene family recommended for the further research.

Soon after their discovery (Medzhitov et al. 1997) it became clear that TLRs represent one of the basic and evolutionary most original components of the animal pathogen-recognition system (Rich 2005). Each vertebrate species is equipped with a set of 9 to 20 *TLR* genes, some of which are maintained through the entire vertebrate phylogeny (Wang et al. 2016b). Individual members of the TLR family differ in their antigen-binding features provided by their leucine-rich repeat exodomains that evolved to recognize different microbe-associated molecular patterns (MAMPs) or host-derived endogenous antagonists (so called damage-associated molecular patterns, DAMPs; Kawai and Akira 2010; Figure 1). The microbe-derived MAMPs (e.g. lipopolysaccharide, lipoprotein, peptidoglycan etc.) are typically largely conservative in their presence in specific pathogen taxa, because the microbes are usually unable to avoid their expression without significant costs (Wang et al. 2015). Given the capacity of individual TLRs to recognise specific conservative microbe-derived ligands, the TLRs were originally expected to represent conservative structures with mostly invariant functionality (Medzhitov and Janeway 1998). Therefore, *TLR* evolution was interpreted mainly on the basis diversification of the functionally distinct *TLR* genes and gene sub-families (Roach et al. 2005).

Indeed, much of the sequence of each gene is under strong purifying selection which maintains the conserved structure and general function of the receptors (Wang et al. 2016b). Yet, from the very first records on the *TLR* variation between species it became also clear that individual sites in the genes may be under positive selection which differentiates the species (Smirnova et al. 2000). Furthermore, while most attention

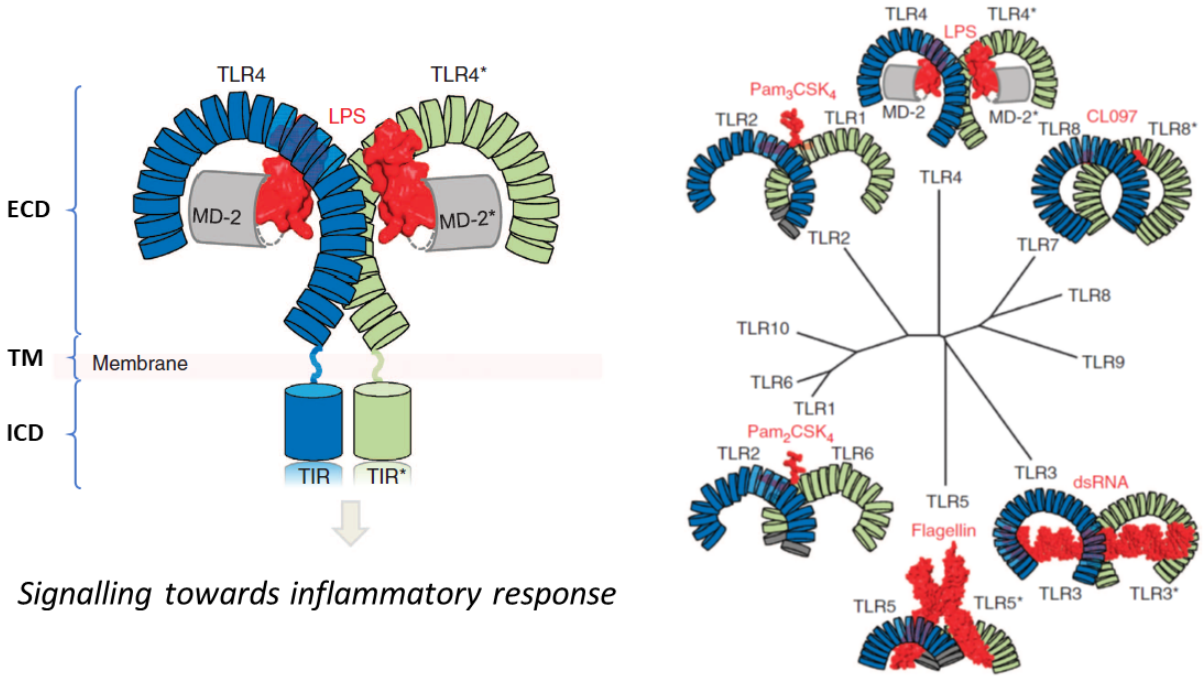

Figure 1. Schematic representation of the Toll-like receptor (TLR) structure and ligand binding. TLRs are type I integral membrane glycoproteins that are formed by an extracellular/endosomal ligand-binding domain (ECD), transmembrane domain (TM) and an intracellular cytoplasmic domain (ICD) which consists of the signalling Toll/IL-1R homology (TIR) domain. TLR4-MD-2 heterodimer-mediated detection of bacterial lipopolysaccharide (LPS) triggers a signalling cascade leading towards activation of an inflammatory immune response (left-hand side). Each TLR homo/hetero-dimer (shown in a phylogenetic tree, right-hand side) recognises a specific set of ligands – several crystal structures of TLRs and their ligands are already known to facilitate interpretation of the functional significance of the genetic variation observed at the interspecific and intraspecific levels in *TLRs*. The ligands are coloured in red, TLRs are shown in blue and green (adapted from Park and Lee 2013).

was paid to description of the level of conversation in *TLR* gene content in vertebrate genomes (Temperley et al. 2008) or their generally conserved functionality (Keestra et al. 2013), certain reports appeared which indicated functional differences even between *TLR* alleles at the intraspecific level in traditional model species such as the chickens (Leveque et al. 2003) or in humans (Ferwerda et al. 2007). The PAPER 1 was probably the first article which accented the importance of the PRR population polymorphism among conspecifics for disease resistance in wild animals. This article of ours was motivated mainly by our previously gained results from sequencing and basic structural and functional characterisation of the zebra finch (*Taeniopygia guttata*) *TLR4*. Here sequencing of a single individual revealed dramatic differences between the two alleles (described in PAPER 2; please note that this was before the next-generation sequencing, NGS, era fully started and the information on the zebra finch genome was not yet available). As a part of the study we performed the analysis of natural selection acting in *TLR4* in vertebrates to support the strong influence of positive selection at certain sites mainly in the ectodomain. The fascinating idea of the genetic variation in TLRs finely adapted to optimise the quality as well as quantity of the immune signalling motivated the development of the research of my slowly-forming research group for many years ahead, supporting with new ideas the grant proposals of mine as well as those of my close collaborators.

Population polymorphism in avian TLRs

Before more information on avian genomes was gained by the Avian Phylogenomics consortium (http://avian.genomics.cn/en/), it was difficult to develop the above-mentioned type of research in the passerine birds. Therefore, (while also attempting to explore a bit the mammalian models, Fornuskova et al. 2013, 2014) I gladly accepted the invitation by prof. Miroslav Šálek to attempt to answer my research questions in a European free-living galliform game bird - the grey partridge. My ambition was to use the molecular tools available for the domestic chicken (reviewed e.g. in Schat et al. 2014) to test variability in function of the grey partridge *TLR* alleles. In this species we investigated three *TLR* genes that are true orthologues to their mammalian counterparts with conserved ligand specificity: *TLR4*, *TLR5* and *TLR7* (Keestra et al. 2013). In parallel to our own immunogenetic description of the *TLR* population polymorphism, multiple groups in the world started to explore the avian *TLR* diversity (Alcaide and Edwards 2011; Grueber et al. 2014). Although neglecting the importance to study the whole coding DNA sequences (CDS) or at least some well-defined functional regions (such as the ligand-binding regions) of the genes, their studies confirmed in birds the evidence which accumulated continuously in mammals (Wlasiuk and Nachman 2010; Areal et al. 2011; Smith et al. 2012) showing that (i) there is a significant positive selection acting in *TLRs*, that (ii) this positive selection can be detected mainly in the ligand-binding regions and that (iii) the distinct *TLR* genes differed in their levels of variation and selection. Some studies also suggested that the level of population polymorphism in *TLRs* may be reasonably high (Alcaide and Edwards 2011). Unfortunately, our findings showed that in the grey partridge the levels of *TLR* genetic polymorphism were only very moderate and the single nucleotide variants (SNVs) revealed did not suggest much functional diversification (PAPER 3). This was, similar to the situation in a bottlenecked population of the New Zealand's Stewart Island robin (*Petroica australis rakiura*; Grueber et al. 2012) and very likely caused by the decrease in the grey partridge population size combined with fragmentation of its populations and a putative local inbreeding. Yet, apart from the truly disappointing finding that the grey partridge is probably not a suitable model for investigation of the functional consequences of *TLR* polymorphism, we still learned that different *TLR* genes may harbour distinct levels of the population polymorphism, with the highest variation detected in *TLR4* and lowest in *TLR7* (partially consistent with lesser kestrel, Falco naumanni, population polymorphism shown previously by Alcaide and Edwards 2011).

To further explore the ranges of *TLR* population polymorphism in birds, we returned to the original description of the TLR4 function reported by Leveque et al. (2003) and their reference on *TLR4* functional

polymorphism in the domestic chicken. However, being based at the department of zoology, we selected for our investigation the traditional fancy breeds of chickens, rather than the laboratory chicken lines or the commercial flocks. Despite it was already Darwin himself who pointed out that variation in domestic fowl populations provides an excellent system for evolutionary investigation of natural and artificial selection (Darwin 1868), the domestic chicken breeds have remained virtually unstudied from the perspective of evolutionary immunology. In a long and elaborative project (again, the sequencing started before NGS technology significantly decreased the sequencing labour and costs) we sequenced CDS regions of *TLR3*, *TLR4*, *TLR5* and *TLR7* in 110 domestic chickens from 25 breeds. For each gene we detected between 19 and 38 SNVs that encoded between 22 to 70 distinct alleles (PAPER 4). Positive selection was detected previously in chicken *TLRs* (Downing et al. 2010). Interestingly, part of the variable sites that were under positive selection in our dataset were also identified previously as positively selected on interspecific level (PAPER 5) and others were neighbouring these sites (Areal et al. 2011; Grueber et al. 2014). The variation revealed in chicken TLRs was surprisingly high compared to the previously published results for other species (Alcaide and Edwards 2011; Grueber et al. 2012; where, however, much fewer individuals were sequenced). Since no sufficiently good data were available for any other animal species to compare, we compared our results with the variability in a corresponding human dataset (again 110 individuals from 25 populations). Although phylogenetically distant, this comparison makes sense, because the two species share their environment for several thousand years (Tixier-Boichard et al. 2011; Larson and Fuller 2014), facing several major joint pathogenic challenges such as avian influenza (Van Reeth 2007), salmonellosis (Guard-Petter 2001) and others (Saif 2008). We have shown that the chicken *TLRs* exhibit in average nine-times higher nucleotide diversity than human *TLRs* and increased non-synonymous variability has been found in chickens compared to humans. The contrast between the species is best documented by the difference in *TLR4* allele numbers where in the fowl there are 70 alleles, while in humans there are only 11 alleles in the entire data set (Figure 2). Therefore, the two species are clearly very differentially equipped to face the pathogens. We do not consider these differences to represent general distinctions between birds and mammals – different species may harbour different levels of genetic polymorphism. Although the adaptation through high genetic variation is

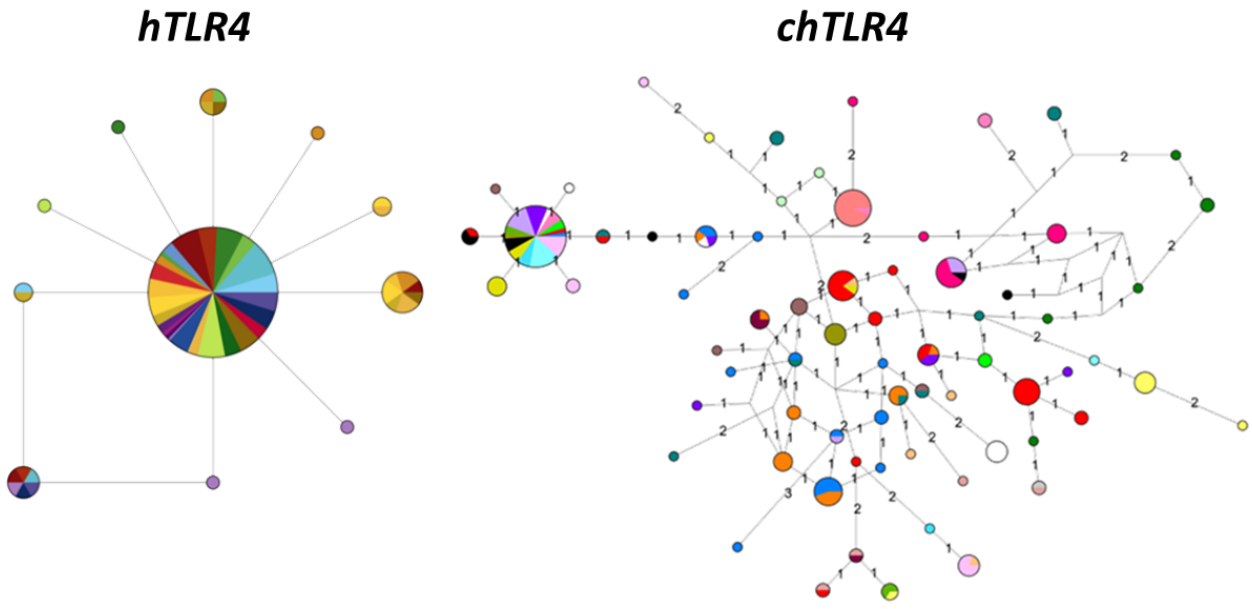

Figure 2. Comparison of *TLR4* allelic variation in humans (*hTLR4*) and chickens (*chTLR4*). Different populations (25) represented in both chickens and humans with 110 individuals in total are highlighted in both haplotype networks with distinct colours. Adapted from PAPER 4.

typical for *MHC* involved in the adaptive immunity, the fact that several sites were detected as under positive selection supports the idea of a functional importance of the intraspecific *TLR* polymorphism in chickens. While my research team together with our collaborators still devote much energy to the description of the level and functionality of the *TLR* intraspecific variation in birds (this time namely in the passerines), my description of our achievements in this direction must regrettably stop at this point: we are too slow (while hopefully not too lazy) to have the results completed and published in the desirable form (although, in my personal opinion, they are highly interesting and relevant).

Evolution of interspecific variation in avian TLRs

Despite the generally conservative nature of the MAMPs recognized by TLRs, different MAMPs still show significant structural variability across the pathogen taxa (see, e.g., Zdorovenko et al. 2007; Wang et al. 2015). For instance, it has been shown for *Yersinia pestis* cell-wall lipopolysaccharide (LPS) that phosphorylation and acylation varies in between bacterial strains (Dentovskaya et al. 2008). In *Pseudomonas aeruginosa* the bacteria can even alter the acylation state of their LPS in response to environmental changes (Hajjar et al. 2002). These changes in LPS structures are then reflected by the intensity of the immune response to infection (Hajjar et al. 2002; Dentovskaya et al. 2008). Since the difference may be dramatic (changing the ligand from immunity-activating agonist to inhibiting antagonist), in evolutionary times this pathogen-specific ligand variation can select for distinct phenotypic changes in TLR ligand-binding regions in different host lineages (Walsh et al. 2008). Before our immunogenetic research in birds started, positive selection acting at different individual sites in the TLR amino acid chains had been shown by several authors on interspecific level in mammals (Smirnova et al. 2000; Ferrer-Admetlla et al. 2008; Jann et al. 2008; Nakajima et al. 2008; Wlasiuk et al. 2009), but without much insight into the functional consequences of the genetic variation. While structural modelling has been adopted in mammals to infer some functional differences between distinct TLRs and their orthologues in different species (Kubarenko et al. 2007), most evolutionary studies concluded only with a statement on positive selection detected at certain sites.

Based on our previous work reported in PAPER 2, my colleagues and I performed an investigation of TLR4, TLR5 and TLR7 phenotypic evolution in Galloanseres (PAPER 5), an ancient clade of birds combining the Galliformes and Anseriformes orders (Jarvis et al. 2014). Galloanseres were selected as an avian clade with the highest number of species with database-reported information on *TLR* immunogenetics. The three *TLRs* were then chosen to represent both bacterial-sensing and viral-sensing TLRs (though simplified categorisation, this division proved useful) with known orthology and functional homology to their mammalian counterparts (Keestra et al. 2013). However, in birds there was virtually no functional understanding of the interspecific sequence variability observed. Thus, this study was innovative by its focus on the comparison of three-dimensional (3D) structures of the proteins, which helped in understanding biologically relevant similarities not detectable at the level of nucleotide sequence information. Using algorithms based on multiple molecular threading (Zhang 2008), we first modelled all the protein structures and then performed comparison of the shape and surface electrostatic potential features with attention being paid to the functionally relevant regions. TLRs are type I integral membrane glycoproteins that are characterized by an extracellular/endosomal ligand-binding domain (ECD) and a cytoplasmic signalling Toll/IL-1R homology (TIR) domain (Palsson-McDermott and O'Neill 2007; Figure 1). We identified pathogen-driven positively selected variability mainly in ECDs of the bacterial-sensing TLR4 and TLR5. The molecular adaptations were not found to change shapes of the tertiary-structures but altered the surface charge distributions, indicating that ligand-binding is much dependent on intensity of the charge interactions. Interestingly, the sites subjected to selection were in the bacterial-sensing TLRs frequently located either in or close to ligand-binding sites reported earlier for model species. Further comparison between human, mouse and chicken TLR4 surface-charge similarities and the resemblance of recombinant receptor responses when stimulated *in vitro*

with a TLR4 ligand panel (Keestra and van Putten 2008; Figure 3) supported the importance of surface charge for ligand-specific binding. This further strengthen our conclusions on the adaptive value of the *TLR* genetic variation and motivated much of the further research we did in this direction.

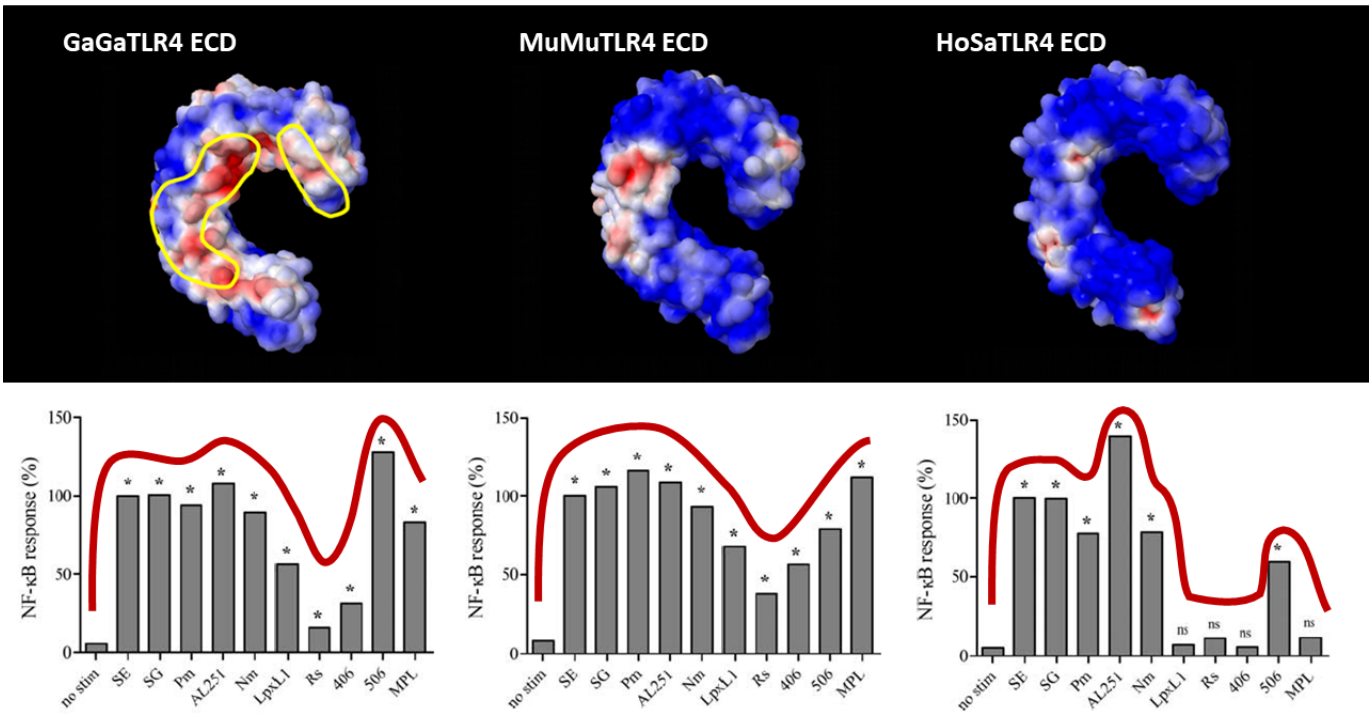

Figure 3. Distribution of the surface electrostatic potential on Toll-like receptor 4 (TLR4) ectodomain (ECD) structure and its association to ligand-binding features. Partial similarity between chicken (GaGa), and mouse (MuMu) TLR4 surface charge is visible in the ligand-binding and MD-2 dimerization region (outlined with a yellow line in the GaGaTLR4 ECD model) when compared to human (HoSa) TLR4 structure (positive surface charge is highlighted in red, negative charge in blue). This is partially mirrored by the pattern of LPS-binding specificity of TLR4/MD-2 when species-specific recombinant proteins were expressed in HeLa 57A cells and stimulated with LPS from *Salmonella enterica* serotype enteritidis (SE), *Salmonella enterica* serotype gallinarum (SG), *Pasteurella multocida* strain PBA885 (Pm), *Pasteurella multocida* ΔwaaQ (AL251), *Neisseria meningitidis* strain H44/76 (Nm), *Neisseria meningitidis* ΔLpxL1 (LpxL1), *Rhodobacter sphaeroides* (Rs), lipid A 406 (406), lipid A 506 (506), or monophosphoryl lipid A (MPL; data presented as the percentage NF-κB luciferase activity; for details see Keestra and van Putten 2008; for clarity of the comparison the pattern is highlighted with a red line). Upper line structures adapted from PAPER 5; bottom line data taken from Keestra and van Putten (2008).

The results reported in PAPER 2 suggested that *TLR4* is a relevant candidate gene to serve as a model for investigation of the host-pathogen molecular co-evolution, while the research reported in PAPER 5 indicated that *TLR5* may be a suitable alternative model to check for general validity of our conclusions. Despite belonging to the same gene family, in avian (and also mammalian) genomes these two genes (and similarly also most other TLRs) are located at different chromosomes (Cormican et al. 2009), resulting in their independent evolutionary histories. Shortly before our work in TLR5 truly started, Alcaide and Edwards (2011) reported TLR5 pseudogene in House Finch (*Haemorhous mexicanus*) and in the White-fronted Amazon (*Amazona albifrons*). We were unconvinced about this result in that time, since in their study the authors did not sequence the whole CDS of the gene, and even if the gene pseudogenisation was confirmed, we assumed this to be only part of allelic variation similar to the one known in humans (Hawn et al. 2003). Yet, TLR5 plays an important role in sensing flagellin, which is, for example, one of the main activators of gut immunity (Zeng et al. 2003; Duan et al. 2013), making any events of *TLR5* pseudogenisation a peculiar evolutionary puzzle. Wlasiuk et al. (2009) suggested that in humans the non-functional *TLR5* allele was likely maintained in the population by genetic drift which avoided the weak selection present in growing human population. However, our subsequent investigation in the passerine birds revealed broad and well-fixed pseudogenisation in the avian *TLR5* (PAPER 6). Out of 47 investigated species 18 possessed a *TLR5* pseudogene,

which (estimated based on the sequence distribution of the substitutions as well as phylogenetic relatedness) emerged at least seven times independently in the passerines. To confirm the lack of any functional copy of the gene, we checked for the *TLR5* mRNA expression pattern in four species with and four species without the *TLR5* pseudogene. Altogether, our results suggested that the non-functional *TLR5* variant is either fixed in different species or alternatively that large numbers of individuals are homozygotes in the *TLR5* pseudogene across different related species. The purifying selection may be weakened in the case of *TLR5* by the redundancy of immunity, where alternative PRRs such as the NLRC4 inflammasome may recognise bacterial flagellin independently on TLR5 (Zhao et al. 2011). The PAPER 6 reported though only a preliminary evidence. Yet, various signals had accumulated indicating potential importance of *TLR5* pseudogenisation, which highlighted that more profound research on a broader taxonomic scale was needed.

There are ten *TLRs* genes present in the chicken genome (Temperley et al. 2008). Most other avian species have maintained the TLR family in the same composition and likely also functionality. Apart from the conservative vertebrate TLRs with shared function between both birds and mammals (TLR3, TLR4, TLR5, TLR7; Iqbal et al. 2005; Schwarz et al. 2007; Keestra et al. 2008; Keestra and van Putten 2008) there are others that may share the function only partially (TLR1 family; Higuchi et al. 2008), or evolved in birds to gain completely novel functionality compared to mammals (TLR15; Higgs et al. 2006; de Zoete et al. 2011; Boyd et al. 2012; Wang et al. 2016a) or even functionally converged to other mammalian receptors (TLR21; Brownlie et al. 2009; Keestra et al. 2010). While much conserved in this way, as shown in the PAPER 6, there are species lacking functional *TLR5* and others were reported to possess duplicated *TLR7*, contributing to the avian interspecific variation. *TLR7* duplication was described first from the zebra finch genome sequence (Cormican et al. 2009) and later some authors observed the same pattern in some other species (Grueber et al. 2012; Raven et al. 2017). However, it was not clear whether the two *TLR7* copies identified in the genomes could not represent artefacts derived from missed-assembled highly divergent alleles.

Gene gain through duplication is an important mechanism for evolving novel functions and pseudogenisation may be a mechanism for avoiding certain misfunctions (Ellegren 2008; Wang et al. 2012). To improve our comprehensive understanding of the evolutionary history of avian *TLRs*, in collaboration with Dave Burt from University of Edinburgh (who in the meanwhile changed for the far-off located University of Queensland, Australia) we analysed all members of the *TLR* family to which the sequence information was available by that time. In this ambitious project my doctoral student Hana Velová screened *TLR* sequences from the whole genome assemblies and target sequence data of 63 bird species covering all major avian clades (PAPER 7). First, we attempted to disentangle the story of the widely duplicated *TLR1* gene family, where the *TLR1* and *TLR2* copies (two in both the genes) were suggested to had duplicated either before (Huang et al. 2011) or after the mammalian-avian divergence (Temperley et al. 2008; Cormican et al. 2009; Wang et al. 2016b). After controlling for the effect of gene conversion in the genes, our results showed that the gene duplication event in *TLR1* probably had occurred before the mammalian-avian divergence, which justifies re-naming avian *TLR1A* gene to *TLR10* and avian *TLR1B* gene to *TLR1*. However, despite the large amount of data collected, in the case of *TLR2* evolution it was not possible for us to arrive to any final conclusions on the validity of the two scenarios. This was partially also given by the only faint traces of the putative mammalian pseudogene of the second *TLR2* copy. Then, we focused on the *TLR7* duplication event. In the genome sequences of the investigated avian species we frequently found the two *TLR7* copies. However, the numbers of amino acid substitutions differentiating the two putative loci were low and ranged between 3 and 21. The sequence analysis as well as the PCR-based quantitative copy number variation analysis performed in selected species brought supportative evidence to *TLR7* gene duplication. Surprisingly, both copies of *TLR7* always clustered separately for each species, indicating that the duplication evens arose independently in several avian lineages. Furthermore, detecting the *TLR5* pseudogenes in Passeriformes, as well as Psittaciformes, Cariamiformes, Trogoniformes, Phaethontiformes, Eurypygiformes and Apodiformes, our study revealed the pattern of independent *TLR5* pseudogenisation in birds on much wider taxonomic scale than previously reported in our PAPER 6. Apart from this basic description of *TLR* gain and loss

in birds, we paid much attention to the analysis of diversifying selection acting in these genes. Consistent with some previous findings (PAPER 5; Wang et al. 2016b), our analysis revealed stronger positive selection acting in *TLR5* and the three-domain *TLRs* (*TLR1*, *TLR10*, *TLR2A*, *TLR2B*, *TLR4*) that encode receptors binding complex (mainly bacterial) ligands than in single-domain *TLR15* and endosomally-expressed *TLRs* (*TLR3*, *TLR7*, *TLR21*). We were able to identify many positively selected substitutions that dramatically change the amino acid physicochemical properties in the receptor proteins. Consistent with previously reported findings (Mikami et al. 2012; PAPER 5) many of the positively selected sites were located in the known functionally-relevant TLR regions and several evolved probably in convergence to similar changes in mammals. The main virtue of the comparative study presented in PAPER 7 can be seen in its unique scale providing us with increased statistical power that allowed us to formulate sufficiently strong conclusions concerning the *TLR* evolution in birds.

The pattern of convergent evolution we reported in *TLRs* (PAPER 7) may result from shared microbial communities represented in different taxa. Although our understanding of the diversity in avian microbiota is still much limited (Waite and Taylor 2015; Evans et al. 2017), the analysis of TLR phenotypic variation might indicate interspecific variation in microbe-driven selective pressures that shape the host PRR system. In principle, this was the underlying hypothesis for the study we reported in PAPER 8, where we tracked the evolution of *TLR4* in passerine birds on a sample of 55 species from several different geographical regions. We focused specifically at the ligand-binding region of TLR4 where previous research identified high levels of putatively adaptive variation (Wlasiuk and Nachman 2010; Alcaide and Edwards 2011; Areal et al. 2011; Grueber et al. 2014; Fornuskova et al. 2014; and also PAPER 2 and PAPER 5). The sequence variability we described resulted in the 3D models of the receptors in variation in the distribution of the surface electrostatic potential that could be clustered into four main distinct patterns. We succeeded in identification of the positively selected positions that determine identity of the charge clusters. Based on comparison with the previously described crystalographic structures (Kim et al. 2007; Ohto et al. 2012), some of these sites appear to directly or indirectly affect LPS binding. Similar to some of our previous studies, also in passerines we found strong consistency in the identified positively selected sites with the results reported for other vertebrates (references above), indicating generally convergent pattern of the selection between taxa. In our study we wished to go a bit further and link this immunogenetic variation with its ecological context. We predicted that ecologically similar groups of species (grouped on the basis of traits associated with parasite richness such as migration, latitudinal distribution, or diet; Schemske et al. 2009; Hannon et al. 2016) would host related microbial communities and, therefore, could be predicted to phenetically cluster based on their TLR4 surface charges. Despite some evidence for convergence among passerine taxa we were unable to find any associations between the TLR4 charge distribution and the ecological characteristics tested. Closely related species mostly belonged to the same surface charge clusters indicating that phylogenetic constraints are key determinants shaping *TLR4* adaptive evolution. This is consistent with the idea of co-speciation between hosts and their microbiota – a phenomenon known as the Fahrenholz's rule (Eichler 1948). Under this hypothesis, pathogens and their hosts continually co-adapt in a phylogenetically corresponding patterns allowing for clade-specific molecular interactions between the hosts and their parasites. This is supported by the recent finding that closely related species of avian hosts are frequently colonized by closely related microbial communities (Kropáčková et al. 2017). However, Kropáčková et al. (2017) also revealed significant effects of some ecological factors on gut microbiota in passerines and in PAPER 8 we showed only imperfect consistency between the TLR4 surface charge clustering and passerine phylogeny, suggesting some contribution of non-phylogenetic factors to TLR4 molecular evolution.

The main function of immune system is to eliminate pathogens and thus maintain host fitness high. Detection of a microbial structure (the issue we have extensively discussed in the previous sections of this habilitation thesis) alone is definitely not enough to fulfil this role (Schmid-Hempel 2011). Inflammation evolved in animals as one of the earliest immune defence effector strategies to kill the pathogens and protect the hosts (Danilova 2006). This complex immunological mechanism is directly induced by certain combinations of PRR-triggered signals involving the NF-κB signalling pathway (Kawai and Akira 2010) which is carefully regulated in its activation, intensity and termination, not to overrespond above the optimal level (Newton and Dixit 2012; Sugimoto et al. 2016). Given that ongoing inflammation brings multiple important costs to the host (Ashley et al. 2012), uncontrolled inflammation may lead to important effects on health and fitness. Immunity evolved to mostly prevent these costs. Activation of inflammation is possible only when MAMP-triggered signal combines with the DAMP-triggered signal informing the host about danger-associated tissue damage (the Danger hypothesis; Matzinger 2002; Piccinini and Midwood 2010; Figure 4). This allows the host not to respond to commensal microbes and other non-pathogenic organisms inhabiting the host body, many of which are equally essential for homeostasis maintenance as the immune system itself (Theis et al. 2016). In the case of pathogen incursion the induced inflammation propagates in the infected tissue where microbial cells are opsonized, phagocyted and killed by macrophages to clear

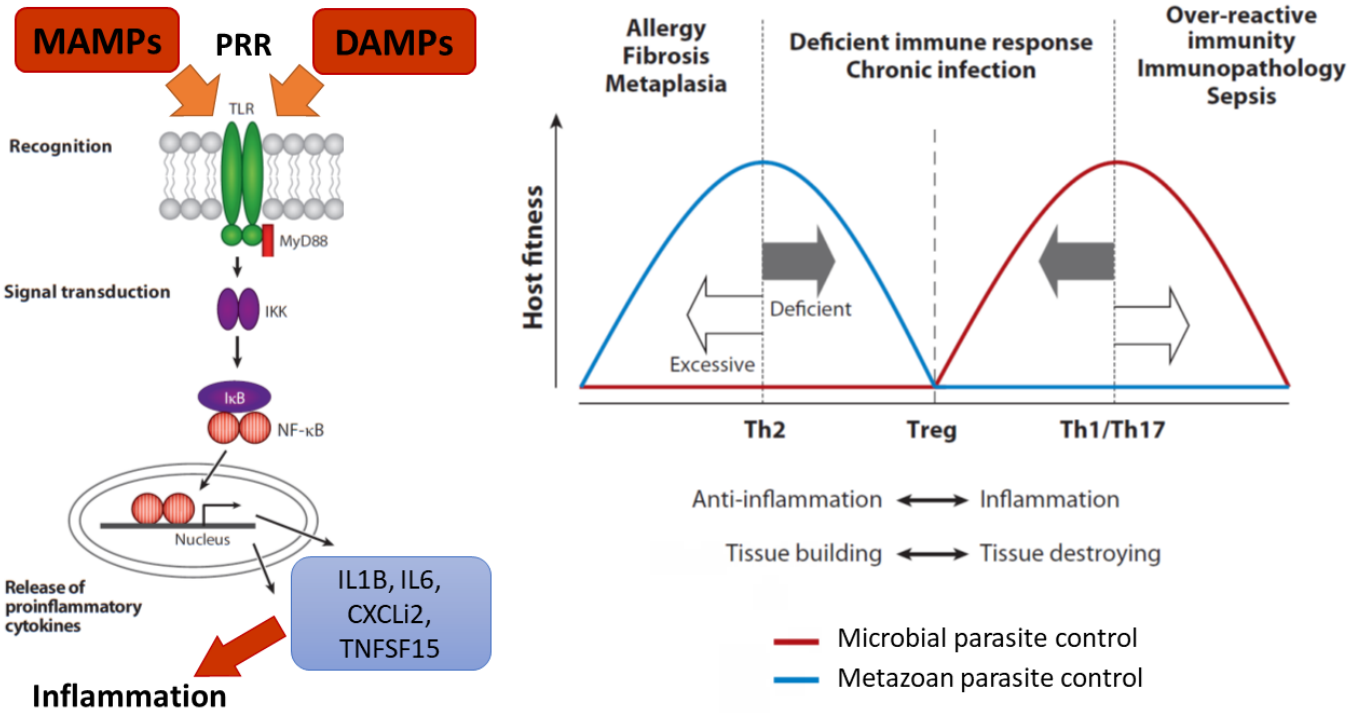

Figure 4. Initiation of inflammation through detection of microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) by Pattern-recognition receptors (PRRs) and balance mediated in pro- and anti-inflammatory immunological regulation. Pathogens induce inflammation through interaction of their ligands (MAMPs) with PRRs (including Toll-like receptors, TLRs) and damage (DAMPs). This triggers a signalling pathway that activates transcription factors (namely NF-κB) in immune cells initiating pro-inflammatory cytokine signalling (involving e.g. IL1β, IL6, CXCLi2 or TNFSF15), which then elicits inflammation (left-hand scheme). Host fitness is evolutionarily optimised for certain level of pressure from different types of pathogens to which certain balance between pro-inflammatory and anti-inflammatory responses is efficient (right-hand scheme). Inflammation regulated namely by Th1 or Th17 cells (red line) is more efficient in controlling microbes (e.g. viruses, bacteria, fungi), while antibody (anti-inflammatory) response regulated by Th2 cells (blue line) is more efficient in controlling large metazoan parasites (helminths, arthropods). Tregs contribute to regulation of this balance. Excessive and deficient responses that decrease host fitness are represented by white and grey arrows, respectively (Th – T helper cells, Treg – regulatory T cells). Please note that the scheme depicting the regulation is highly simplified; adapted from Ashley et al. (2012).

the infection (Ezekowitz 2002). During this process, individual cells are regulated by various signals which include cell-to-cell receptor-ligand interactions as well as signalling through soluble signalling molecules, namely cytokines (Ashley et al. 2012). The eventual microbe killing is associated with production of highly reactive oxygen and nitrogen species (ROS and RNS, respectively) which may alter the local redox balance in the inflamed tissue. If the clearance of the infection fails or the immune response targets inappropriate stimuli, the inflammation-associated tissue damage may give rise to various chronic inflammatory diseases such as metabolic syndromes and autoimmunity (Nathan and Ding 2010; Figure 4B). The formation of the involved immunological components as well as their induction also recruits resource (including micronutrients which many be limited) and energy stores that, hence, cannot be utilised elsewhere, e.g. in growth or reproduction. This trade-off is believed to represent a basic principle of the ecological immunology (Sheldon and Verhulst 1996).

Ecological immunology (or ecoimmunology) develops classical immunology towards a focus on the immune function acting in a changeable ecological and evolutionary context (Adelman et al. 2014). However, by its origin this discipline derived from ecology rather than from immunology (therefore termed by some as immunoecology; Seed 1993; Hasselquist 2007), which determined not only its scope and concepts, but also its terminology, methodology and choice of biological systems that serve as models for the investigation. In 1990s when ecological immunology started to emerge (Folstad and Karter 1992; Seed 1993) the interest of the mainstream immunologists shifted mainly towards biochemical regulation of immune defence, leaving questions concerning the ecology of immune defence vastly neglected. At the same time the interest of ecologists studying host-parasite interactions grew towards the research on host fitness and its maintenance (Hasselquist 2007). With limited immunological background, especially many ornithologists began to conduct ecoimmunological research, building the conceptual framework of the novel discipline without arousing much interest among classical immunologists (Seed 1993). Hence, with few exceptions (e.g. Millet et al. 2007), the dialogue between immunologists and ecologists remained insufficient, which necessarily deepened the misunderstanding between both groups of scientists. To contribute to strengthening this dialogue, Tomáš Albrecht and I analysed the meaning of the pivotal term of ecoimmunology of that time, the 'immunocompetence', to show that it is being used differently both between different ecological studies (Norris and Evans 2000; Owen et al. 2010) as well as between immunologists and ecologists (PAPER 9). This is partially because ecologists frequently operated with a fuzzy concept of general resistance to all pathogens, which is, given regulatory trade-offs within immune defence activation (e.g. Walsh and Mills 2013), a non-existing immunological feature. It appears that the hosts usually cannot adapt equally well to all types of parasites if the intensity of different reciprocally negatively regulated immune responses is dependent on the interaction between host's variable immunogenotype and intruding pathogen variability. Therefore, the relative ability of the host to control infections varies for different pathogens (Viney et al. 2005). How much our article (PAPER 9) served its mission and contributed to the general understanding of these relationships is difficult to judge, yet since its publication the term 'immunocompetence' started to withdraw from ecological literature, being replaced with more precise terminology.

Another problematic aspect of the ecoimmunological investigation is the methodology. Given the molecular tool specificity, requirements on rapid or clean sample processing or high costs, many of the precise immunological methods are frequently inaccessible for the use in heterogeneous wild non-model organisms studied in the field, which are the relevant study subjects for ecological research. Thus, the list of immunological methods that are available for field zoological research is relatively short (Millet et al. 2007; Adelman et al. 2014). Furthermore, the unclear immunological background of several of the remaining tests of immune function often drove ecologists into some misinterpretation of the collected data. It is my sincere hope that our research focused at the characterisation of the immune response behind the phytohaemagglutinin (PHA) skin-swelling test contributed to strengthening of the methodological framework of ecological immunology with a robust interpretational background to this method. The PHA skin-swelling test (Kennedy and Nager 2006) belongs among methodological approaches that have received wide popularity

in ecological immunology thanks to its general applicability and procedural simplicity. This test was originally developed in early medical (Airo et al. 1967) and veterinary (Goto et al. 1978) research as a screening method for *in vivo* immunosuppression. Two decades later this method was adopted in ecological research and has been used for another two decades as a measurement of the cell-mediated immunity in many different vertebrates (Hernandez et al. 2005; Goüy de Bellocq et al. 2006; Hawley et al. 2009; Griggio et al. 2010; Brown et al. 2011). Although generally interpreted as a measurement of the T-cell responsiveness ('T-cell mediated immunocompetence'), our study of the zebra finch responses to different PHA isolectins showed that the inflammation induced by PHA *in vivo* cannot be interpreted as a T-cell specific response (PAPER 10). PHA is a plant lectin with a known T-cell-mitogenic function (Nowell 1960). Yet, it has been shown that in the tetrameric structure of this molecule there are subunits that do not bind and activate T cells (Yachnin and Svenson 1972; Leavitt et al. 1977) and instead cause erythroagglutination (Rigas and Osgood 1955). Our results showed that the isolated erythroagglutinating subunit (PHA-E) triggers stronger response when subcutaneously injected into the wing web (patagium) of zebra finches than the lymphoproliferating subunit (PHA-L), indicating that the immune response is independent of the direct T-cell activation by PHA. Thus, together with the previously published analysis of the histological composition of the inflamed tissue (Martin et al. 2006) our study helped to recognise the non-specific nature of the inflammatory response, refuting interpretations of other studies in the field (Tella et al. 2008). Although this interpretational shift may appear relatively minor from the perspective of ecology, it is undoubtedly important from the immunological point of view: not only we moved the attention from adaptive immunity (T cells) towards innate immunity (inflammation), but we also contributed to better understanding of the nature of existing trade-offs between immune defence and reproduction. These can be predicted to reflect more the redox balance of the organism in the case of inflammatory response, which is associated with oxidative burst (Ashley et al. 2012).

Avian inflammation

Although our experimental and histological work reported in PAPER 10 improved our understanding of the immunological processes investigated in ecoimmunological research, still there has been a dramatic methodological gap between the immunological and ecological research. I fully realised this when, thanks to kind hospitality and attentiveness of Pete Kaiser and Lisa Rothwell, I was allowed to learn some of the immunological approaches myself: first in 2009 at the Institute for Animal Health, Compton, UK and later in 2013 at the Roslin Institute, Edinburgh, UK. Their research in 'chickenology' (a joke term used by Pete to refer to avian immunology conducted in chickens) motivated my desire to understand the regulatory processes within the inflamed tissues of the free-living birds we investigated in field. To optimally protect the host, immune response is carefully regulated to achieve balance between immunity and immunopathology (Graham et al. 2005). During inflammation in vertebrates this balance is mediated by pro-inflammatory and anti-inflammatory cytokine signalling (Ashley et al. 2012; Figure 4). Over the years of the systematic immunological research mainly in chickens, the regulatory function of many avian cytokines has been described (Kaiser 2007; Kaiser and Stäheli 2014). This knowledge allowed us to basically describe some of the processes involved in regulation of the immune response to PHA in grey partridge (PAPER 11), where we assessed the cytokine expression profile of the skin inflammation. Since we did not find any differential expression of *IL2* (the T-cell growth factor), our results brought further support to the conclusion that T-cell proliferation does not contribute to the immune response measured in the PHA skin-swelling test. On the other hand, we detected differential expression in the general pro-inflammatory cytokines (*IL1B*, *IL6*), that are involved in directing the Th17 response, and anti-inflammatory (*TGFB*) cytokines, which supported the evidence for non-specific type of the inflammatory immune response. Surprisingly, we did not find any strong link between the swelling response and the cytokine expression in the tissue, suggesting that the immunological regulatory processes do not manifest into the metrically measurable outer traits that are typically measured in the ecological research. To my knowledge, this study together with the one by Adelman et al. (2013b) were

the first to investigate pro-inflammatory cytokine signalling in free-living birds.

The bi-annual Avian Immunology Research Group Meeting is really a great melting pot of the immunological research in birds. It is a big shame that this meeting is typically not attended by any researchers devoted to ecological immunology. When I first attended this meeting in 2010 when the conference was held in Budapest, Hungary, I chanced to meet there Karl A. Schat from the Cornell University, Ithaca, USA. He was actually the only other person at that conference except for myself who was not presenting a research conducted in chickens, ducks or turkeys, but in passerines. As a professor emeritus Ton Schat was by that time slowly retreating from the active research, but (being still very much involved) he encouraged me to write to Dana M. Hawley from Virginia Tech, Blacksburg, USA and discuss with her possibilities for collaboration. Although I did this immediately, it took us many years and several unsuccessful grant applications before we found a way how to truly collaborate in our research. This chance came in 2015 when I succeeded in the Fulbright Visiting Scholar Program with a project titled 'Cytokine expression in *Mycoplasma gallisepticum* infected tissue in house finches (*Haemorhous mexicanus*)' and was awarded a scholarship that covered my eight-month research in Dana's lab.

The house finch is a sparrow-sized omnivorous North American passerine belonging to the family Fringillidae which can be presently recognised as one of the key model species of avian evolutionary ecology (Hill 2002). *Mycoplasma gallisepticum* is then a small bacterium which is, lacking a cell wall, resistant to various antibiotic treatments. The bacterium represents an economically significant horizontally transferred poultry pathogen which infects mainly the upper respiratory tract, decreasing agricultural production (Ley 2008). In 1990s a novel strain of this pathogen rapidly mutated to colonise house finch as its novel host (Dhondt et al. 2005). In house finches, the symptoms of the disease are different from those in poultry: the infected passerines show swelling and lesions in the ocular area. This malady known as mycoplasmal conjunctivitis importantly decreases survival of the house finches (Faustino et al. 2004). The pathogenic bacterium further rapidly evolves in the host (Hawley et al. 2013) and transmission to other passerine species has been recorded (Luttrell and Fischer 2007). Thanks to foresightedness of the American researchers a wide spectrum of isolates from different time points and geographic areas has been collected over the past two decades and these are presently available at the *Mycoplasma* Diagnostic and Research Laboratory of the North Carolina State University College of Veterinary Medicine for experimental research (Ley et al. 2016), enabling direct investigation of an ongoing host-parasite coevolution. Namely two *M. gallisepticum* isolates that differ in their co-evolutionary history with the host were recognised to cause conjunctivitis with a distinct course of the illness: the most original VA1994 isolate causing a more rapid and milder self-healing form of the disease, and the evolved NC2006 isolate which more frequently induces a chronic disease (Grodio et al. 2012). While much research has focused on the description of the dynamics of the epidemic (Hochachka and Dhondt 2000; Dhondt et al. 2005), relationships between host phenotypic traits and probability of recovery (Hill and Farmer 2005) or description of the pathogen, its transmission and virulence evolution (Hawley et al. 2013; Adelman et al. 2013a; Fleming-Davies et al. 2018), only a limited number of studies have investigated the immunology of the host. Those studies show that house finches do respond to *M. gallisepticum* by antigen-specific antibody production (Grodio et al. 2009), which, however, does not appear to be protective (Grodio et al. 2013). Furthermore, during mycoplasmal conjunctivitis the haematological (Davis et al. 2004) and gene expression (Wang et al. 2006) profile of the host is altered. Importantly, Adelman et al. (2013b) provided first evidence of the association between M. gallisepticum infection and inflammation in house finches. In my project conducted at the Virginia Tech I focused on the variation in expression profiles of key pro-inflammatory and anti-inflammatory cytokines during the course of the *M. gallisepticum* infection across fifteen different tissues. In PAPER 12 we have reported the development of seven new probe-based one-step RT-qPCR assays to investigate mRNA expression of house finch cytokine genes (*IL1B*, *IL6*, *IL10*, *IL18*, *TGFB2*, *TNFSF15* and *CXCLi2*, syn. *IL8L*). We used these assays to describe differences in tissue-specific cytokine expression during *M. gallisepticum* infection with the original VA1994 and evolved NC2006 isolates. Our results indicated strong pro-inflammatory cytokine expression during

the infection especially in the periocular tissues (the nictitating membrane, conjunctiva and the Harderian gland) where the more derived and virulent isolate NC2006 triggered stronger inflammatory response than VA1994. Consistently with the situation in poultry (Bradbury 2005), the overly strong inflammation was linked to pathology: the extent of pro-inflammatory *IL1B* signalling was correlated with conjunctival loads of the bacteria (Figuer 5) and the extent of clinical signs of conjunctivitis. Thus, it appears that *M. gallisepticum* evolves in the house finch to trigger stronger inflammation to facilitate its transmission, which is in agreement with some previous reports suggesting that the passerine-specific lineage of *M. gallisepticum* adapts to its passerine host with increasing virulence (Hawley et al. 2013; Osnas et al. 2015; Figure 5), which, however, weakens its virulence for the original poultry host (O'Connor et al. 1999; Pflaum et al. 2017; Figure 5). Since the house finch populations with distinct evolutionary history with the pathogen seemingly differ in their responsiveness to the mycoplasmal infection (Bonneaud et al. 2011, 2012; Adelman et al. 2013b), future research should tell us more about the precise adaptations optimising the balance in their inflammatory signalling.

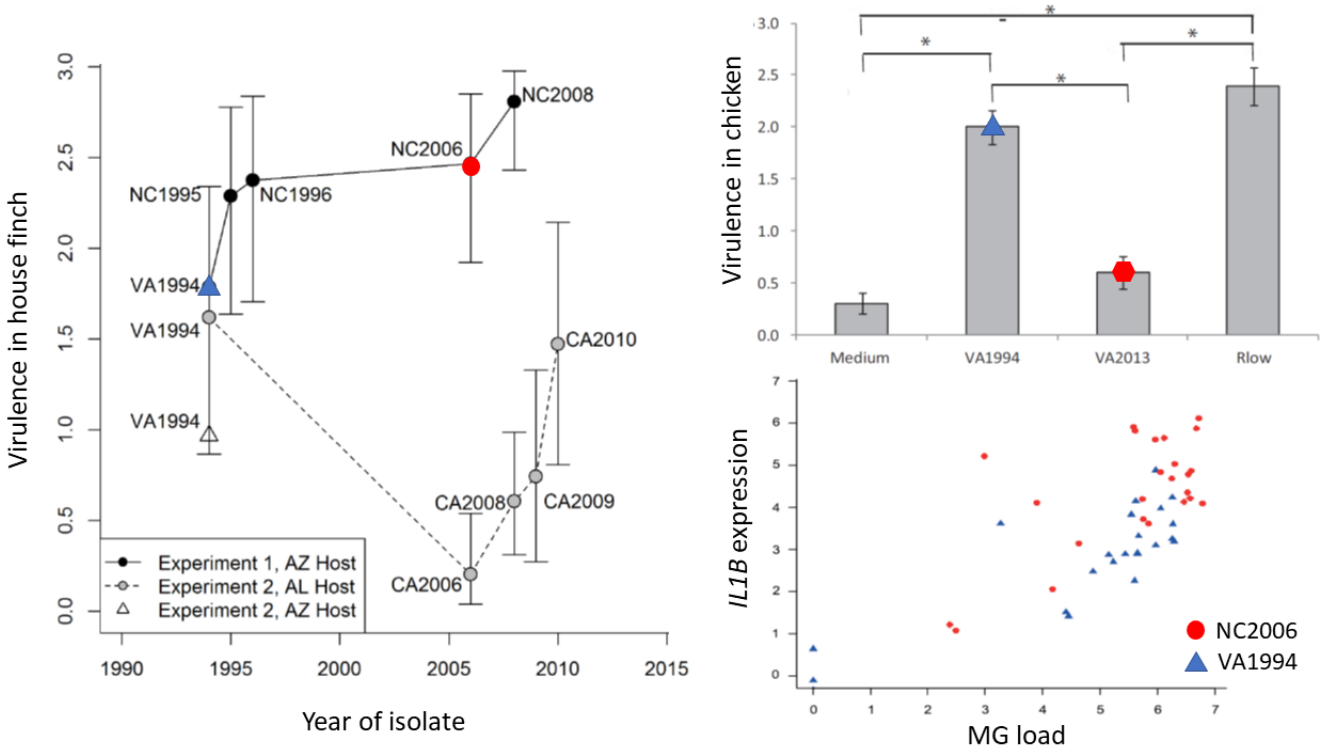

Figure 5. Evolution of inflammation-related virulence is *Mycoplasma gallisepticum*. In house finch, the average virulence index (calculated based on the intensity of periorbital pathology) for the *M. gallisepticum* isolates grow with evolutionary time (left-hand graph; the research was done with two host populations: AZ = Arizona, AL = Alabama). In poultry, in contrast, the virulence (assessed based on tracheal lesions) of the house finch *M. gallisepticum* isolates decrease with evolutionary time (right-hand top graph; error bars indicate standard errors of the means and asterisks indicate significantly different groups; Medium = control, Rlow = poultry strain of MG). Our results indicate that *M. gallisepticum* adapted to house finch by triggering stronger pro-inflammatory response, measured as *IL1B* expression in internal eyelid (right-hand bottom graph). MG isolates: blue circle = original VA1994; red triangles = evolved NC2006; red hexahedron = evolved VA2013. Adapted from Hawley et al. 2013 Pflaum et al. 2017 and PAPER 12.

Traits associated with inflammatory responsiveness and general health in birds

While most forms of inflammation investigated in the ecoimmunological research represent a non-lethal local response, the progression of the inflammation is dependent on systemic organismal factors and simultaneously this inflammation affects the systemic homeostasis. This is given by the fact that inflammation is a costly process which consumes resources and hence it is tightly linked to condition of the organism (Ashley et al. 2012). Strategies for resolving trade-offs arising from resource and energy allocation into pathogen resistance and investments into growth, reproduction and other life-history traits are of a paramount interest of the ecoimmunological research (Sheldon and Verhulst 1996; Adelman et al. 2014). The Indicator model of sexual selection postulates that the opposite sex can use ornaments to assess the health status of their bearers. Much of the ecoimmunological research has focused on carotenoid-based ornaments as model systems for testing this hypothesis (Lozano 1994). According the Hamilton and Zuk hypothesis (Hamilton and Zuk 1982; Figure 6), individuals with elaborately developed ornaments are predicted to be in good condition and health state, which may result from some genetic advantage decreasing their susceptibility to parasites ('good genes'). Since a considerable part of the variability in disease resistance is highly hereditable, determined by immune genes (such as *MHC* genes or *TLRs*), sexual selection allowing increasing in frequency of advantageous protective alleles seems to be a significant mechanism accelerating selective sweeps or maintaining balancing selection that is promoting genetic diversity in host-parasite co-evolution (Woolhouse et al. 2002).

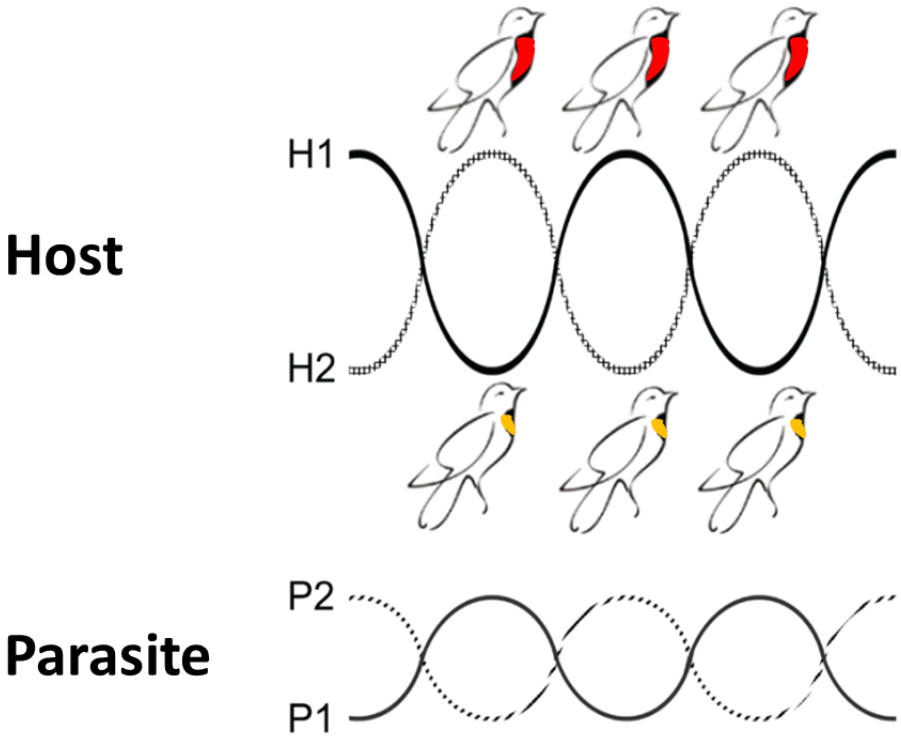

Figure 6. Parasite-mediated sexual selection. A schematic representation of cyclical changes in host (H1, H2) and parasite (P1, P2) allele frequencies. Parasites possessing alleles compatible to host genotype (P1 to H1 and P2 to H2) are more efficient in infecting the host, thus lowering its condition and ornamentation. Therefore, the level of ornament expression reflects the infection status and contemporary protective advantage of the host genotype. Allele frequencies in host and its parasite change in cycles. This is based on contemporary selection advantage of host genotype increasing the allele frequency and subsequent spread of parasite genotype co-adapted to this host genotype and decreasing the allele frequency. Scheme designed according to the model proposed by Hamilton & Zuk (1982); adapted from Vinkler et al. (2011).

In my research, I attempted to test the Indicator hypothesis both in passerine (PAPER 13) and galliform (PAPER 14, PAPER 15 and also PAPER 11 mentioned above) birds, where we used the PHA skin-swelling test to investigate how the peripheral pro-inflammatory responsiveness was related to the condition-dependent ornamental colouration. In passerines the research was oriented at scarlet rosefinches in which the male breast plumage colour ranges from yellow to red (PAPER 13). Surprisingly, in terms of the interpretational framework of that time and unlike in the galliform grey partridges we studied (PAPER 11, PAPER 15), in the rosefinches we showed negative association between the plumage ornamentation and inflammatory responsiveness. This is, the inferior males and not the superior ones mounted stronger immune responses. Such a result apparently contrasted much of the research previously published (e.g. Blount et al. 2003; Faivre et al. 2003; Mougeot 2008) and also the general good-genes concept (Hamilton and Zuk 1982), while it was potentially partially consistent with the immunosuppression effect predicted by the Immunocompetence handicap hypothesis (Folstad and Karter 1992; Sheldon and Verhulst 1996). To better understand the possible mechanism of this relationship, we performed a histological analysis of the inflamed tissue in part of the study individuals and revealed that the increase in magnitude of the swollen tissue, which is measured in the test, is dependent on basophil infiltration. In birds, the immune response to PHA is sometimes (though rarely) termed as an cutaneous basophil hypersensitivity (Koutsos et al. 2007), given the large infiltration of basophiles into the stimulated skin (Goto et al. 1978; McCorkle et al. 1980; Martin et al. 2006). Highly ornamented healthier individuals recruited fewer basophils into the inflamed tissue, which resulted in less intensive swelling response. However, such a mechanistic relationship does not necessarily disprove the Hamilton-Zuk hypothesis or support the Immunocompetence handicap hypothesis (as suggested by some authors; Gonzalez et al. 1999), because, unfortunately, the methods that are commonly used to measure immune responsiveness may not differentiate the adaptations for immunity to act against pathogens from the actual activation state of immune system caused by the pathogens (health).

To better understand the health effects on inflammatory responsiveness in passerine birds, we performed a simple experiment in which we described the relationship between changes in haematological traits (peripheral blood leukocyte composition before and after stimulation), tissue leukocyte infiltration and strength of the inflammation after a PHA subcutaneous stimulation in zebra finches (PAPER 16). We were able to show that the course and intensity of the skin inflammatory response is dependent on the haematological state of an individual – higher initial basophil and lymphocyte frequencies were associated with an increased swelling response. Particularly, the lymphocytes were more readily recruited into the inflamed tissue when represented in blood in higher proportions. Contrariwise, the PHA-induced local inflammation changes the frequencies of blood-borne leukocytes. This appears to correspond with the dynamics of leukocyte trafficking and alterations in haematopoiesis in response to stimulation (mainly in basophils). Since both lymphocytes (more frequent in blood of healthy individuals) and basophils (more frequent in blood of diseased individuals) have promoted the inflammatory response, our results suggest two different pathways linking the inflammatory response and health. Either healthier individuals may respond stronger to stimulation due to their increased capacities to mobilise lymphocytes (as observed e.g. by Horak et al. 1999; Dlugosz et al. 2014 or in our PAPER 15) or alternatively diseased individuals may respond stronger due to their increased activation of pre-stimulated basophils (e.g. PAPER 13). It is also important to realise that different species differ in their frequencies of leukocyte types represented in the peripheral blood (Campbell and Ellis 2007). One of our recent studies (PAPER 17) that applied a newly adjusted method of flow cytometry to quantify the avian blood-borne cells indicates that even different populations of the same species (in this case the domestic chicken breeds) may vary in their blood cellular composition, despite common rearing environment. Therefore, we may assume that also different populations of free-living species may significantly differ in their haematological traits, affecting the capacity of the individuals to mount inflammatory responses. In any case, our PAPER 16 suggested a strong effect of the haematological variability on the mode of avian inflammation measured in ecoimmunological research and we believe that our results justify the requirement of haematological data accompanying the PHA skin-swelling test and related methods.

In scarlet rosefinch we performed a complex haematological screening to evaluate the health state of the birds in our study population. This research indicated an interesting aspect of the rosefinch immunology: most individuals showed increased levels of peripheral basophils (PAPER 18). This is very unusual in birds or mammals (Campbell and Ellis 2007), although it appears that several passerine birds may have generally higher basophil levels (http://wildlifehematology.uga.edu/). Contrary to mammals, avian basophils are important cells involved in various inflammatory responses (Maxwell and Robertson 1995). In rosefinches we have shown a positive association between blood peripheral basophils and *Haemoproteus* infection status (PAPER 18). This trend is not exceptional - similar one was observed, e.g., by Garvin et al. (2003) in blue jays (*Cyanocitta cristata*). Our results thus indicate that the ornamentation-associated inflammatory responsiveness may be eventually linked to blood borne diseases. Therefore, the results of our study appear to support the Indicator model and are consistent with the 'good genes' hypothesis proposed by Hamilton and Zuk in 1980s (Hamilton and Zuk 1982).

However, health-related traits may also be linked to cryptic sexual selection occurring on the level of gamete production, their survivorship and fertilisation success (Jennions and Petrie 2000). It has been even predicted that ornamental traits may serve to females to identify functional fertility in males (the phenotype-linked fertility hypothesis, Sheldon 1994), which may be advantageous especially in cases where females do not randomly copulate with multiple males. We have tested the relationships between carotenoid- and melanin-based plumage ornaments, haematological traits and sperm morphological traits in great tit males (PAPER 19). In passerines it has been shown that increased sperm competition leads to a decrease in variation of sperm length within ejaculates (Kleven et al. 2008; Immler et al. 2008). This is likely because sperm selection favours optimal sperm lengths. Our results support the association between sperm traits and blood cellular composition, suggesting that either individuals maintaining low sperm variability may afford to invest more into heterophil production or there could be a trade-off between individual investment into reproduction (ejaculate quality) and long-term physiological stress (indicated by increased heterophil frequencies). However, unlike for example Helfenstein et al. (2010), we were unable to identify in great tits any association between sperm-related traits and male ornament expression.

The haematological traits are in birds indicative of both condition changes and long-term stress (Davis et al. 2008). Thus, there is a strong environmental component in the inter-individual variation in these traits (Krams et al. 2011; Frigerio et al. 2017). Urban environmental pollution results in contamination of synanthropic organisms with health-impairing toxic trace elements. This pattern is observable especially in birds that may serve as bioindicators of such pollution. In various experimental setups and correlational studies, organic and especially inorganic pollutants have been shown to affect blood cellular composition in birds (e.g. Llacuna et al. 1996; Geens et al. 2010; Jara-Carrasco et al. 2015; Cid et al. 2018). To elucidate the urban environmental pollution effects on health-related traits in great tits, we performed an association study across 13 cities in the Czech Republic, linking heavy metal contamination (lead Pb, cadmium Cd, copper Cu, chromium Cr, and arsenic metalloid As) with selected haematological traits (PAPER 20). Our results showed that males with higher concentrations of heavy metals in blood had a lower heterophil/lymphocyte ratio, assumingly due to the direct toxicity of heavy metals in heterophils. Furthermore, we found decreased absolute erythrocyte count (a trait indicative of anaemia) in these males. These results partially differ from those recently reported by Cid et al. (2018) in house sparrows, where Pb intoxication have not resulted in any decrease in heterophil frequencies (although haematocrit as a measure of the erythrocyte count was decreased in birds receiving the highest Pb doses). While correlational in their nature, our results indicate that despite current strict regulation on air pollution that have allowed general improvements in atmospheric heavy metal contamination, on a large geographic scale non-degradable heavy metals persistently contaminate animal bodies at levels that may have physiological effects related to immune function and health.

INTERIM CONCLUSION AND CRITICAL SELF-REFLECTION

Here, we have reached the final point of the overview introductory part of the habilitation thesis and some critical self-reflection would be probably pertinent. Looking back at the past decade of my (more or less) independent investigation, I find certain satisfaction in the focus and breadth of the research. My colleagues and I have contributed to strengthening the basis of the field of evolutionary and ecological immunology conceptually as well as methodologically, revealing quite many interesting and relevant facts about avian immune system evolution and its functioning under changeable environmental conditions. We have shown the diversity of putative adaptations in certain immune genes in birds, attempting to indicate their functional meaning. We have described specific regulatory mechanisms of avian inflammation, an immunological double-edged sword that is harmful not only to the pathogens, but also to the host. We have also identified condition-dependent traits associated with inflammation and haematological traits in birds, showing the ecological variation in these traits. Unfortunately, many relevant results we gained (even from the early days of my professional career) have not yet been published, waiting for their finalization. Most importantly, the link between immunogenetic variation and the immunological performance of avian individuals in changeable environmental context is still elusive, waiting for its description. This is an ambitious task which is, nevertheless, the only logical outcome of the work we have been doing so far. I sincerely hope that adopting this goal as an outline for the future research will take us to new and exciting discoveries that will promote the field of evolutionary and ecological immunology and also our active role in its global development.

REFERENCES

Own publications included into the thesis

PAPER 1: Vinkler M. & Albrecht T. (2009): The question waiting to be asked: Innate immunity receptors in the perspective of zoological research. Folia Zoologica 58 (Suppl. 1): 15–28

PAPER 2: Vinkler M., Bryjová A., Albrecht T. & Bryja J. (2009): Identification of the first Toll-like receptor gene in passerine birds: TLR4 orthologue in Zebra finch (*Taeniopygia guttata*). Tissue Antigens 74: 32-41; DOI: 10.1111/j.1399-0039.2009.01273.x

PAPER 3: Vinkler M., Bainová H., Bryjová A., Tomášek O., Albrecht T. & Bryja J. (2015): Characterization of Toll-like receptors 4, 5 and 7 and their polymorphism in grey partridge. Genetica 143: 101–112; DOI: 10.1007/s10709-015-9819-4

PAPER 4: Świderská Z., Šmídová A., Buchtová L., Bryjová A., Fabiánová A., Munclinger P. & Vinkler M. (2018): Avian Toll-like receptor diversity far exceeds human polymorphism: an insight from domestic chickens. Scientific Reports 8: 17878; DOI: 10.1038/s41598-018-36226-1

PAPER 5: Vinkler M., Bainová H. & Bryja J. (2014): Protein evolution of Toll-like receptors 4, 5 and 7 within Galloanserae birds. Genetics Selection Evolution 46: 72; DOI: 10.1186/s12711-014-0072-6

PAPER 6: Bainová H., Králová T., Bryjová A., Albrecht T., Bryja J. & Vinkler M. (2014): First evidence of independent pseudogenization of Toll-like receptor 5 in passerine birds. Developmental and Comparative Immunology 45(1): 151–155; DOI: 10.1016/j.dci.2014.02.010

PAPER 7: Velová H., Gutowska-Ding M. W., Burt D. W. & Vinkler M. (2018): Toll-like receptor evolution in birds: gene duplication, pseudogenisation and diversifying selection. Molecular Biology and Evolution 35(9): 2170–2184; DOI: 10.1093/molbev/msy119

PAPER 8: Králová T., Albrecht T., Bryja J., Hořák D., Johnsen A., Lifjeld J.T., Novotný M., Sedláček O., Velová H. & Vinkler M. (2018): Signatures of diversifying selection and convergence acting on passerine Toll-like receptor 4 in an evolutionary context. Molecular Ecology 27: 2871-2883. DOI: 10.1111/mec.14724

PAPER 9: Vinkler M. & Albrecht T. (2011): Handling 'immunocompetence' in ecological studies: Do we operate with confused terms? Journal of Avian Biology 42: 490-493. DOI: 10.1111/j.1600-048X.2011.05499.x

PAPER 10: Vinkler M., Bainová H. & Albrecht T. (2010): Functional analysis of the skin-swelling response to phytohaemagglutinin. Functional Ecology 24: 1081-1086; DOI: 10.1111/j.1365-2435.2010.01711.x

PAPER 11: Vinkler M., Svobodová J., Gabrielová B., Bainová H. & Bryjová A. (2014): Cytokine expression in phytohaemagglutinin-induced skin inflammation in a galliform bird. Journal of Avian Biology 45: 43–50; DOI: 10.1111/j.1600-048X.2011.05860.x

PAPER 12: Vinkler M., Leon A. E., Kirkpatrick L., Dalloul R. A. & Hawley D. M. (2018): Differing house finch cytokine expression responses to original and evolved isolates of *Mycoplasma gallisepticum*. Frontiers in Immunology 9: 13. DOI: 10.3389/fimmu.2018.00013

PAPER 13: Vinkler M., Schnitzer J., Munclinger P. & Albrecht T. (2012): Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Animal Behaviour 83: 17-23; DOI: 10.1016/j.anbehav.2011.10.001

PAPER 14: Svobodová J., Gabrielová B., Synek P., Maršík P., Vaněk T., Albrecht T. & Vinkler M. (2013): The health signalling of ornamental traits in the grey partridge (*Perdix perdix*). Journal of Ornithology 154: 717–725. DOI: 10.1007/s10336-013-0936-5

PAPER 15: Svobodová J., Gabrielová B., Hyršl P., Albrecht T. & Vinkler M. (2016): Melanin and carotenoid ornaments are related to the individual condition in free-living grey partridges (*Perdix perdix*). Journal of Ornithology 157: 1007–1015; DOI: 10.1007/s10336-016-1349-z

PAPER 16: Bílková B., Vinklerová J. & Vinkler M. (2015): The relationship between health and cell-mediated immunity measured in ecology: Phytohaemagglutinin skin-swelling test mirrors blood cellular composition. Journal of Experimental Zoology Part A 323:767–777; DOI: 10.1002/jez.1990

PAPER 17: Bílková B., Bainová Z., Janda J., Zita L. & Vinkler M. (2017): Different breeds, different blood: cytometric analysis of whole blood cellular composition in chicken breeds. Veterinary Immunology and Immunopathology 188: 71–77; DOI: 10.1016/j.vetimm.2017.05.001

PAPER 18: Vinkler M., Schnitzer J., Munclinger P., Votýpka J. & Albrecht T. (2010): Haematological health assessment in a passerine with extremely high proportion of basophils in peripheral blood. Journal of Ornithology 151 (4): 841–849; DOI: 10.1007/s10336-010-0521-0

PAPER 19: Svobodová J., Bauerová P., Eliáš J., Velová H., Vinkler M. & Albrecht T. (2018): Sperm variation in Great Tit males (*Parus major*) is linked to a haematological health-related trait, but not ornamentation. Journal of Ornithology 159: 815–822. DOI: 10.1007/s10336-018-1559-7

PAPER 20: Bauerová P., Vinklerová J., Hraníček J., Čorba V., Vojtek L., Svobodová J. & Vinkler M. (2017): Association of urban environmental pollution with health-related physiological traits in a free-living bird species. Science of the Total Environment 601–602: 1556–1565; DOI: 10.1016/j.scitotenv.2017.05.276

Other references

Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? Trends Ecol Evol 21:433–438

Adelman JS, Ardia DR, Schat KA (2014) Ecoimmunology. In: Schat KA, Kaspers B, Kaiser P (eds) Avian Immunology. Academic Press, San Diego, pp 391–411

Adelman JS, Carter AW, Hopkins WA, Hawley DM (2013a) Deposition of pathogenic *Mycoplasma gallisepticum* onto bird feeders: host pathology is more important than temperature-driven increases in food intake. Biol Lett 9:. doi: 10.1098/rsbl.2013.0594

Adelman JS, Kirkpatrick L, Grodio JL, Hawley DM (2013b) House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of *Mycoplasma gallisepticum* infection. Am Nat 181:674–89. doi: 10.1086/670024

Airo R, Mihailes E, Astaldi G, Meardi G (1967) Skin reactions to phytohaemagglutinin. Lancet 1:899–900

Alcaide M, Edwards SV (2011) Molecular evolution of the Toll-like receptor multigene family in birds. Mol Biol Evol 28:1703–1715. doi: 10.1093/molbev/msq351

Areal H, Abrantes J, Esteves PJ (2011) Signatures of positive selection in Toll-like receptor (TLR) genes in mammals. Bmc Evol Biol 11:. doi: 10.1186/1471-2148-11-368

Ashley NT, Weil ZM, Nelson RJ (2012) Inflammation: Mechanisms, Costs, and Natural Variation. In: Futuyma DJ (ed) Annual Review of Ecology, Evolution, and Systematics, Vol 43. Annual Reviews, Palo Alto, pp 385–406

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Bonneaud C, Balenger SL, Russell AF, et al (2011) Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. Proc Natl Acad Sci U S A 108:7866–7871. doi: 10.1073/pnas.1018580108

Bonneaud C, Balenger SL, Zhang JW, et al (2012) Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. Mol Ecol 21:2628–2639. doi: 10.1111/j.1365-294X.2012.05551.x

Boyd AC, Peroval MY, Hammond JA, et al (2012) TLR15 is unique to avian and reptilian lineages and recognizes a yeast-derived agonist. J Immunol 189:4930–4938. doi: 10.4049/jimmunol.1101790

Bradbury JM (2005) Poultry mycoplasmas: sophisticated pathogens in simple guise. Br Poult Sci 46:125–136. doi: 10.1080/00071660500066282

Brown GP, Shilton CM, Shine R (2011) Measuring amphibian immunocompetence: validation of the phytohemagglutinin skin-swelling assay in the cane toad, *Rhinella marina*. Methods Ecol Evol 2:341–348. doi: 10.1111/j.2041-210X.2011.00090.x

Brownlie R, Zhu JZ, Allan B, et al (2009) Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 46:3163–3170. doi: 10.1016/j.molimm.2009.06.002

Bustamante CD, Fledel-Alon A, Williamson S, et al (2005) Natural selection on protein-coding genes in the human genome. Nature 437:1153–1157. doi: 10.1038/nature04240

Campbell TW, Ellis CK (2007) Avian and Exotic Animal Hematology and Cytology. Blackwell Publishing Professional, Ames

Carnero-Montoro E, Bonet L, Engelken J, et al (2012) Evolutionary and functional evidence for positive selection at the human

CD5 immune receptor gene. Mol Biol Evol 29:811–823. doi: 10.1093/molbev/msr251

Cid FD, Fernandez NC, Perez-Chaca MV, et al (2018) House sparrow biomarkers as lead pollution bioindicators. Evaluation of dose and exposition length on hematological and oxidative stress parameters. Ecotoxicol Environ Saf 154:154–161. doi: 10.1016/j.ecoenv.2018.02.040

Clayton DH, Moore J (1997) Host-Parasite Evolution: General Principles and Avian Models. Oxford University Press, Oxford

Cormican P, Lloyd AT, Downing T, et al (2009) The avian Toll-Like receptor pathway-Subtle differences amidst general conformity. Dev Comp Immunol 33:967–973. doi: 10.1016/j.dci.2009.04.001

Danilova N (2006) The evolution of immune mechanisms. J Exp Zool Part B-Mol Dev Evol 306B:496–520

Darwin C (1868) The variation of animals and plants under domestication, 1st ed. John Murray

Davis AK, Cook KC, Altizer S (2004) Leukocyte profiles in wild house finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. EcoHealth 1:362–373. doi: 10.1007/s10393-004-0134-2

Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22:760–772. doi: 10.1111/j.1365-2435.2008.01467.x

de Zoete MR, Bouwman LI, Keestra AM, van Putten JPM (2011) Cleavage and activation of a Toll-like receptor by microbial proteases. Proc Natl Acad Sci U S A 108:4968–4973. doi: 10.1073/pnas.1018135108

Dehove A, Commault J, Petitclerc M, et al (2012) Economic analysis and costing of animal health: a literature review of methods and importance. Rev Sci Tech Int Off Epizoot 31:605–617, 591–604

Dentovskaya SV, Bakhteeva IV, Titareva GM, et al (2008) Structural diversity and endotoxic activity of the lipopolysaccharide of *Yersinia pestis*. Biochem-Mosc 73:192–199

Dhondt AA, Altizer S, Cooch EG, et al (2005) Dynamics of a novel pathogen in an avian host: Mycoplasmal conjunctivitis in house finches. Acta Trop 94:77–93

Dlugosz EM, de Bellocq JG, Khokhlova IS, et al (2014) Age at weaning, immunocompetence and ectoparasite performance in a precocial desert rodent. J Exp Biol 217:3078–3084. doi: 10.1242/jeb.106005

Downing T, Lloyd AT, O'Farrelly C, Bradley DG (2010) The differential evolutionary dynamics of avian cytokine and TLR gene classes. J Immunol 184:6993–7000. doi: 10.4049/jimmunol.0903092

Duan QD, Zhou MX, Zhu LQ, Zhu GQ (2013) Flagella and bacterial pathogenicity. J Basic Microbiol 53:1–8. doi: 10.1002/jobm.201100335

Dye C (2014) After 2015: infectious diseases in a new era of health and development. Philos Trans R Soc B Biol Sci 369: 20130426. doi: 10.1098/rstb.2013.0426

Eichler W (1948) XLI.—Some rules in Ectoparasitism. Ann Mag Nat Hist 1:588–598. doi: 10.1080/00222934808653932

Ellegren H (2008) Comparative genomics and the study of evolution by natural selection. Mol Ecol 17:4586–4596. doi: 10.1111/j.1365-294X.2008.03954.x

Evans JK, Buchanan KL, Griffith SC, et al (2017) Ecoimmunology and microbial ecology: Contributions to avian behavior, physiology, and life history. Horm Behav 88:112–121. doi: 10.1016/j.yhbeh.2016.12.003

Ezekowitz RA (2002) Local opsonization for apoptosis? Nat Immunol 3:510–512. doi: 10.1038/ni0602-513

Faivre B, Preault M, Salvadori F, et al (2003) Bill colour and immunocompetence in the European blackbird. Anim Behav 65:1125–1131

Fauci AS (2001) Infectious Diseases: Considerations for the 21st Century. Clin Infect Dis 32:675–685. doi: 10.1086/319235

Faustino CR, Jennelle CS, Connolly V, et al (2004) *Mycoplasma gallisepticum* infection dynamics in a house finch population: seasonal variation in survival, encounter and transmission rate. J Anim Ecol 73:651–669. doi: 10.1111/j.0021-8790.2004.00840.x

Ferrer-Admetlla A, Bosch E, Sikora M, et al (2008) Balancing selection is the main force shaping the evolution of innate immunity genes. J Immunol 181:1315–1322

Ferwerda B, McCall MBB, Alonso S, et al (2007) TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. Proc Natl Acad Sci U S A 104:16645–16650

Fleming-Davies AE, Williams PD, Dhondt AA, et al (2018) Incomplete host immunity favors the evolution of virulence in an emergent pathogen. Science 359:1030–1033. doi: 10.1126/science.aao2140

Folstad I, Karter AJ (1992) Parasites, Bright Males, and the Immunocompetence Handicap. Am Nat 139:603–622

Fornuskova A, Bryja J, Vinkler M, et al (2014) Contrasting patterns of polymorphism and selection in bacterial-sensing toll-like receptor 4 in two house mouse subspecies. Ecol Evol 4:2931–2944. doi: 10.1002/ece3.1137

Fornuskova A, Vinkler M, Pages M, et al (2013) Contrasted evolutionary histories of two Toll-like receptors (Tlr4 and Tlr7) in wild rodents (MURINAE). Bmc Evol Biol 13:. doi: 10.1186/1471-2148-13-194

Frazer KA, Ballinger DG, Cox DR, et al (2007) A second generation human haplotype map of over 3.1 million SNPs. Nature 449:851–861. doi: 10.1038/nature06258

Frigerio D, Ludwig SC, Hemetsberger J, et al (2017) Social and environmental factors modulate leucocyte profiles in free-living Greylag geese (*Anser anser*). Peerj 5:e2792. doi: 10.7717/peerj.2792

Fumagalli M, Sironi M, Pozzoli U, et al (2011) Signatures of environmental genetic adaptation pinpoint pathogens as the main selective pressure through human evolution. PLOS Genet 7:e1002355. doi: 10.1371/journal.pgen.1002355

Garvin MC, Homer BL, Greiner EC (2003) Pathogenicity of *Haemoproteus danilewskyi*, Kruse, 1890, in blue jays (*Cyanocitta cristata*). J Wildl Dis 39:161–169

Geens A, Dauwe T, Bervoets L, et al (2010) Haematological status of wintering great tits (*Parus major*) along a metal pollution gradient. Sci Total Environ 408:1174–1179. doi: 10.1016/j.scitotenv.2009.11.029

Gonzalez G, Sorci G, de Lope F (1999) Seasonal variation in the relationship between cellular immune response and badge size in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 46:117–122. doi: 10.1007/s002650050600

Goto N, Kodama H, Okada K, Fujimoto Y (1978) Suppression of phytohemagglutinin skin response in thymectomized chickens. Poult Sci 57:246–250

Goüy de Bellocq J, Krasnov BR, Khokhlova IS, Pinshow B (2006) Temporal dynamics of a T-cell mediated immune response in desert rodents. Comp Biochem Physiol -Mol Integr Physiol 145:554–559

Graham AL, Allen JE, Read AF (2005) Evolutionary causes and consequences of immunopathology. Annu Rev Ecol Evol Syst 36:373–397. doi: 10.1146/annurev.ecolsys.36.102003.152622

Griggio M, Zanollo V, Hoi H (2010) UV plumage color is an honest signal of quality in male budgerigars. Ecol Res 25:77–82

Grodio JL, Buckles EL, Schat KA (2009) Production of house finch (*Carpodacus mexicanus*) IgA specific anti-sera and its application in immunohistochemistry and in ELISA for detection of *Mycoplasma gallisepticum*-specific IgA. Vet Immunol Immunopathol 132:288–294. doi: 10.1016/j.vetimm.2009.06.006

Grodio JL, Hawley DM, Osnas EE, et al (2012) Pathogenicity and immunogenicity of three *Mycoplasma gallisepticum* isolates in house finches (*Carpodacus mexicanus*). Vet Microbiol 155:53–61. doi: 10.1016/j.vetmic.2011.08.003

Grodio JL, Ley DH, Schat KA, Hawley DM (2013) Chronic *Mycoplasma* conjunctivitis in house finches: Host antibody response and M. gallisepticum VlhA expression. Vet Immunol Immunopathol 154:129–137. doi: 10.1016/j.vetimm.2013.05.010

Grueber CE, Wallis GP, Jamieson IG (2014) Episodic positive selection in the evolution of avian Toll-like receptor innate immunity genes. PLOS ONE 9:e89632. doi: 10.1371/journal.pone.0089632

Grueber CE, Wallis GP, King TM, Jamieson IG (2012) Variation at innate immunity Toll-like receptor genes in a bottlenecked population of a New Zealand robin. PLOS ONE 7:. doi: 10.1371/journal.pone.0045011

Guard-Petter J (2001) The chicken, the egg and *Salmonella enteritidis*. Environ Microbiol 3:421–430

Guivier E, Galan M, Salvador AR, et al (2010) Tnf-α expression and promoter sequences reflect the balance of tolerance/resistance to Puumala hantavirus infection in European bank vole populations. Infect Genet Evol 10:1208–1217. doi: 10.1016/j.meegid.2010.07.022

Hajjar AM, Ernst RK, Tsai JH, et al (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol 3:354–359. doi: 10.1038/ni777

Hamilton WD, Zuk M (1982) Heritable True fitness and bright birds - A role for parasites. Science 218:384–387

Hannon ER, Kinsella JM, Calhoun DM, et al (2016) Endohelminths in bird hosts from Northern California and an analysis of the role of life history traits on parasite richness. J Parasitol 102:199–207. doi: 10.1645/15-867

Hasselquist D (2007) Comparative immunoecology in birds: hypotheses and tests. J Ornithol 148:S571–S582

Hawley DM, Hallinger KK, Cristol DA (2009) Compromised immune competence in free-living tree swallows exposed to mercury. Ecotoxicology 18:499–503. doi: 10.1007/s10646-009-0307-4

Hawley DM, Osnas EE, Dobson AP, et al (2013) Parallel patterns of increased virulence in a recently emerged wildlife pathogen. PLoS Biol 11:e1001570. doi: 10.1371/journal.pbio.1001570

Hawn TR, Verbon A, Lettinga KD, et al (2003) A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. J Exp Med 198:1563–1572

Helfenstein F, Losdat S, Moller AP, et al (2010) Sperm of colourful males are better protected against oxidative stress. Ecol Lett 13:213–222. doi: 10.1111/j.1461-0248.2009.01419.x

Hellgren O (2015) Allelic variation at innate immune genes (avian β-defensins), within a natural population of great tits. J Avian Biol 46:113–118. doi: 10.1111/jav.00370

Henneberg M, Saniotis A (2012) How can evolutionary medicine inform future personalized medicine? Pers Med 9:171–173. doi: 10.2217/PME.11.99

Hernandez A, Yager JA, Wilkie BN, et al (2005) Evaluation of bovine cutaneous delayed-type hypersensitivity (DTH) to various test antigens and a mitogen using several adjuvants. Vet Immunol Immunopathol 104:45–58

Higgs R, Cormican P, Cahalane S, et al (2006) Induction of a novel chicken toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infect Immun 74:1692–1698

Higuchi M, Matsuo A, Shingai M, et al (2008) Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. Dev Comp Immunol 32:147–155

Hill GE (2002) A red bird in a brown bag: the function and evolution of colorful plumage in the House finch. Oxford University Press, New York

Hill GE, Farmer KL (2005) Carotenoid-based plumage coloration predicts resistance to a novel parasite in the house finch. Naturwissenschaften 92:30–34

Hillier LW, Miller W, Birney E, et al (2004) Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432:695–716

Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. Proc Natl Acad Sci U S A 97:5303–5306. doi: 10.1073/pnas.080551197

Horak P, Tegelmann L, Ots I, Moller AP (1999) Immune function and survival of great tit nestlings in relation to growth conditions. Oecologia 121:316–322

Huang YH, Temperley ND, Ren LM, et al (2011) Molecular evolution of the vertebrate TLR1 gene family - a complex history of gene duplication, gene conversion, positive selection and co-evolution. Bmc Evol Biol 11:. doi: 10.1186/1471-2148-11-149

Immler S, Calhim S, Birkhead TR (2008) Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543. doi: 10.1111/j.1558-5646.2008.00393.x

Iqbal M, Philbin VJ, Withanage GSK, et al (2005) Identification and functional characterization of chicken Toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar typhimurium. Infect Immun 73:2344–2350

Janeway CA (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54 Pt 1:1–13

Jann OC, Werling D, Chang JS, et al (2008) Molecular evolution of bovine Toll-like receptor 2 suggests substitutions of functional relevance. Bmc Evol Biol 8:

Jara-Carrasco S, Gonzalez M, Gonzalez-Acuna D, et al (2015) Potential immunohaematological effects of persistent organic pollutants on chinstrap penguin. Antarct Sci 27:373–381. doi: 10.1017/S0954102015000012

Jarvis ED, Mirarab S, Aberer AJ, et al (2014) Whole-genome analyses resolve early branches in the tree of life of modern birds.

Science 346:1320–1331. doi: 10.1126/science.1253451

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biol Rev 75:21–64

Kaiser P (2007) The avian immune genome a glass half-full or half-empty? Cytogenet Genome Res 117:221–230

Kaiser P, Stäheli P (2014) Avian cytokines and chemokines. In: Avian Immunology, 2nd edition. Academic Press, London, pp 189–204

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373–384. doi: 10.1038/ni.1863

Keestra AM, de Zoete MR, Bouwman LI, et al (2013) Unique features of chicken Toll-like receptors. Dev Comp Immunol 41:316–323. doi: 10.1016/j.dci.2013.04.009

Keestra AM, de Zoete MR, Bouwman LI, van Putten JPM (2010) Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. J Immunol 185:460–467. doi: 10.4049/jimmunol.0901921

Keestra AM, de Zoete MR, van Aubel RAMH, van Putten JPM (2008) Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol 45:1298–1307. doi: 10.1016/j.molimm.2007.09.013

Keestra AM, van Putten JPM (2008) Unique properties of the chicken TLR4/MD-2 complex: Selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol 181:4354–4362

Kennedy MW, Nager RG (2006) The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trends Ecol Evol 21:653–655. doi: 10.1016/j.tree.2006.09.017

Kim HM, Park BS, Kim JI, et al (2007) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist eritoran. Cell 130:906–917. doi: 10.1016/j.cell.2007.08.002

Kleven O, Laskemoen T, Fossoy F, et al (2008) Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. Evolution 62:494–499. doi: 10.1111/j.1558-5646.2007.00287.x

Kloch A, Wenzel MA, Laetsch DR, et al (2018) Signatures of balancing selection in toll-like receptor (TLRs) genes – novel insights from a free-living rodent. Sci Rep 8:8361. doi: 10.1038/s41598-018-26672-2

Koch M, Camp S, Collen T, et al (2007) Structures of an MHC class I molecule from B21 chickens illustrate promiscuous peptide binding. Immunity 27:885–899. doi: 10.1016/j.immuni.2007.11.007

Koutsos EA, Lopez JCG, Klasing KC (2007) Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (*Gallus gallus domesticus*). Comp Biochem Physiol B-Biochem Mol Biol 147:87–92

Krams I, Cirule D, Krama T, Vrublevska J (2011) Extremely low ambient temperature affects haematological parameters and body condition in wintering Great Tits (*Parus major*). J Ornithol 152:889–895. doi: 10.1007/s10336-011-0672-7

Kropackova L, Tesicky M, Albrecht T, et al (2017) Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence. Mol Ecol 26:5292–5304. doi: 10.1111/mec.14144

Larson G, Fuller DQ (2014) The evolution of animal domestication. In: Futuyma DJ (ed) Annual Review of Ecology, Evolution, and Systematics, Vol 45. Annual Reviews, Palo Alto, pp 115–136

Leavitt RD, Felsted RL, Bachur NR (1977) Biological and biochemical properties of *Phaseolus vulgaris* isolectins. J Biol Chem 252:2961–2966

Leveque G, Forgetta V, Morroll S, et al (2003) Allelic variation in TLR4 is linked to susceptibility to *Salmonella enterica* serovar typhimurium infection in chickens. Infect Immun 71:1116–1124. doi: 10.1128/IAI.71.3.1116–1124.2003

Ley DH (2008) *Mycoplasma gallisepticum* infection. In: Saif YM (ed) Diseases of Poultry, 12th Edition. Blackwell Publishing, Ames, Iowa, USA, pp 807–834

Ley DH, Hawley DM, Geary SJ, Dhondt AA (2016) House finch (*Haemorhous mexicanus*) conjunctivitis, and *Mycoplasma* spp. isolated from North American wild birds, 1994-2015. J Wildl Dis 52:669–673. doi: 10.7589/2015-09-244

Llacuna S, Gorriz A, Riera M, Nadal J (1996) Effects of air pollution on hematological parameters in passerine birds. Arch Environ Contam Toxicol 31:148–152

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98.