Charles University

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Neuro-immune crosstalk during peripheral inflammation in avian models

Neuroimunologické změny vyvolané periferním zánětem u ptáků

Doctoral Thesis

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List of publications

PAPER I

Melepat, Balraj, Tao Li, and Michal Vinkler. "Natural selection directing molecular evolution in vertebrate viral sensors." Developmental & Comparative Immunology (2024): 105147.

PAPER II

Balraj Melepat, Daniel Divín, Kateřina Marková, Tao Li, Nithya Kuttiyarthu Veetil, Eleni Voukali, Lucie Schmiedová, Martin Těšický and Michal Vinkler "The neuro-immune crosstalk between CNS and periphery during acute immune response to virus-mimicking RNA in parrots" (Submitted in Veterinary Research).

PAPER III

Eleni Voukali, Daniel Divín, Mercedes Goméz Samblas, Nithya Kuttiyarthu Veetil, Tereza Krajzingrová, Martin Těšický, Tao Li, **Balraj Melepat**, Pavel Talacko, Michal Vinkler "Subclinical peripheral inflammation has systemic effects impacting central nervous system proteome in budgerigars" (Published: Developmental and Comparative Immunology, June 2024).

PAPER IV

Veetil, Nithya Kuttiyarthu, Haniel Cedraz de Oliveira, Mercedes Gomez-Samblas, Daniel Divín, **Balraj Melepat**, Eleni Voukali, Zuzana Świderská et al. "Peripheral inflammation-induced changes in songbird brain gene expression: 3'mRNA transcriptomic approach." Developmental & Comparative Immunology 151 (2024): 105106.

PAPER V

Kuttiyarthu Veetil, Nithya, Amberleigh E. Henschen, Dana M. Hawley, **Balraj Melepat**, Rami A. Dalloul, Vladimír Beneš, and Michal Vinkler. "Varying conjunctival immune response adaptations of house finch populations to a rapidly evolving bacterial pathogen." Frontiers in Immunology 15 (2024): 1250818.

PAPER VI

Balraj Melepat, Amberleigh E. Henschen, Nithya Kuttiyarthu Veetil, Dana M. Hawley, Rami A. Dalloul, James S. Adelman and Michal Vinkler "Cytokine regulation of the house finch population-specific immune responses to an evolving pathogen, *Mycoplasma gallisepticum*". (Manuscript draft).

Abstract (English)

Birds play a multitude of roles within ecosystems, functioning as predators, scavengers, pollinators and seed dispersers. With an estimated population of approximately 50 billion individuals, birds are among the most populous animal classes on Earth. They inhabit diverse ecosystems, including forests, deserts, wetlands, grasslands, savannas, and mountains. Some bird species are highly specialised in their habitats and exhibit minimal movement, while others undertake extensive migration across the globe. Notably, certain birds are synanthropic, thriving in close association with human settlements, while others remain strictly wild. Given their widespread distribution, species richness, and ecological diversity, birds are primary targets and reservoirs for various pathogens. A recent study found that birds are associated with approximately 18.4% of emerging infectious diseases in the world, and nearly half of the world's bird species are in decline. This underscores the critical need to study avian immune systems and disease mechanisms.

Similar to other vertebrates, the avian immune system also comprises innate and adaptive components. During an infection, the pathogen recognising receptors in the avian innate immune system initiates an inflammatory response to eliminate pathogens. This process involves a tightly regulated interplay of immune cells and related molecules, including cytokines, to prevent self-damage. An unchecked inflammatory response can escalate to systemic inflammation, potentially breaching the blood-brain barrier and causing neuroinflammation. Despite the importance of innate immunity, research on avian innate immune receptors is comparatively underdeveloped. It is also to be noted that most immune studies in avians are done on chicken models, which has its advantages. However, the chicken immune system does not fully represent the vast diversity of avian species. Therefore, it is imperative to extend the research to other bird groups. Passeriformes and Psittaciformes are closely related and together they constitute more than half of the total bird population. However, these orders are underrepresented in avian immunology studies.

This PhD thesis aims to bridge the research gap in the innate immune responses of birds during inflammation and extend our understanding of the avian immune system beyond poultry. The thesis work begins with a comprehensive overview of vertebrate virus-sensing innate immune receptors, highlighting the significant research gap in birds. The next part of the thesis covers the experiments where my colleagues and I investigated the effect of sterile viral peripheral inflammation in budgerigars and sterile bacterial peripheral inflammation in both budgerigars and zebra finches and tested their effects on the central nervous system. Our study showed that peripheral inflammation can induce neuroinflammation. We also found that parrots are highly susceptible to neuroinflammation. In the later parts of my thesis, my colleagues and I used the host-pathogen system of house finches (*Haemorhous mexicanus*) and *Mycoplasma gallisepticum* to investigate the role of evolutionary history in the immune response during pathogen infection. In this study, we found that evolutionary history indeed plays an important role in the host immune response to the pathogen. To conclude our experiments, we analysed differential expression patterns of the immune-related genes to understand the underlying inflammatory response, employing an interdisciplinary approach for this analysis.

Abstract (in Czech)

Ptáci mají v ekosystémech mnoho rolí, fungují jako predátoři, mrchožrouti, opylovači a pomáhají šířit semena rostlin. S odhadovanou populací přibližně 50 miliard jedinců patří ptáci k nejpočetnějším zvířecím třídám na Zemi. Obývají různorodé ekosystémy, včetně lesů, pouští, mokřadů, travních porostů, savan a hor. Některé ptačí druhy jsou vysoce přizpůsobení svým biotopům a vykazují minimální pohyb, zatímco jiné podnikají rozsáhlé migrace po celém světě. Někteří ptáci jsou synantropní, což znamená, že se jim daří v těsné blízkosti lidských sídel, zatímco jiní zůstávají striktně divocí. Vzhledem k jejich širokému rozšíření, druhové bohatosti a ekologické rozmanitosti jsou ptáci hlavními cíli a rezervoáry různých patogenů. Nedávná studie zjistila, že ptáci jsou spojováni s přibližně 18,4 % nově vznikajících infekčních onemocnění na světě a téměř polovina všech ptačích druhů ubývá na početnosti. Studium ptačích imunitních systémů a mechanismů onemocnění je proto kritické.

Podobně jako u jiných obratlovců, i ptačí imunitní systém se skládá ze složek vrozené a adaptivní imunity. Během infekce rozpoznávající receptory ptačího vrozeného imunitního systému patogeny a zahajují zánětlivou reakci vedoucí k jejich eliminaci. Tento proces zahrnuje pečlivě regulovanou spolupráci imunitních buněk a příslušných molekul, včetně cytokinů, aby se předešlo poškození vlastních tkání. Nekontrolovaná zánětlivá reakce může eskalovat do systémového zánětu, který může narušit hematoencefalickou bariéru a způsobit zánět i v nervové soustavě. Navzdory svému významu je výzkum ptačích receptorů vrozené imunity relativně málo rozvinutý. Je také třeba poznamenat, že většina imunitních studií u ptáků je prováděna na kuřatech, které mají jakožto modelový organismus své vlastní výhody. Avšak imunitní systém kuřat ne zcela dobře reprezentuje obrovskou rozmanitost ptačích druhů. Proto je nezbytné rozšířit výzkum i na jiné skupiny ptáků. Pěvci (Passeriformes) a papoušci (Psittaciformes) jsou blízce příbuzní a dohromady tvoří více než polovinu celkové ptačí populace. Přesto jsou tyto řády v imunologických studiích nedostatečně zastoupené.

Tato dizertační práce si klade za cíl doplnit dosud chybějící informace o vrozených imunitních reakcích ptáků během zánětu a rozšířit naše chápání ptačího imunitního systému nad rámec studií orientovaných na drůbež. Práce začíná komplexním přehledem virových receptorů vrozené imunity u obratlovců, s poukazem na nedostatek informací týkajících se ptáků. Další části práce jsou zaměřeny na experimenty, ve kterých jsme s kolegy zkoumali účinky sterilního virového periferního zánětu u andulek a sterilního bakteriálního periferního zánětu u andulek a zebřiček a testovali jejich vliv na centrální nervový systém. Naše studie ukázala, že periferní zánět může vyvolat zánět v mozku. Také jsme zjistili, že papoušci jsou vysoce náchylní k těmto zánětům nervové soustavy. V dalších částech mé dizertační práce jsme s kolegy použili jako systém hostitel-patogen hýla mexického (*Haemorhous mexicanus*) a bakterii *Mycoplasma gallisepticum* k výzkumu role evoluční historie v imunitní reakci během infekce patogenem. V této studii jsme zjistili, že evoluční historie skutečně hraje důležitou roli v imunitní reakci hostitele. Na závěr jsme v našich experimentech analyzovali míru exprese imunitních genů, abychom pochopili proces zánětlivé reakce, přičemž k této analýze jsme využili interdisciplinární přístup.

General Introduction

Humans are deeply connected to our environment, including plants and animals. While the importance of plants and the environment is well recognized due to our direct reliance on resources like air and water, the critical connections to animals, particularly in the context of infectious diseases, are often less appreciated. The emergence of diseases like SARS-CoV-2 and avian influenza has underscored the link between human health and the welfare of both wild and domestic animals (Agarwal et al., 2020; Cyranoski, 2020; Malik Peiris, 2009). The United Nations reports about 75% of all infectious diseases are of animal origin highlighting the significance of animal health in the context of human survival (Cleaveland et al., 2001; Taylor et al., 2001). The World Health Organization's "One World, One Health" concept emphasizes a transdisciplinary approach to understanding the interconnectedness of humans, animals, plants and the environment (Kahn, 2009; Monath et al., 2010). Therefore, understanding animal health, particularly their immune systems, is as crucial as understanding human health (Du Pasquier, 1992; Gerardo et al., 2020).

Innate and Adaptive immune systems

The vertebrate immune system comprises the innate and the adaptive components (Dempsey et al., 2003; Janeway and Medzhitov, 2002). The innate immune system is the first line of defence and includes cells like macrophages, dendritic cells and natural killer cells, which recognize pathogens through pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002; Xiao, 2017). PRRs detect the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), initiating immune responses such as phagocytosis and cytokine production (Chen et al., 2018; Gleichmann et al., 2020; Hansen et al., 2011). The PRRs are categorised into families like Toll-like receptors (TLR), C-type lectin receptors (CLR), retinoic acid-inducible gene I-like receptors (RLR), nucleotide-binding oligomerization domain-like receptors (NLR), and Absent in Melanoma-2 (AIM2)-like receptors (Akira et al., 2006). These receptors not only trigger innate immune responses but also activate the adaptive immune system (Akira et al., 2006).

The adaptive immune system consists of T cells and B cells, responsible for cell-mediated and antibody-mediated immunity, respectively (Bennett et al., 2018; Merlo and Mandik-Nayak, 2013). While the MHC receptors are well-studied in adaptive immunity, there is less research on PRRs of innate immunity, beyond the TLR gene in animals (Acevedowhitehouse and Cunningham, 2006; Outlaw et al., 2019). Hence this thesis focuses on the innate immune system and PRRs.

Inflammatory response

Acute inflammation helps eliminate pathogens and remove damaged cells, characterised by redness, swelling, heat, pain and loss of tissue function (Medzhitov, 2010). Controlled inflammation benefits the host, but uncontrolled inflammation can lead to conditions like septic shock and chronic inflammation, causing severe damage (Ashley et al., 2012). Inflammation must be tightly regulated, involving cytokines that mediate cell-cell communication. Cytokine are classified into interleukins (IL), interferons (IFN), transforming growth factor-beta (TGF-β), tumour necrosis factor superfamily (TNFSF), colony-stimulating factors (CSF) and chemokines (Dinarello, 2007; Holtmann and Resch, 1995). Pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α promote inflammation, while cytokines like IL-10 and IL-4 have anti-inflammatory effects (Dinarello, 2000; Sprague and Khalil, 2009). Even the various immune-related and immune modulatory genes such as the B-cell lymphoma gene 10 (BCL10) or the Caspase genes are directly or indirectly modulating the inflammation by influencing the downstream signalling of cytokine production (Gehring et al., 2018; Scharschmidt et al., 2004). The PRRs and cytokines play a critical role in the regulation and outcome of inflammation (Dinarello, 2000).

Pathogen Recognition and PRR Evolution

PRRs, especially TLR genes, recognize various pathogens and initiate inflammatory responses (Alcaide and Edwards, 2011; Gay and Gangloff, 2007; Outlaw et al., 2019). TLRs detect microbial components and nucleic acids, leading to the activation of transcription factors like NF-kB and IRF3, which induce cytokine production (Akira and Takeda, 2004). PRRs within the same family show ligand detection specificity, influenced by natural selection processes like positive, negative and balancing selection (Karlsson et al., 2014). Positive selection sites in protein domains indicate evolutionary adaptation to pathogen pressures (Wagner, 2007).

Model systems in Immunological studies

Traditional immunological studies use laboratory-bred animals like rats and pigs (Bryda, 2013; Meurens et al., 2012). However, understanding the complete disease process requires studying animals in natural settings, when we consider the evolutionary aspects and other aspects of nature influencing the disease outcome (Buchmann, 2014). Birds, particularly passerines and parrots, offer valuable models due to their diverse habitats, and cognitive abilities (Barker et al., 2004; Williams et al., 2023). Due to this abundance and diversity, they also serve as one of the primary targets and reservoirs for various viral and bacterial infections (Fleming-Davies et al., 2018; Schmidlin et al., 2019). Passerines, like house finches, are significant for studying host-pathogen interaction, as seen in the house finch-Mycoplasma gallisepticum (MG) system (Dhondt et al., 1998; Vinkler et al., 2018).

Research focus

This comprehensive study aims to enhance our understanding of the immune systems in passerines and parrots, contributing to evolutionary immunology and providing insights into how these birds manage infections. The research leverages next-generation sequencing advanced transcriptomics and RT-qPCR tools to explore immune gene expression and molecular models in these avian models. This thesis comprises several research papers. The first paper reviews the evolutionary dynamics of the PRRs in vertebrates, highlighting gaps in our understanding of immune system evolution. The second paper studies peripheral-neuro-immune interactions in parrots during viral mimicking inflammation. The third paper examines the peripheral-neuroimmune response in parrots during bacterial mimicking inflammations using proteomics and RT-qPCR analysis. The fourth paper describes the zebra finches during bacterial mimicking inflammation using transcriptomics and RT-qPCR analysis. The fifth paper investigates the immunological response in house finches with different co-evolutionary histories with MG. The sixth paper extends the fourth paper by using RT-qPCR to detail gene expression patterns and by including the samples which failed during the transcriptomics analysis.

General Aims

In my doctoral project, I focused on answering the following research questions:

1. **How the natural selection affect the molecular evolution of pathogen-sensing receptors in vertebrates?**

   The immune receptors of the innate immune system recognise pathogens with specialised receptors, the Pathogen Recognition Receptors (PRRs). In this work, my co-authors and I surveyed the available literature on the PRRs and prepared a comparative review of molecular evolutionary studies in vertebrate viral sensors. My colleagues and I reviewed the natural selection acting on the

vertebrate PRRs recognising viruses and positive selection targets of ligand binding and signalling domains of the PRRs.

2. **How do immune responses differ in peripheral tissues versus the brain during peripheral viral mimetic ligand-induced inflammation in parrots?**

   In this experiment, my colleagues and I injected viral RNA, which mimics poly(I:C), into the peritoneum of budgerigars (*Melopsittacus undulatus*) to trigger the inflammatory response. We then sampled tissue from the periphery (ileum) and brain and analysed the expression of inflammasome genes (*IL6*, *IL1B*), the ligand identifying receptor *TLR3* and inflammasomes genes (*NLRP3* and *CASP1*) using the RT-qPCR technique.

3. **How do immune responses differ in peripheral tissues versus the brain during peripheral bacterial mimetic ligand-induced inflammation in parrots?**

   In this study, my co-authors and I investigated the systemic and central nervous system (CNS) response to subclinical acute peripheral inflammation induced by dextran sulphate sodium (DSS) and lipopolysaccharide (LPS) in budgerigar (*Melopsittacus undulatus*). Our main objectives of this study were to examine the impact of DSS on the histology of the gastrointestinal tract in budgerigar, to analyse the proteomic profiles of budgerigar plasma (PL) and cerebrospinal fluid (CSF), and to evaluate and compare the effects of acute low-grade peripheral inflammation caused by DSS and lipopolysaccharide in parrots.

4. **How do immune responses differ in peripheral tissues versus the brain during peripheral bacterial mimetic ligand-induced inflammation in passerines?**

   By injecting lipopolysaccharide, a marker for bacterial pathogen invasion that is recognised by the immune system, my co-authors and I induced peripheral inflammation in zebra finches. We then sampled brain tissue from the CNS and skin tissue from the periphery and analysed the expression of immune-related genes and inflammatory marker cytokines in both tissues using RNA-Seq and QuantSeq transcriptomics methods, followed by RT-qPCR analysis.
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5. **How do the immune responses vary during a bacterial infection in passerines with differing co-evolutionary histories with the bacterial pathogen?**

   In this experiment, house finches from four different populations VA, IA, AZ and HI with different evolutionary histories were infected with the mycoplasma pathogen from two different evolutionary time points (VA1994 and VA2013), and the tissues were collected during the first days of infection. From the collected tissues, my co-authors and I used the conjunctival tissue to isolate the RNA and identify the differentially expressed immune-related genes using the QuantSeq method.

6. **How can the validation and in-depth analysis of differentially expressed immune genes selected from the transcriptomic analysis of bacterial-infected passerines with differing co-evolutionary histories with the bacterial pathogen, be achieved?**

Based on our previous analysis of QuantSeq RNA transcriptomics sequencing data from house finches, we identified differential expressions of numerous immune-related genes. To validate and further elucidate these expression patterns within house finch populations, my c-authors and I conducted RT-qPCR experiments targeting the pro-inflammatory cytokine *IL1B*, anti-inflammatory gene (*IL10*) selected immunoregulatory gene (*BCL10*) and cytokines. In this study, we also included the bird samples which were previously excluded in our QuantSeq analysis due to library failure. We hypothesized that the BCL10 expression affects the IL1B/IL10 expression levels, underlying variation in tolerance among the house finch populations.

General Material and methods

Model Organisms

The avian orders Psittaciformes and Passeriformes were central to our study.

Psittaciformes

- Budgerigar (*Melopsittacus undulatus*)

1. A native of Australia, budgerigars exhibit diverse colouration, including wild-type and domesticated varieties.
2. Known for their social monogamy and notable cognitive abilities, they are increasingly utilised in studies on problem-solving and vocal learning.
3. Our experiments involving poly(I:C) and DSS/LPS-induced inflammation in parrots aimed to understand neuroimmune interactions using budgerigar models.

Passeriformes

- Zebra finch (*Taeniopygia guttata*)

1. Indigenous to sub-tropical regions of Australia, Africa, and Southeast Asia, zebra finches are socially monogamous and renowned for their vocal learning abilities.
2. With a fully sequenced genome, they serve as a crucial model organism in biomedical research, particularly in neurology studies.
3. Our study involving sterile bacterial peripheral inflammation aimed to elucidate neuroinflammatory responses in their brains.

- House Finch *(Haemorhous mexicanus*)

1. Residing across western North America, these birds exhibit adaptability to diverse habitats and are known for their specialized feeding habits.
2. Infected by Mycoplasma gallisepticum (MG), leading to severe conjunctivitis, first reported in 1993-1994 in Washington DC and Virginia (VA) caused house finch population decline.
3. Later the disease spread from the eastern part of the USA to other parts of the mainland such as Iowa (IA), and Arizona (AZ) sparing some of the island population of house finches like the Hawaii (HI) populations.
4. In our experiments, we used the house finch population from four locations in the USA, Virginia, Iowa, Arizona, and Hawaii, in chronological order based on their exposure to the MG infection.

5. We used the mycoplasma strain isolated in 1994 (VA1994) and another strain from a disease outbreak in 2013 (VA2013) to infect these four bird populations.
6. All our infectious work was conducted in the USA with the help of our collaborators.

Summary of key methods

Experimental design:

In the parrot experiments, the budgerigars were maintained in the animal facility of the Faculty of Science at Charles University. For the poly(I:C) experiment, the 27 birds were divided into three-time groups (3 hours, 6 hours,24 hours) each group consisting of 9 individuals, in which 3 were administered with low dose poly(I:C) (approximately 12.5mg/kg), 3 with high dose poly(I:C)(50mg/kg) and 3 controls injected with 0.9% saline. Based on their time groups, the birds were euthanized by decapitation, at the time intervals of 3, 6 and 24 hours. After the post-mortem blood collection from carotids, blood smears were made, and different selected tissues were immediately collected (including the brain and ileum used in this study) and placed into the RNA-later solution where they were stored at +4°C overnight and then frozen at -80°C until analysis.

For our DSS experiment in 35 parrots, the birds were divided into four experimental groups: 1)DSS treatment (low dose-3; high dose-3; very high dose-3) 2) LPS treatment-3, 3) Combined DSS and LPS treatments (low DSS+LPS-7; high dose+ LPS-7), 4) controls-3. The animals were administered DSS at different dosages: low dose at 25 mg/day, high dose at 50 mg/day and very high dose at 75 mg/day. For the LPS experiment, the parrots were injected subcutaneously into the left wing patagium with 0.2 mg LPS suspended in 20 µl sterile saline. The LPS was administrated one day after the DSS, or control treatments were finished. Six hours after LPS treatment, the blood was taken from all the experimental birds and all the experimental animals were sacrificed by CO2 and collected CSF, brain and other tissues including the intestine. The collected tissues were immersed in RNAlater and stored at +4 °C for 24 h and then frozen at −80 °C.

The zebra finch birds were also collected from the local bird facilities and maintained in the animal facility of the Faculty of Science at Charles University. The zebra finches got intra-abdominal administration of LPS at a dosage equivalent to 6 micrograms of body weight and the controls were injected with sterile Dulbecco's phosphate-buffered saline.

For the house finch experiment, the experimental design involved capturing 60 young and healthy house finches using mist and feeder traps in Virginia, Iowa, Arizona and Hawaii between June and September 2018 by our USA collaborators. All the birds were maintained in the Iowa State University animal facility. During the month of October 2018, the 15 individuals representing the four different house finch populations underwent divisions into three experimental groups. For each population, there were 5 individuals designated as (controls), treated with Frey's medium containing 15% swine serum alone, 5 individuals as treatment subjects inoculated with the MG isolate VA1994 and the rest 5 were treated with the evolved MG isolate VA2013.

Three days post-infection following the eyesore reading the birds were euthanized by rapid decapitalization and a panel of nine tissues was collected. All tissue samples were promptly submerged in RNA protectant within 15 minutes after euthanasia and refrigerated immediately. The frozen brain and conjunctiva-

associated lymphatic tissue samples were transported within 48 hours to Charles University in Prague, Czech Republic, where they were stored at -80 °C until further processing.

Throughout the experiment, all birds were housed individually in medium-sized flight cages and provided *ad libitium* access to food and water. Environmental conditions, including the light-dark cycle (12:12h) and temperature (approximately 22°C) were maintained consistently.

RNA isolation, preparation, and quantitative real-time polymerase chain reaction (RT-qPCR) were conducted using a HighPure RNA Tissue Kit. The quality and quantity of RNA were assessed using a Nanodrop instrument. RT-qPCR considered the gold standard for mRNA quantifications, was performed using gene-specific double-quenched probe methods to reduce background fluorescence and improve the signal-to-noise-ratio. The 28S r RNA gene served as the reference gene for RT-qPCR. Data obtained from RT-qPCR were analysed using both standard gene expression quantity (Qst) and relative gene expression ratio ® methods, enabling a comparison of gene expression between treatments and controls.

Next-generation Sequencing (NGS) and Transcriptomics analysis were carried out at the European Molecular Biology Laboratory (EMBL) in Germany. RNA-seq libraries were generated using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and the Lexogen QuantSeq 3' polyadenylated RNA Library Prep Kit FWD for Illumina. Sequencing was performed on the Illumina NextSeq 500 platform, and subsequent transcriptomics analysis was conducted using the BAQCOM pipeline for adapter trimming, alignment, and read count. Differential gene expression (DGE) analysis was performed using the limma and DESeq2 packages in R. All statistical analyses were conducted using the respective versions of R and R-Studio software.

General Results and Discussion

The health and immunity of animals are of paramount importance due to conservative, economic and zoonotic risks (Cleaveland et al., 2001; Cyranoski, 2020; Dawkins, 2019). While immunological studies have focused primarily on laboratory-grown rodents or primates, these studies often overlook critical factors influencing animal immunity, such as evolutionary context (Bryda, 2013; Buchmann, 2014; Fleming-Davies et al., 2018; Meurens et al., 2012). Hence, it is imperative to examine animal-centric studies to gain insights with significant implications for economics, conservation efforts, and human health (Cleaveland et al., 2001; Kirby et al., 2008). In this research, we selected the avian immune system as our focal model to explore its genetic diversity and review available studies on immunity and evolutionary immunology.

Our study focused on birds of the orders Passeriformes and Psittaciformes, which are understudied despite being suitable candidate models for wildlife immunology and comparative immunology. Ur studies examined the expression patterns of immune-related genes during inflammation induced by diverse stimulants, including sterile inflammation and bacterial infection.

Paper 1: Molecular Evolution of Innate Immune Receptors

In this article, we analysed the molecular evolution of receptors in the innate immune system responsible for recognising viral pathogens. We found that virus-recognising Toll-like receptors (TLR) show less population and interspecific variation compared to other TLR family genes. Specifically, the *TLR7* subfamily (*TLR7*, *TLR8*, and *TLR9*) exhibited stronger selection compared to *TLR3*. Additionally, the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) family showed evolutionary conservation, with *NLRP1* being the most conserved. Positive selection in *NLRP3* was observed in the leucine-rich repeats (LRR) in mammals and the linker region in birds. Other receptors, such as RIG-1-like receptors (RLRs) and *MDA5*, also showed positive selection in specific domains.

Neuroinflammation studies

Neuroinflammation is linked to several diseases such as Alzheimer's, dementia, autism spectrum disorders, schizophrenia and major depressive disorder in humans. In birds also it can cause social isolation, depression and other behavioural issues. Neuroinflammation can have multiple reasons such as viral diseases, peripheral inflammation, gut inflammation or autoimmune disorders. Among these, peripheral inflammation causing neuroinflammation is the least studied. In our **Papers 2 and 3** we have checked the immunological effects of viral as well as bacterial stimulants on peripheral inflammation causing neuroinflammation.

Paper 2: Peripheral inflammation with poly(I:C) in parrots

we used the viral mimicking poly(I:C) to induce a peripheral inflammation in parrots and evaluated the peripheral as well as the brain inflammation using the ileum and the brain tissues respectively. Our results showed that the pro-inflammatory cytokine expression in the ileum and brain are correlated with each other. Surprisingly we did not find any difference in the *TLR3* gene expression (the receptor detecting poly(I:C) in the periphery. However, the *IL6* gene expression was upregulated in the periphery and peaked at 3 and 6 hours after stimulation. In the brain, *TLR3* and both pro-inflammatory cytokines (*IL1B* and *IL6*) were upregulated at 3 to 6 hours after stimulation. This is suggestive of the immune response in the periphery triggering neuroinflammation. Our results also showed a positive correlation between the intestinal as well as the brain expressions of *IL1B*, *IL6* and *CASP1*.

To summarise, this study is to our knowledge the first one to explore the in vivo immune response to poly(I:C) in parrots and also the first one in birds to check the expression patterns of *NLRP3* and *CASP1* genes during poly(I:C) treatment in both ileum and brain. The time dynamics and expression patterns of the pro-inflammatory cytokines revealed in our study suggest the immune crosstalk between the periphery and CNS during the poly(I:C) stimulation. Our results also demonstrate that parrots are highly susceptible to severe neuroinflammation induced by peripheral viral infections.

Paper3: Histopathology, Proteomics and RT-qPCR in DSS and LPS treated birds

In this paper my co-authors and I examined the histopathology of the gastrointestinal tract following DSS treatment, observing significant structural alterations, including erosion of the epithelial layer, irregular crypts, and shortened mucosal layer. Proteomics analysis of plasma (PL) and cerebrospinal fluid (CSF) identified 180 proteins in PL and 978 proteins in CSF, with 155 overlapping proteins showing co-structuring between the Pl and CSF proteomes. mRNA expression levels of pro-inflammatory cytokines showed significant increases following LPS treatment. This study demonstrated that the peripheral immune response changes brain metabolism in parrots, with LPS causing systemic inflammation within 6 hours.

Paper 4: Transcriptomics approaches in zebra finches

We compared QuantSeq and RNAeq approaches followed by RT-qPCR to confirm the expression of inflammatory cytokines in skin and brain tissue from zebra finches after 24 hours of peripheral LPS injection. Our initial RNA-Seq analysis in the skin showed fewer immune-related genes in the skin analysis. However, with the QuantSeq approach in the skin, we found upregulation of a maximum number of immune-related genes, and it was reflected similarly in the RT-qPCR analysis. So, we did only the QuantSeq analysis for the brain and found upregulation of some of the immune-related genes and cytokines. Our findings also revealed evidence of transcriptomic alterations in peripheral tissues, specifically the skin, following both local and systemic inflammatory stimuli, subsequently influencing gene expression regulation within the brain. It was also noted that 24 hours post-stimulation, visible pro-inflammatory regulatory patterns manifest within the periphery, while exerting minimal impact on the gene expression landscape of the zebra finch brain, which predominantly exhibits anti-inflammatory signalling pathways.

Further clarification is needed regarding the temporal dynamics governing the transition from neuroinflammatory to anti-inflammatory states, along with explaining the specific contributions of individual genes and associated pathways. Moreover, our study, incorporating RT-qPCR validation, underscores the utility of cost-effective methodologies such as QuantSeq, particularly beneficial for investigations within non-model, genetically diverse species, thereby facilitating the identification of crucial inflammation-related markers with broad species applicability.

House finch-mycoplasma system

The naturally occurring wild host-pathogen systems enabling studies of immune evolution are rarely established in vertebrates (Vinkler et al., 2023). In our **papers 5 and 6** we have used a best-studied evolutionary system in birds the house finch-mycoplasma system (Hawley et al., 2013). In our initial experiments, we analysed the brain tissue samples using RT-qPCR analysis, for the *IL1B* expression profile (unpublished result). Since our bird samples are from the post-3-day infection, we did not find any upregulation of inflammatory cytokines in the brain (unpublished result). So, for our experiment, we used conjunctival tissue, which is the tissue directly affected during mycoplasmal infection.

Paper 5: **Differential gene expression in conjunctival tissue post mycoplasma infection in house finches.**

Here we did the QuantSeq sequencing, and elucidated gene expression alterations in house finch conjunctival tissue at 3 days post-inoculation (DPI) with MG. This investigation concentrated on differentially expressed genes (DEGs) pertinent to the immune response, especially those exhibiting variability among house finch populations with divergent co-evolutionary histories with MG. Notable up-regulation of inflammatory genes associated with Th1/Th17 pathways was identified, including *TLR1B*, *CXCL12*, *IL17R*, and *CD74*. Remarkably, *BCL10*, a pivotal regulator of *NFKB* signalling, demonstrated down-regulation in the Virginia population with prolonged MG exposure, suggesting an adaptive mechanism to enhance infection tolerance by mitigating inflammation. Our findings diverge from previous research, revealing population-specific immune response adaptations in the Harderian gland tissue from the same birds (Henschen et al., 2023). Thus, our results underscore the intricacy of immune regulation, proposing that extended co-evolution with MG may foster a more balanced immune response, augmenting infection tolerance.

Paper 6. Correlation and in-depth analysis of house finch transcriptomics data using RT-qPCR

Initially, we tested the correlation between the expression patterns of the *IL1B*, *IL10* and *BCL10* genes from our transcriptomics and RT-qPCR analyses (Fig.12). Our proinflammatory *IL1B* and anti-inflammatory *IL10* genes showed significant correlations between the transcriptomics RT-qPCR data. The *BCL10* gene did not show any significant correlations between the transcriptomics and RT-qPCR data. However, the *BCL10* gene expression was linked to the *IL1B* gene expression. Notably, the genes *IL1B*, *IL10* and *BCL10*, displayed significant differences in their expression patterns across the four distinct house finch populations (VA, IA, AZ, HI), dependent upon the treatment type (VA1994 or VA2013). The Virginia house finch population, with a long evolutionary history with mycoplasma, managed the inflammation by downregulating both pro-inflammatory (*IL1B*) and anti-inflammatory (*IL10*) gene expressions. This was comparable to our previous studies in the house finches (Henschen et al., 2023; Kuttiyarthu Veetil et al., 2024). To our surprise, the Iowa population of house finches, with almost similar evolutionary history with the mycoplasma showed a marked upregulation of *IL1B* gene expression and significant downregulation of the *BCL10* gene, particularly noticeable when compared to the Virginia population. Overall, our findings offer clearer insight into the house finch adaptation against the MG-induced immunopathology and contribute to the general understanding of the host's evolutionary response to pathogen virulence increase.

General Conclusion

During my PhD research, my co-authors and I investigated innate immune system-related genes and their role in inflammation in birds. Our findings were consistent with recent immunological studies in model organisms and the experimental design and methods were also comparable with them. We analysed gene expression patterns to identify the underlying immune response utilising various immune stimulants, including sterile bacterial viral mimicking poly(I:C), DSS, LPS, and actual bacterial infection caused by Mycoplasma gallisepticum.

Our review paper on vertebrate viral-sensing genes highlighted significant research gaps in this field emphasizing the need to broaden studies on different receptors beyond just MHC and TLR receptors. We discovered considerable variation in vertebrate virus-sensing receptor systems; sensors recognizing viral nucleic acids are more conserved, while those detecting complex ligands show greater diversity. The limited data available for comparison across gene families necessitates caution in drawing conclusions. Despite these limitations, existing studies suggest potential adaptations in virus sensors, including evolutionary arms races, gene loss, and convergent evolution. Further research is essential to explore phenomena like parallel evolution among vertebrate taxa, which could benefit from standardized methodologies despite the abundance of genomic data.

In our studies, we employed less utilised model systems in immunology and inflammation, such as passerines and parrots, to conduct our research. Our research is the first to investigate the expression of inflammatory complex genes, such as *NLRP3*, and *CASP1*, during viral inflammation in parrots. Additionally, our studies in both zebra finch and parrots explore how peripheral inflammation, whether bacterial or viral, affects neurons in these respective models, an underexplored area of research. We found that peripheral inflammation induces a similar inflammatory response in the brain in both models. Notably, after 24 hours of acute inflammation, both peripheral and brain tissues were able to regulate and control the inflammation. Our comparative study on DSS and LPS treatment in parrots showed that the LPS treatment had a significantly greater influence on systemic immune gene expression compared to the DSS treatment. In our zebra finch transcriptomics study by comparing two different transcriptomics methods, we identified that the QuantSeq method can act as a cost-effective alternative method for the classical RNA-seq method in identifying key markers of inflammation-related genes.

In our house finch-mycoplasma model system study**,** our findings shed light on potential immunological mechanisms underlying the enhanced tolerance to Mycoplasma gallisepticum (MG) observed in birds from Virginia (VA) population in comparison to other house finch populations. Specifically, they suggest a pivotal role for the equilibrium between the Th1 and Th17 pathway activation during the initial conjunctival response to MG infection in house finches. Population with no or recent exposure to MG may exhibit a propensity for upregulation of *IL-17*-associated pathway, as observed in the Arizona population of birds. Conversely, populations with a long-standing co-evolutionary history with MG, such as the Virginia population, may favour *IL12* signalling to bolster Th1 and/or anti-inflammatory immune response. Our investigation also indicates that a more recent MG isolate (VA2013) elicits stronger expression of immune genes in the conjunctiva compared to infection with the original isolate (VA1994). Given that this regulation may extend beyond immune pathways, affecting non-lymphoid tissue functions, including the sickness behaviour, which could influence MG transmission among finches. Our future investigation will concentrate on clarifying the role of various immune cell subsets in the immune response to MG alongside examining changes in gene expression encompassing various functions in non-lymphoid tissues. Similar to the QuantSeq analysis RT-qPCR also showed that the Virginia population of house finches with a long evolutionary history displayed increased tolerance to the disease compared to other bird populations.

To conclude, in all these studies, my co-authors and I used an interdisciplinary approach, integrating zoology, immunology, molecular biology, ecology, evolutionary biology, and bioinformatics to understand the fundamental molecular mechanisms underlying the peripheral inflammation that leads to neuroinflammation in the immunologically understudied bird models.

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