Charles University

Faculty of Science

Department of Zoology


# Neuro-immune effects of peripheral inflammation in avian models

Neuroimunologické změny vyvolané periferním zánětem u ptáků

Doctoral Thesis

Balraj Melepat, MSc

Supervisor: doc. RNDr. Michal Vinkler, Ph.D.

Prague, 2024

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**Declaration of originality**

I declare that this thesis or its substantial part has not been submitted to obtain the same or any other academic degree. I have written it independently based on the material cited in the text and in consultation with my supervisor and colleagues.

In Prague on Date: 3/7/2024 Balraj Melepat

**PROHLÁŠENÍ**

Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Tuto práci jsem zpracoval samostatně na základě uvedených informačních zdrojů a literatury.

V Praze dne Date: 3 /7/2024 Balraj Melepat

## Statement of contribution

The results presented in the dissertation of Balraj Melepat, MSc. are a joint work of the Laboratory of Evolutionary and Ecological Immunology and collaborative teams. The author statements included in the individual articles and manuscripts included in this thesis reflect my participation in the research described in this dissertation. All the literary works I consulted for this thesis have been appropriately cited.

## PROHLÁŠENÍ O PODÍLU NA PUBLIKACÍCH

Výsledky prezentované v dizertační práci Mgr. Balraj Melepat jsou společným dílem členů Laboratoře evoluční a ekologické imunologie a spolupracujících týmů. V každém článku či rukopisu obsaženém v této práci je uvedeno prohlášení o autorském podílu, které definuje mou účast na výzkumu popsaném v této doktorské práci. Veškerá literatura, která sloužila jako základ pro tuto práci, byla řádně citována.

My contribution to all publications included in the doctoral thesis is as follows:

***Information gathering, experimental design, reviewing of the manuscript, Statistical analysis, data interpretation and reviewing of the manuscript and fund acquisition.***

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

   ***Conceived and drafted the manuscript, literature review, and writing-review and editing preparation.***

2) **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)

   ***Formal analysis, investigation, methodology, project administration, funding acquisition, writing–review and editing preparation.***

3) 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).

   ***Investigation***

4) Nithya Kuttiyarthu Veetil, Haniel Cedraz de Oliveira, Mercedes Goméz Samblas, Daniel Divín, **Balraj Melepat**, Eleni Voukali, Zuzana Świderska, Tereza Krajzingrová, Martin Těšický, Ferris Jung, Vladimír Beneš, Ole Madsen and Michal Vinkler. "Peripheral inflammation-induced changes

in songbird brain gene expression: 3' mRNA transcriptomic approach". (Published: Developmental and Comparative Immunology February 2024).

***Formal analysis, writing– review& editing preparation, funding acquisition.***

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6) **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)

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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**

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**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**

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## 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

We humans are deeply connected to our environment, the plants, and our animal co-inhabitants. Although we can easily understand our connection to the environment through our direct needs for fresh air, and water as well as other resources, and with plants as the primary producers, our connections to animals are often less appreciated. However, with the recent increase in infectious diseases originating from wild animals, including Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) or avian influenza causing immense harm to humans, we have come to realise that human survival is closely linked to the welfare of both wild and domestic animals [1–3]. A recent United Nations study, which found that around seventy-five per cent of all infectious diseases are of animal origin, reinforces this concern [4, 5]. The World Health Organization has recognised the importance of this issue and has developed the concept of "One World, One Health", which uses a transdisciplinary approach to understand the link between humans, animals, plants, and the environment [6, 7]. All of this shows that understanding animal health is just as important as understanding human health. It should also be noted that like humans, one of the determining factors for animal health is how well their immune system resists or copes with an invasion of pathogens [8–10].

The immune system of vertebrates consists of two basic components: the innate immune system and the adaptive immune system [11–13] (A more detailed illustration of cells of the innate and adaptive responses is given in Figure 1). Although both the adaptive and innate immune systems play a combined role in fighting pathogens, the innate immune system is considered the first line of defence against pathogens [13, 14]. The most important cells representing innate immunity are macrophages, dendritic cells and natural killer cells [13, 15]. The macrophages and dendritic cells have pattern recognition receptors (PRR) and can identify pathogen-associated molecular structures (PAMS) and threat-associated molecular patterns (DAMP) [16, 17]. The PRRs, which are germline-coded proteins in the host cell, can detect various kinds of pathogens regardless of their life cycle [18]. The PRRs found in the host immune cells are the very first and fast in reacting with the pathogen or cell damage and initiate the first defence steps such as phagocytosis, killing infected cells or pathogens and initiating cytokine production [19]. Though all the PRRs have a similar immunological role, they are grouped into different protein families based on the distinct protein domains, ligand detection mechanisms, cellular locations, and downstream signalling pathways [20, 21]. The five different types of PRRs, which are mainly found in vertebrate immune cells, include the Toll-like receptors (TLR), the C-type lectin receptors (CLR), the retinoic acid-inducible gene I-like receptors (RLR), the nucleotide-binding oligomerisation domain (NOD)-like receptor families (NLR) and the Absent in Melanoma-2 (AIM2)-like families [18]. The PRR-based recognition of pathogens is crucial not only to innate immune system initiation but also they initiate the cytokine cascades and lead to a secondary signal for the activation of the adaptive immune system [20].

The most important cells of adaptive immunity are T cells and B cells [22, 23]. T cells are responsible for cell-mediated immunity, while B cells are responsible for antibody-mediated immunity [22, 23]. Like the PRRs of the innate immune system cells the adaptive immune system cells use the major histocompatibility complex (MHC) receptors to identify and eliminate the pathogens [24]. While the MHC of animals has been extensively studied, there are comparatively fewer studies available for the PRRs other than the TLR gene [24–26]. Thus, in my thesis, I am focusing more on the innate immune system and the PRRs.

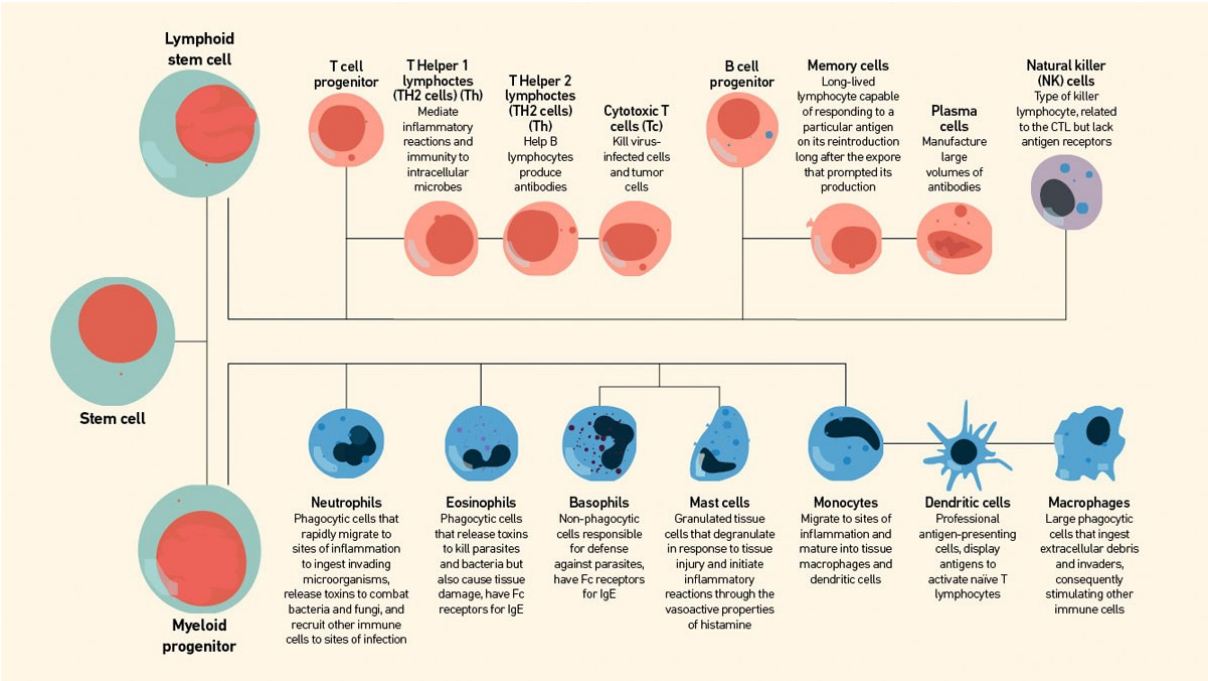


**Figure 1. Cells of the innate and adaptive immune system, their origins and roles in the immune response. Credit: Technology Networks. Taken from** [27].

An acute inflammatory response not only helps to eliminate pathogens but also to remove damaged cells and toxic compounds [16, 28]. The inflammatory response at the tissue level is characterised by redness, swelling, heat, pain, and loss of tissue function [28]. A controlled inflammatory response is always in favour of the host, helping to eliminate the pathogen, but an uncontrolled one leads to detrimental immune pathological conditions like septic shock [29]. Such a long-term effect can lead to chronic inflammation and cause severe irreparable damage to the host cells [29–31]. Sometimes a peripheral inflammatory response can even cause neuroinflammation in the central nervous system, which damages the neurons [32–34]. This leads to further problems, as the adult neuronal cells in the central nervous system are not regenerated [35–37]. The immune system therefore has several control steps to balance the inflammatory response [28, 38]. The cytokines play a major role in the cell-cell communication between all types of cells including immune cells, making them important molecules in the inflammation outcome [39, 40]. 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 [41–43]. Based on the structural homology the cytokines are classified into interleukins (IL), interferons (IFN), transforming growth factor-beta (TGF- *β)*, tumour necrosis factor superfamily (TNFSF), colony-stimulating factors (CSF) and chemokines [40, 44–46]. Each of this family contains an enormous number of proteins [45, 46]. Some cytokines like TGF-beta show pleiotropic antagonism in nature, making it difficult to relate their expression with inflammation [47, 48]. However, cytokines such as interleukin-1-beta (*IL-1β*), *IL-6*, and tumour necrosis factor-alpha (*TNF-α*) are inherently pro-inflammatory and involved in the upregulation of inflammation, while cytokines such as *IL10*, *IL4*, *IL11* mostly have an anti-inflammatory effect [49, 50]. Thus, by examining the expression of these pro-inflammatory and anti-inflammatory cytokines, we can comprehend the molecular nature of inflammation [51, 52]. Inflammation is controlled by tightly regulating the activation of PRRs on immune cells and their downstream signalling and production of pro-inflammatory cytokines and anti-inflammatory cytokines [53–55].

With this understanding of the crucial role of PRRs in eliminating pathogens, let us examine how these PRRs recognise pathogens and trigger the inflammatory response. The most extensively studied PRR genes belong to the family of TLR genes that are located in the cell membrane, both in the extracellular membrane (recognising microbial components) and in the endosomal membrane (recognising nucleic acids) [26, 56, 57]. The ligand-binding leucine-rich repeat ectodomain of these proteins forms a horseshoe-shaped structure and upon activation they dimerise and the cytoplasmic Toll-like (IL-1) receptor (TIR) homologous domain is bypassed and the downstream signalling cascades are initiated, leading to the activation of transcription factors such as NF-kB, IRF3 and ultimately leading to the production of pro-inflammatory cytokines and interferons [58, 59]. Although there are slight differences in intermediary signalling pathways and signalling cascades, most PRRs have a similar downstream effect [60]. But for all these similar downstream effects, even within the same PRR families, ligand detection is different [20, 61]. For example, *TLR3* is activated by double-stranded RNA [62], whereas *TLR7* from the same family is only activated by single-stranded RNA [63]. Another example is the variations in the pathogen binding region of a single PRR gene among the same population of animals [64, 65]. So how do they achieve this kind of specificity with almost similar structural and functional domains within the same family? Natural selection is the most compelling explanation. The natural selection pressure exerted by pathogens on immune genes can lead to three possibilities, namely positive selection, which promotes the frequency of beneficial alleles [66, 66], negative selection, which removes deleterious alleles [67], and balancing selection, which promotes the diversity of alleles [68, 69]. The amino acid codons that change in the particular protein domain to provide some fitness benefit to the individual are known as positive selection sites [70]. They are products of the selection process acting on that specific protein domain [70, 71]. If more positive selection sites are present, this means that the domain is subject to stronger positive selection [70, 71]. Through these different selection processes, an immune gene within the same family will achieve its diversified function with high specificity for the pathogen. To study such a complex immunoregulatory system and its development, the selection of the most appropriate model was therefore of utmost importance.

Most in vivo immunological studies in humans or animals use laboratory-bred animals such as rats and pigs as model organisms, which has its advantages in studies such as the discovery and testing of vaccines and new drugs, cancer studies and gene silencing studies [72–76]. These laboratory animal studies are sufficient to get an idea of the general structure and function of the immune system [77, 78]. However, this may not be sufficient to understand the entire disease process. An example of this is avian influenza in wild waterfowl, which are the reservoirs for the viruses but are the least affected by it, while it kills several other bird species [79]. So, if the immune system is similar how some are more affected than others? One possible answer is that although the basic components of the immune system are the same in all animals, some additional components in nature influence the final disease outcomes [76, 80, 81]. These include the evolutionary history of host-pathogen interaction and general behavioural and social changes during infection [82, 83].

Let us delve into the evolutionary aspect of infection in detail. During an infection the pathogen and the host will undergo an arms race, the host will try to resist the infection and the pathogen will counter and this cycle will go on as suggested by the Red Queen hypothesis “It takes all the running you can do, to keep in the same place”. This co-evolutionary history can do several phenotypic changes in the host (e.g.: avoiding a particular site with several pathogens) as well as genetic modification in the host immune genes and also the counter-acting genes of the pathogen (such as pathogen receptor modification) [84–86]. However not always the host and pathogen interaction are antagonistic, sometimes the host tries to co-exist with the pathogen by tolerating it instead of resisting it [87]. When the host's immune system attempts to resist the pathogen, it rigorously recognises, neutralises and eliminates the pathogen from the organism [88]. However, this response can also cause damage to the host tissues [89]. However, during disease tolerance,

the immune system focuses less on reducing the pathogen load and instead attempts to reduce damage to the host's tissues by tightly controlling the host's immune response [87, 88]. Thus, to uncover such a complex immunological and evolutionary perspective of a host-pathogen system a comparative study is needed in animals that live in more realistic situations, such as wild hosts that act as virus and bacterial reservoirs.

As a model system, birds can fulfil some of the above-mentioned criteria, as they can be found in a variety of habitats from forests, deserts, wetlands, grasslands, savannas and mountains [90, 91]. Some of these birds are synanthropic, meaning they are highly adapted to urban, suburban rural areas [92, 93]. Among them, certain species are kept as pet birds or poultry, while others remain as commensal wild birds [94–96], and are cognitively advanced (e.g. Courtship behaviour, navigation over long distances, spatial memory, flying and singing) [97, 98]. Furthermore, some additional factors make birds a suitable candidate for an in-depth study of the wildlife immune system. Firstly, as mentioned earlier some wild birds are a reservoir for various pathogenic bacterial and viral species that can lead to mass mortality of both wild and domesticated birds, causing serious conservation and economic problems [79]. One such example is the West Nile virus (WNV), which caused several outbreaks in different parts of the world, killing birds and other animals, including humans [99]. Most of these outbreaks occurred on bird migration routes. So, this brings us to our second point, the highly social and mobile nature of birds which facilitates the dispersion of diseases rapidly over long distances. Thirdly, they possess a diversified and highly evolved immune system compared to their reptilian ancestors [100]. Finally as stated by Jesicca Bolker in her article about selecting model organisms, "There is more to life than rats and flies" [101].

Among birds, most biomedical and immunology studies have focused on the chicken (*Gallus gallus*), [102–104]. Extensive research on chickens has helped to breed genetically modified chickens such as hypertensive or hyperglycaemic breeds, which are of great use in biomedical research [105, 106]. However, it is also important to look beyond the chicken model when we look at immunological studies. The chicken model immune system can represent the Galliformes family, but it cannot represent the entire avian immune system [103, 107]. An example is the absence of the previously mentioned RIG-I (retinoic acid-inducible gene I), in chickens, which is present in ducks, geese and finches [108, 109]. In addition to this, it also should be noted that chickens cannot represent cognitively advanced birds for behavioural and social experiments [110, 111]. So, we need immune models from birds beyond chickens to explore the vast possibility of immune studies in avians [103, 107, 112].

The Passeriformes are the most diverse and species-rich order of birds, representing the majority of the avian taxa [113–115]. They make up more than 56% of the total bird population [90]. Passerines include birds such as zebra finches and house finches which occur in the wild and are also found in a variety of habitats from urban to rural areas and as pets [116–118]. Due to this abundance and diversity, they also serve as one of the primary targets and reservoirs for various viral and bacterial infections [91, 119, 120]. One additional advantage of Passeriformes as a model system in evolutionary immunology is the availability of a naturally occurring host-pathogen system the house finches and mycoplasma. Among vertebrates naturally occurring host-pathogen systems are rare. Some of the best-documented examples are European rabbits (*Oryctolagus cuniculus*) and Myxoma virus [121], lowland leopard frog (*Lithobates yavapaiensis*) and fungus (*Batrachochytrium dendrobatidis*) [122], common frogs (*Rana temporaria*) and rana virus [123]. Among them, house finches ( *Haemorhous mexicanus*) and the bacterium (MG) (*Mycoplasma gallisepticum*) serve as an important model system [124, 125].

The house finch and the mycoplasma system is an ecologically and evolutionarily well-researched system, but there is less knowledge about the underlying molecular mechanisms of the finch immune system [126]. Mycoplasmas are bacteria with a low genome content and lacking their cell wall, belonging to the class of

mollicutes [127]. Mycoplasmas are generally known for their ability to manipulate the host's inflammatory response and cause damage to vertebrate host tissue [128, 129]. The *Mycoplasma gallisepticum* (MG) was first discovered as a pathogen in chickens that caused severe respiratory infections [130]. Later in 1994, it was reported for the first time in the USA Maryland and Virginia, that the pathogen had jumped from chickens to house finches, causing severe conjunctivitis and deaths [131]. MG later spread to different parts of the US and affected the house finch population in different parts of the country [132]. Scientists have continuously monitored the spread of the disease over the past 20 years and have obtained the original MG strain, and subsequent infections at different time points [124, 133]. The availability of these strains from different evolutionary time points and from house finch populations with different evolutionary histories with MG make them a unique wild host-pathogen to study the evolution of immune systems in a non-mammalian model.

The other group of birds which have almost similar living conditions and are the closest living relatives of the Passeriformes are the Psittaciformes [134]. Both are pet birds living closely associated with humans. Nearly 60% of all parrot species are experiencing a global population decline, highlighting the urgent need for conservation efforts focused on captive populations [135]. They also serve as the reservoir for various bacterial and viral pathogens including the West Nile virus [136, 137]. Parrots have exceptional cognitive abilities with complex social interaction [97, 138]. A recent study on parrots showed that they are highly prone to neuroinflammatory diseases due to the loss of a cannabinoid receptor, CNR2 [139]. Hence birds from these two orders can serve as the best model for neuro-immune studies in animals.

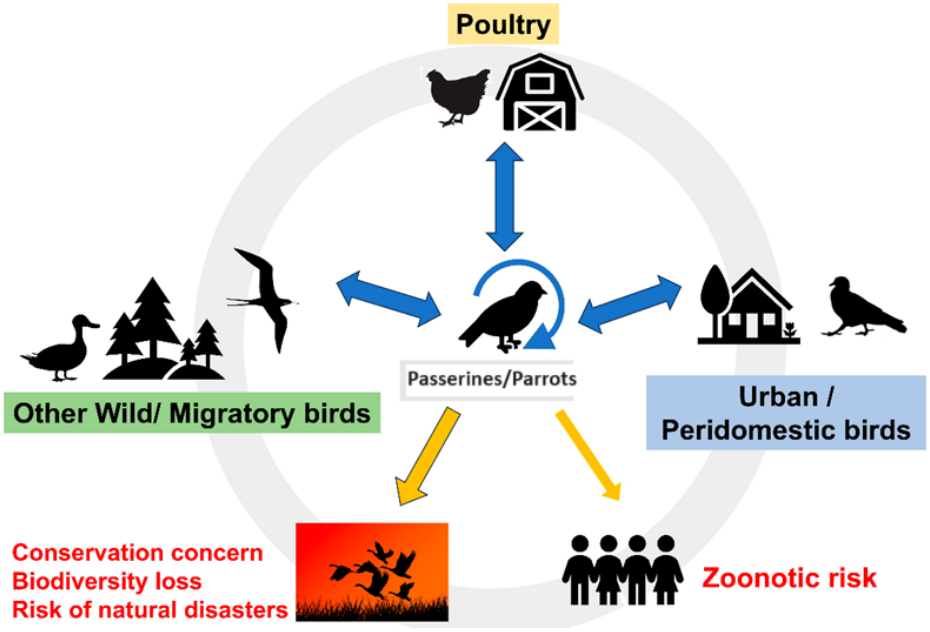


**Figure 2. The theoretical role of passerine and parrots in the circulation and potential spillover of viruses and other infectious agents among the passerines and parrots (blue semi-circular arrow around the bird in the centre), domestic, peridomestic and other wild birds (blue bidirectional arrows) and their relation to potential zoonotic risk or threats to biodiversity (orange unidirectional arrows). The relation of passerines and parrots to theoretical consequences, such as zoonotic risk or threats to biodiversity, are depicted using orange unidirectional arrows. The One Health concept is represented by the light grey ring. (Courtesy:** [91] **(license (https://creativecommons.org/licenses/by/4.0/).**

Although there are evolutionary and immunological studies on these two populations, which together make up a large proportion of the avian population, they are underrepresented, not only in research on avian evolutionary immunology but also in immunological research in birds as a whole [91]. It is indeed surprising to learn that little research has been done on the receptors of the innate immune system, the genes associated with the immune system signalling and their expression during inflammation, and the molecular evolution of the immune system in birds with such a large number of pathogens. Therefore, in our study, we used the avian models of these two groups to understand the immune system of the animals in detail.

The online availability of genetic sequences from humans and other animals has changed the entire field of biology in a very positive way [140]. The new fields of next-generation sequencing (NGS) have further accelerated genetic and genomic studies [140, 141]. Therefore, we took advantage of the availability of NGS data from passerines and parrots for our studies. The recent NGS tools, the two transcriptomics analysis approaches, namely the QuantSeq (sequencing focuses only on the 3' end of the transcript) and RNA-Seq (sequencing covers the entire transcript), combined with real-time quantitative polymerase chain reaction, were used in our research to identify and confirm differential gene expression [141, 142].

My research thesis commences with a comprehensive review paper focusing on the evolutionary dynamics of vertebral viral sensing PRRs within the immune gene system. Through my investigation, I have inquired into the positive selection sites situated within the ligand binding and signalling domains of these receptors. This study has shed light on a significant gap in our understanding regarding the overall molecular evolution of the vertebrate immune system. First, this review has formed a basis to place the later experimental tasks into an immunological context. My second research paper explains the neuroimmune cross talk in parrots during peripheral infection caused by viral mimicking synthetic RNA. This is the first paper to study the NLR family PRR along with other immune-related genes in budgerigars during viral mimicking inflammation. In my third research paper, my colleagues and I investigated the difference in the peripheral and central nervous system immune-related gene expression pattern during acute inflammatory response caused by bacterial mimicking dextran sulphate sodium (DSS) and lipopolysaccharide (LPS in budgerigar. In my fourth research paper, we examined the neuro-immune cross-talk during the bacterial mimicking synthetic LPS-caused peripheral inflammation in zebra finch birds. Here we performed RNA sequencing using two different transcriptomics approaches, the comparatively expensive RNA-Seq and the less expensive QuantSeq analysis. Further, we selected some of the immune-related genes from the transcriptomics analysis and performed real-time quantitative polymerase chain reaction (RT-qPCR) analysis to confirm the results from transcriptomics analysis and to obtain a more comprehensive understanding of immune gene expression. In the fifth research paper, my co-authors and I used the naturally occurring avian host-pathogen system the house finches and mycoplasma. This host-pathogen system was used to understand the difference in the immunological response during mycoplasma infection in the house finches with varying co-evolutionary histories with the mycoplasma. The sixth paper extended our previous work presented in the fifth paper, by performing RT-qPCR analysis to gain a deeper understanding of the gene expression patterns identified as differentially expressed in our transcriptomics study. Additionally, we included the bird samples that were previously excluded due to library preparation failures during QuantSeq analysis. So, I have completed these studies and all these research articles are either published, submitted or nearing submission in reputed journals.

## General aims

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

1. **How the natural selection affects 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.

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 Methods

In this section, the objective is to present a comprehensive overview of our methodologies, emphasising their distinctive features, rather than delving into intricate details already available in the respective individual manuscripts.

### Model Organisms

The model organisms I used in my study are from the avian order Psittaciformes and Passeriformes.

### Psittaciformes

#### Budgerigar (*Melopsittacus undulatus*) (Order: Psittaciformes, Sub order: Loriinae, Family: Psittaculidae)

The budgerigar, a native bird from Australia is coloured as wild types as well as in several domesticated colours [138, 143]. The wild-type birds are found to have six black round spots arranged in a row across the throat [143]. The yellow-coloured feathers were present in the crown and throat, which can be identified distinctively from the surrounding green plumage [143]. The wild adult males have rich blue ceres, and bluish legs and feet, while the females have pale brown to dark brown ceres and pinkish legs and feet [143]. In captivity, they are socially monogamous and the females are socially dominant and aggressive [144].

Parrots are used increasingly in problem-solving and vocal learning studies, due to their incredible cognitive abilities. Parrots are also shown human-like depressive behaviours such as anxiety, and overeating which are observed by veterinarians [145, 146]. It is also shown that they are highly prone to neuroinflammation due to the loss of cannabinoid receptor 2 (CNR2) [139]. In our **paper 2** we used 27 budgerigars and in our **paper 3** we used 35 budgerigars. The birds were purchased from Vyškov Zoo and from local hobby breeders.

**Figure 3. Parrots (Courtesy: Martin Těšický)**

**Passeriformes**

**Zebra Finch (*Taeniopygia guttata*) (Order: Passeriformes, Sub order: Oscines, Family: Estrildidae)**

These are granivorous birds located in the sub-tropical parts of Australia, Africa, and Southeast Asia [147]. They are highly social and monogamous birds [147, 148]. Male zebra finches typically possess vibrant orange cheek feathers, a feature absent in their female counterparts [148]. The female zebra finch is characterized by an orange beak, while the male of the species exhibits a red beak and only the male zebra finches can engage in singing behaviour [144, 147, 148]. After chickens, they were the second species of birds whose genome was fully sequenced [149].

Zebra finches are considered to be an important model in biomedical research especially in neurology studies[149]. Initially, they were used as the best model for sexual behaviours and later used to study vocal development [148]. They can serve as a model for human vocal studies, as they share a molecular, anatomical, and physiological similarity with the human vocal circuit [144]. So, in our **paper 4** we have used 24 adult zebra finches from local hobby breeders.

**Figure 4. Zebra finch (Courtesy: Oldřich Tomasek)**

**House finch (*Haemorhous mexicanus*) (Order: Passeriformes, Sub order: Oscines, Family: Fringillidae)**

The house finches are the resident species of western Canada, the western United States and Mexico [150]. In the wild, they live in a variety of habitats from the open coniferous forest, desert scrubs, pine-oak forest, coastal areas and elevations of 3500 meters [150]. They are also found to be living very close to humanly populated areas [150]. These birds are highly specialized in obtaining their food and nestling from human neighbourhoods. They primarily feed on weed seeds, berries and grains and occasionally eat small insects like aphids [151]. The male exhibits a vibrant red colouring on their head and chest, complemented by brown and white stripes that adorn their body, tail and wings [151]. The females lack distinctive red hues and showcase a predominantly light brown plumage with dark brown edges on their wings and tails [151]. Both genders share a similar size, measuring around 13 centimetres in length. The female lays 4 to 5 eggs of pale blue colour with black and lavender dots on them [151].

**Figure 5. House finch (Courtesy: Bonnie Fairbanks Flint)**

These birds were brought in captivity to New York in 1940 and later set free [116]. After initial struggles, they showed exponential population growth and spread throughout the USA [116, 152]. During 1993-94 the first report of severe conjunctivitis was reported in Washington DC and Virginia [116]. The pathogen causing this disease *Mycoplasma gallisepticum* was isolated from the eastern USA birds in 1994 [130]. The disease was characterized by mild to severe ocular swelling, conjunctivitis and ocular and nasal discharge [153]. It caused the decline of more than half of the house finch population during that time [154]. The disease later 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) population (Details in Figure:4) [124]. As mentioned above the mycoplasma samples of the original strain from 1994 and strains of other subsequent infections from the last 20 years from various parts of the USA were preserved [124, 133]. In our **papers 5 and 6** we used a total of 60 house finches of four different populations from 4 locations in the USA, based on the time of their contact with the MG infection, namely Virginia (VI) with the longest history of mycoplasma infection, followed by Iowa, and Arizona and finally the population from Hawaii Island which had with no history of infection with the mycoplasma. 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. All our infectious work was conducted in the USA with the help of our collaborators. The tissues were then later transported to the Czech Republic and further experiments are conducted here.

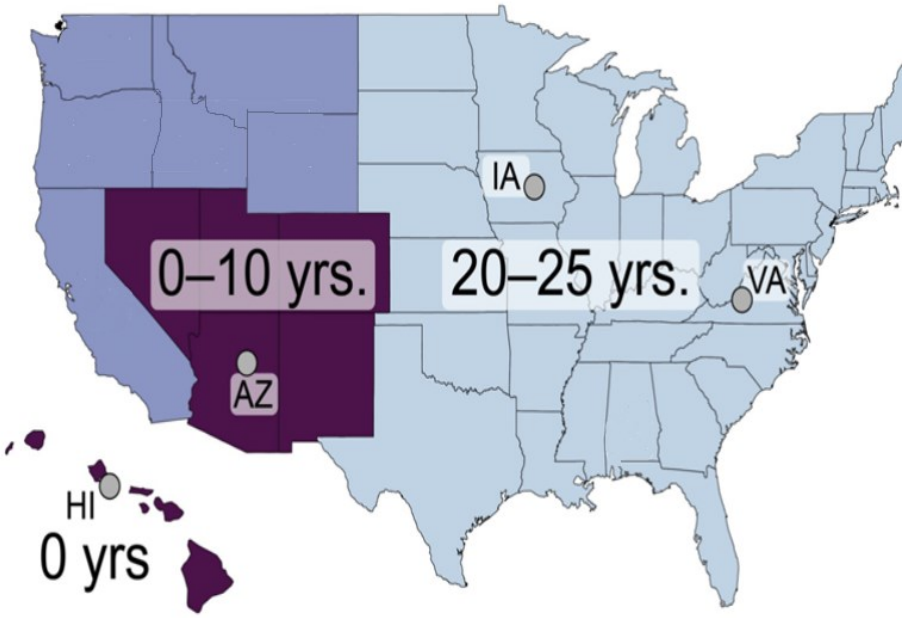


**Figure 6. The house finches from four different populations spanning the temporal invasion gradient of the bacterial pathogen, *Mycoplasma gallispeticum*. From the oldest to the most recent these are Virginia (VA), Iowa ( IA), Arizona (AZ) and Hawaii (HI). (Courtesy:** [124] **(license (https://cran.r-project.org/web/license/GPL-2).**

**Summary of the 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)**

The RNA from different tissue samples were isolated using a High Pure RNA Tissue Kit (Roche, Basel, Switzerland) and the quality and quantity of RNA was measured using a Nanodrop instrument (NanoDrop ND-1000). The extracted RNA was diluted in molecular grade water enriched with carrier transfer RNA (Qiagen, cat. No. 1068337), in a 1:5 ratio for the target genes and 1:500 for the 28S rRNA gene which was used as the reference gene for the RT-qPCR.

The real-time quantitative polymerase chain reaction (RT-qPCR) is the golden standard for mRNA quantification [155, 156]. The RNA quantification can be done by either absolute or relative methods. Absolute quantification determines expression levels in a total number of copies, it is particularly used to get the precise measurements of the gene expression levels. The relative quantification identifies the fold of expression change between two samples. It is used to compare gene expression patterns under different experimental conditions, such as treatment versus control. The relative quantification needs data normalization using housekeeping gene expression [157].

There are three major steps in a single cycle of real-time PCR, the first step is a denaturation step (High temperature to melt the DNA strands, the second step is an annealing step (when the primers hybridize to the DNA sequence) and the third step is Extension step (by the Special DNA polymerase). If the sample is RNA there will be an additional initial step called reverse transcription, where the cDNA is synthesized from

the RNA. In one step of RT-qPCR, the reverse transcription and amplification are done in the same tube, and in two-step RT-qPCR both are done in two separate tubes [158].

Here for RT-qPCR, I have used the gene-specific double-quenched probe method, which reduces the background fluorescence and improves the signal-to-noise ratio compared to the single fluorescently labelled probes [159]. Initially, when the fluorophore and quencher in the probe are sitting nearby, the fluorescent signals produced from the fluorophore will be absorbed by the quencher by fluorescent resonance energy transfer (FRET). During the extension step when the fluorophore and quencher molecules are physically separated by the 5'-3' exonuclease activity of Taq-Polymerase, the FRET is disrupted, and the fluorescent signals will be emitted from the fluorophore and the RT-qPCR machine will detect the signal. (Figure.5) [159].

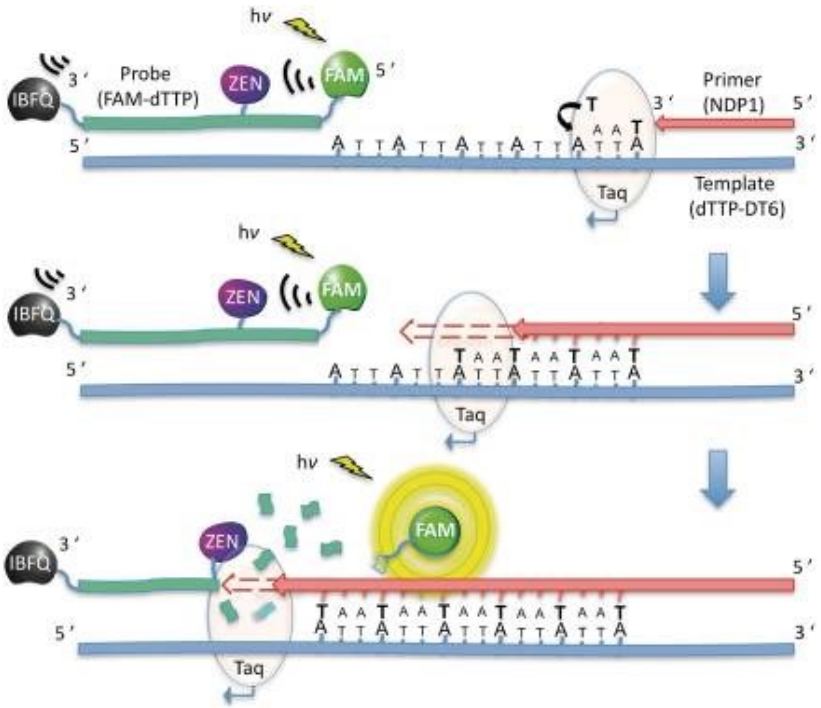


**Figure7. Representation of double quenched probe method in RT-qPCR** The IBFQ and ZEN are the two fluorescent quenchers and FAM is the fluorophore **(http://creativecommons.org/licenses/by-nc/3.0).**

The cross point value (Cp), quantification cycle value (Cq) or cycle threshold (Ct) is the number of amplification cycles at which a fluorescent signal exceeds the minimum threshold set for the detection [160, 161]. The Ct value is inversely proportional to the quantity of amplicon in the reaction, which means a low Ct value for an amplicon means it is high in amount [162].

To conclude, in all our experiments we used the one-step RT-qPCR procedure and double-quenched probe detection method. We have used the 28S gene as our reference gene. In our assay, we used either the standard gene expression quantity (Qst) method, which helps to compare the gene expression between treatments and controls or the relative gene expression ratio (R) which provides the measure of gene expression fold

change in the treatments against the controls [32]. In the Qst method, the gene expression can be quantified using the formula $Q = E^{\Delta Cq}$ where E is the mean amplification efficiency of that assay and ΔCq is the difference between the arbitrary Cq value chosen for the gene such as the difference between the lowest Cq value and the sample Cq [126, 163]. For the relative gene expression method we used the formula $R = (E_{target})^{\Delta Cq\ target(control-sample)} / (E_{reference})^{\Delta Cq\ reference(control-sample)}$. Where $E_{target}$ is the efficiency of the target gene, $E_{reference}$ is the efficiency of the reference gene, $\Delta Cq_{target}$ is the Cq deviation of control minus sample of the target gene and $\Delta Cq_{reference}$ is the Cq deviation of control minus sample of the reference gene [164].

**Next-Generation Sequencing and Transcriptomics analysis**

All the NGS-related work in our experiment was performed at the European Molecular Biology Laboratory (EMBL) in Germany. The RNA-Seq libraries were generated with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, while QuantSeq libraries utilised the Lexogen QuantSeq 3' polyadenylated RNA Library Prep Kit FWD for Illumina. Sequencing for both was performed on the Illumina NextSeq 500 platform. All the transcriptomics analysis included in our publsignallingas was done by the bioinformatician from our group. Briefly, the BAQCOM pipeline (https://github.com/hanielcedraz/BAQCOM) Toll-like receptors trimming, alignment and read count estimation. The respective reference genomes were downloaded from the Ensembl (https://www.ensembl.org/index.html). The differential gene expression (DGE) was done using the limma (Linear Models for Microarray Data) package [165] and the DESeq2 package in R.

**Statistical analysis**

All the statistical analysis was performed using the respective versions of R and R-studio software.

## General results and discussion

Animal health and immunity are of paramount importance due to the conservative, economic and, above all, zoonotic risks [2, 167]. Although there are immunological studies in animals, most of them are focused on laboratory-grown rodents, pigs or primates [72, 73, 168]. While these comparative studies are invaluable in the field of human biomedical field, they often overlook critical factors influencing animal immunity, such as the evolutionary context of the variation observed [80, 100, 119, 169]. With this in mind, a thorough examination of animal-centric studies has become imperative. Such research offers profound insights with far-reaching implications for economics, conservation efforts, and human health. Hence, we selected the avian immune system as our focal model, recognizing its potential to address the diverse applications mentioned above. So, we first tried to understand the genetic diversity of the avian immune system and reviewed the available studies on immunity and evolutionary immunology in the respective models.

We used birds of the order Passeriformes and Psittaciformes, which are understudied, although they represent the most suitable candidate models for the study of wildlife immunology and molecular evolutionary and comparative immunology. In general, our experimental results addressed the different expression patterns of immune-related genes during inflammation induced with diverse stimulants (both sterile inflammation and bacterial infection) in the respective avian model systems. Our studies were mostly comparing the different aspects of gene expression. Our studies were not limited to comparing gene expression in the periphery and central nervous system during bacterial and viral inflammation. But also explored variations in immune gene expression across house finch populations with varying evolutionary histories with the pathogen.

**Paper 1:** In this article, we have analysed the molecular evolution of the receptors of the innate immune system for the recognition of viral pathogens. First, we looked at the genes of the virus-recognising Toll-like receptor (TLR) family, which show less population and interspecific variation compared to other TLR family genes. Among vertebrate virus-sensing TLRs, the *TLR7* subfamily, which includes *TLR7*, *TLR8* and *TLR9*, shows evidence of stronger selection compared to *TLR3*. We also included the relatively understudied viral-sensitive TLR genes such as *TLR13*, *TLR19* and *TLR22*. The second family of genes located in the cytoplasm are the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The NLR family is also evolutionarily conserved in vertebrates. Among them, *NLRP1* is the most conserved with the fewest positively selected sites, but with a strongly positively selected site in the linker region connecting the nucleotide-binding oligomerization domain-like receptor with pyrin domain (PYD) to the nucleotide-binding domain (NBD) in mammals. In the case of *NLRP3*, the strongest selection site is in the leucine-rich repeats (LRR) in mammals, and the linker region connecting the NBD to the LRR in birds. In *NOD1* and 2, the linker regions and the CARD region also showed moderate selection that spared the LRR region. In *NLRC3* and *NLRC5*, the linker region together with the LRR region showed strong selection in birds. In the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in birds, the CARD domain (caspase recruitment domain) plays a role in downstream signalling and contains the most positively selected sites. In the *MDA5* gene from the same family, the most highly selected amino acid sites are in the helicase domain, which plays a role in RNA binding. Similar to *NLRP1*, strong positive selection in the linker region was also found for the IFI16 protein located in the nucleus in mammals, while no studies are available for this gene in birds. The *AIM2*, *OAS* and *CLR* genes showed weak to moderate positive selection in their respective domains. Further details are shown in figure 10.

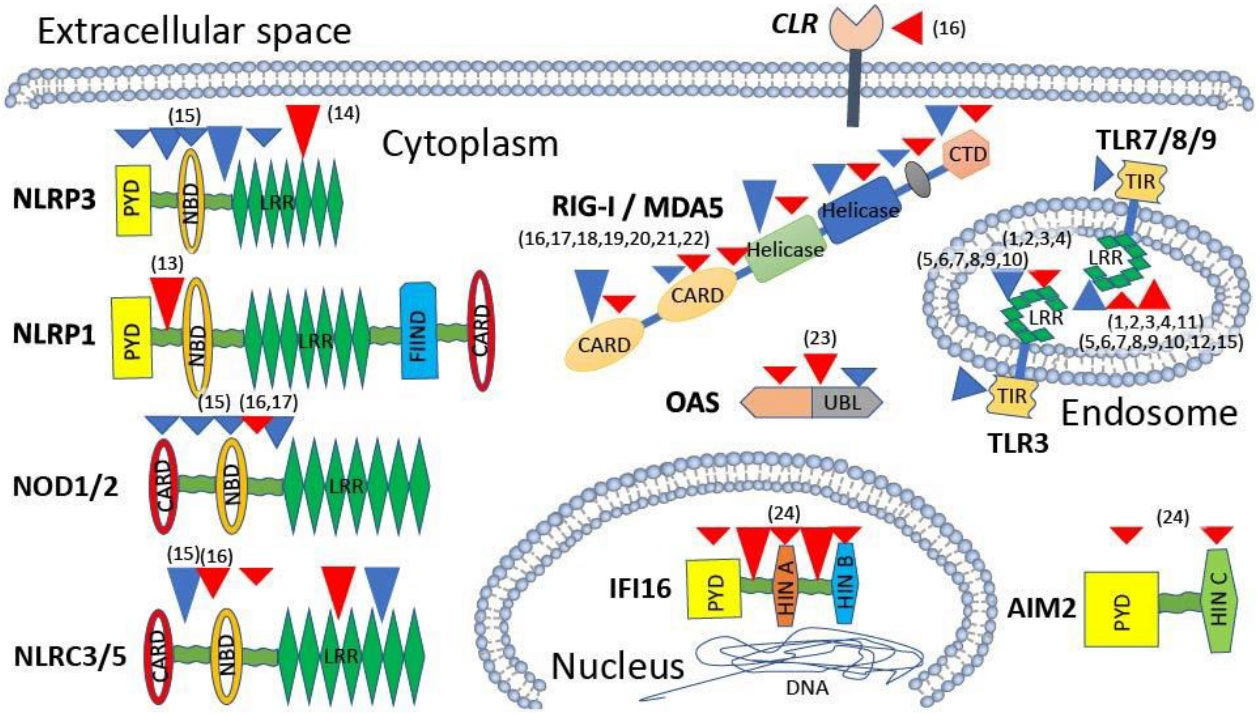


**Figure 8. Positive selection acting in vertebrate viral sensing pattern recognition receptors (PRRs).** PRR gene families were selected based on information available about their molecular evolution. Triangles

indicate domains and molecular regions (not sites) under positive selection; triangle colour indicates taxon in which positive selection was detected: red = mammals, blue = birds; triangle size indicates the intensity of positive selection in terms of numbers of positively selected sites detected (weak, medium or strong selection; for details see Table 1). TLR = Toll-like receptor, NLRP = nucleotide-binding oligomerization domain-like receptor with pyrin domain (PYD), NLRC = nucleotide-binding oligomerization domain-like receptor with caspase-recruitment domain (CARD) protein, NOD = nucleotide-binding oligomerization domain protein, RIG-I = retinoic acid-inducible gene I, MDA5 = melanoma differentiation-associated protein 5, OAS = oligoadenylate synthetase, AIM2 = Absent in melanoma-2, IFI16 = Interferon-inducible protein 16, CLR = C-type-lectin receptors. To each protein, different domains are shown: TIR = Toll/interleukin-1 (IL-1) receptor (TIR) domain, LRR = leucine-rich repeat, PYD = pyrin domain, CARD = caspase-recruitment domain, NBD = nucleotide-binding domain, FIIND = function to find domain, CTD = C-terminal domain, UBL = C-terminal tandem ubiquitin-like (UBL) domains, HIN = hematopoietic interferon-inducible nuclear antigens.

Neuroinflammation is linked to several diseases such as Alzheimer's, dementia, autism spectrum disorders, schizophrenia and major depressive disorder in humans [170–172]. In birds also it can cause social isolation, depression and other behavioural issues [145]. Neuroinflammation can have multiple reasons such as viral diseases, peripheral inflammation, gut inflammation or autoimmune disorders [34]. Among these, peripheral inflammation causing neuroinflammation is the least studied [34]. In our **papers 2 and 3** we have checked the immunological effects of viral as well as bacterial stimulants on peripheral inflammation causing neuroinflammation in parrots.

In **paper 2**, 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 (Fig.9). This is suggestive of the immune response in the periphery triggering neuroinflammation.

A previous study showed that when the chickens were injected with the poly(I:C), they displayed a reduction in food intake (anorexia) after 3 hours [173], which was also comparable to the previous reports in rodents [174, 175]. However unlike rodents the anorexia in chicken was not related to the interferon alpha, gamma or tumor necrosis factor in both brain and spleen [173]. This hints at the IFN, and TNF independent inflammation responses in birds treated with poly(I:C).

In this study, we also focused on the receptors identifying the poly(I:C) recognition of both TLR3 and NLRP3. The NLRP3 is assumed to be modulating the IL6 and IL1B, independent of TLR3 and through the CAP1 downstream signaling pathway. Our results showed a positive correlation between the intestinal as well as the brain expressions of *IL1B*, *IL6* and *CASP1*. This is similar to the previous avian studies, showing the consistency in expression patterns of different pro-inflammatory cytokines [126].

In our study both poly(I:C) doses showed a peak of inflammation at 3 to 6 hours and decline to baseline after 24 hours, this was comparable with the previous study conducted in mice by Cunningham et al (2007) [176], where they found that the pro-inflammatory cytokines IL1B and IL6 peaks at 3 hours and decline to baseline after 24 hours in mice injected with poly(I:C).

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.

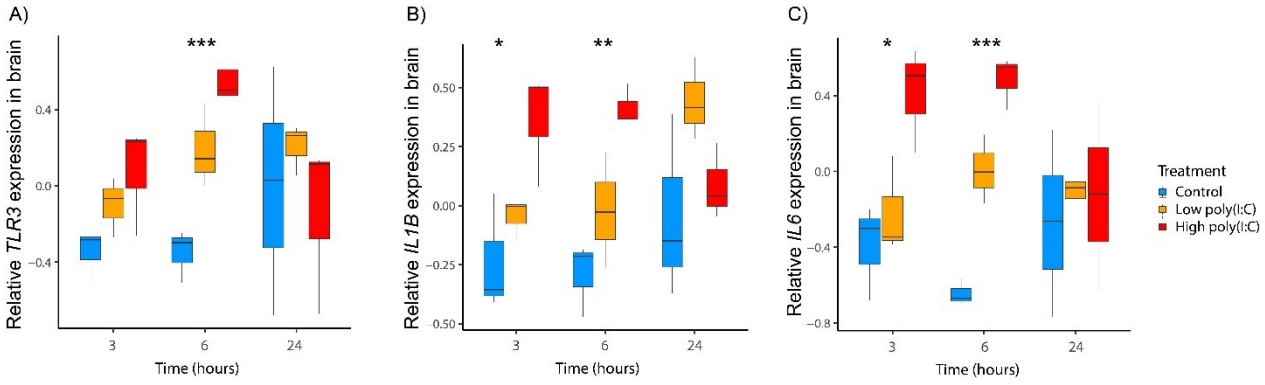


**Figure 9. Changes in relative *TLR3* (A) *IL1B* (B) *IL6* (C) gene expression in budgerigar brain at different time points during a response to poly(I:C).** The *TLR3* gene expression is shown as logQst values on the y-axis, and time across three sampling time points (3, 6 and 24 hours) is plotted on the x-axis. C = controls (green), L = low dose of poly(I:C) (orange), H = high dose of Poly(I:C) (red). The asterisks indicate the significant differences revealed by the TukeyHSD test: * for 0.010<P<0.050, ** for 0.001<P<0.010, *** for P << 0.001 (for details see Table S12 in ESM1).

**In paper 3**, initially, we examined the histopathology of the gastrointestinal tract (GIT) following DSS treatment. It showed that DSS caused significant structural alterations in the intestine, including erosion of the epithelial layer, irregular crypts, and shortened mucosal layer. However, we did not observe bleeding or significant weight loss in birds treated with lower doses of DSS. Next, we performed the proteomics analysis of PL and CSF to understand the changes in the protein composition. We identified a total of 180 proteins in PL and 978 proteins in CSF and an overlap of 155 proteins between PL and CSF. Ten proteins differentially expressed in the PL of the birds treated with DSS compared to the controls were associated with an immune process, coagulation and metabolic pathways. Meanwhile, the 73 proteins differentially expressed in the CSF were involved in oxidative phosphorylation, response to stress, and transport. We also found moderate co-structuring between the PL and CSF proteomes, with similarities between the control and DSS-treated groups as well as between the LPS-treated groups.

To assess the intensity of inflammation in the brain and intestine, we measured the mRNA expression levels of the pro-inflammatory cytokines. We found a significant increase in *IL1B* expression in the intestine and *IL6* expression in the colon following LPS treatment, but no significant effect of DSS treatment. Thus, this experiment showed that the peripheral immune response can change the brain metabolism in parrots. It also demonstrated that a small dosage of subcutaneous LPS can cause systemic inflammation within 6 hours, but a DSS administration for 7 days has no such effect on immunity. Our findings are comparable to Talley et al. (2021) [177], who found that both peripheral inflammation by DSS and LPS can induce

neuroinflammation in the mice. However, in mice, the DSS injection resulted in higher neuro-inflammation than LPS [177], but our study in the budgerigar LPS induced higher neuroinflammation compared to the DSS treatments. We also revealed that for low-grade inflammation in the budgerigar model, LPS rapidly dysregulates the proteome composition in the biological fluids, and this is linked to the pro-inflammatory cytokine transcription profile. Meanwhile, the DSS has a much milder effect on the proteome composition of biological fluids. To conclude we found that the peripheral inflammation caused by LPS and also by DSS (through the gut-brain axis), had a significant impact on the brain physiology of budgerigar.

In **paper 4** we have compared two different transcriptomics approaches namely the QuantSeq and RNA-Seq followed by the RT-qPCR, to confirm the expression of inflammatory cytokine in skin and brain tissue of zebra finches after 24 hours of peripheral LPS injection. Our initial RNA-Seq analysis in the skin showed fewer immune-related genes. However, with the QuantSeq approach in the skin, we observed an upregulation of numerous immune-related genes. Some of these gene expression patterns were also validated with RT-qPCR (Fig 10), which yielded comparable results. So, we did only the QuantSeq analysis for the brain and found upregulation of some immune-related genes and cytokines such as *AVBD10*, *AVD*, CXCLI1, CXCLI2 and *IL1B*. 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. Comparative analyses are necessary for unravelling the fundamental tenets of neuro-immune interplay regulation. 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.

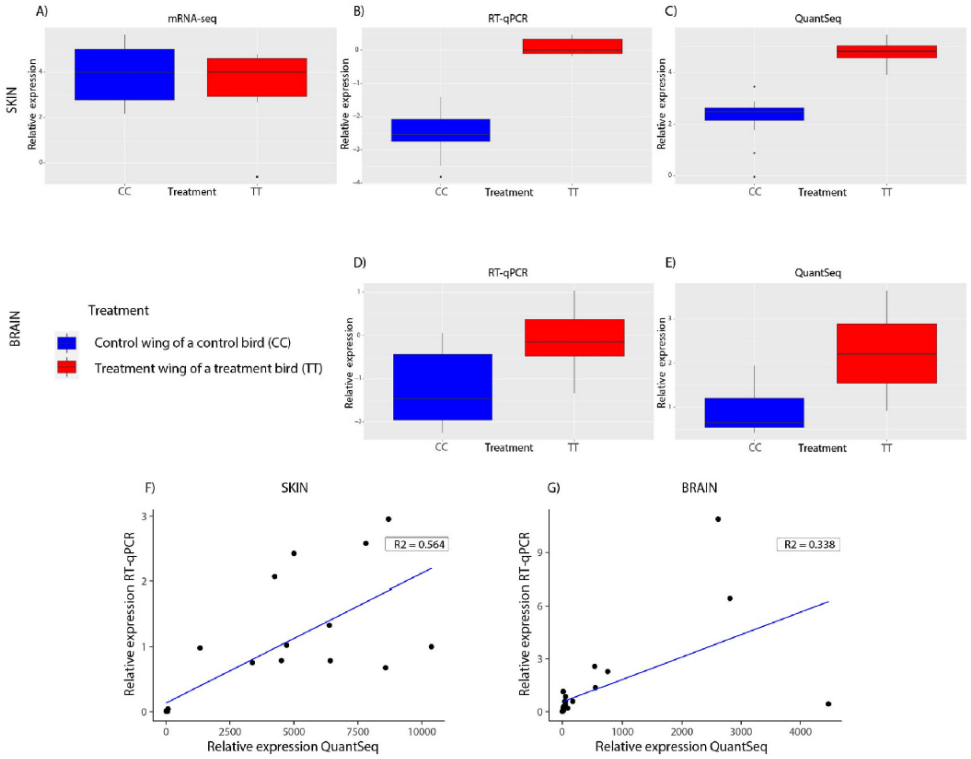

**Figure 10.** Expression changes in the AVD-like gene estimated through (A) RNA-seq (RS1), (B) RT-qPCR, (C) QuantSeq approaches in the skin samples of controls (CC)and treatment individuals (TT) with peripheral response stimulated with bacterial lipopolysaccharide (LPS) in zebra finch. (D) RT-qPCR aI(E) QuantSeq show AVD-like gene expression in the brain during this peripheral response. Correlation between the RT-qPCR and QuantSeq data on the AVD-like gene expression in skin, r = 0.751, p << 0.001 (F) and in the brain, r = 0.581, p = 0.003 (G). ( Courtesy **:** [32]**.**

The naturally occurring wild host-pathogen systems enabling studies of immune evolution are rarely established in vertebrates [125]. In our **papers 5 and 6** we have used a most-studied evolutionary system in birds the house finch-mycoplasma system [178]. 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), which can be compared to some other similar studies [176, 179, 180]. So, for our experiment, we used conjunctival tissue, which is the tissue directly affected during mycoplasmal infection.

In our **paper 5**, using the QuantSeq sequencing, we 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 (Fig.11). 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. It was also found that *ACOD1*, which is a negative regulator of inflammation was also upregulated. Our findings diverge from previous research, revealing population-specific immune response adaptations in the Harderian gland tissue from the same birds [124]. 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.

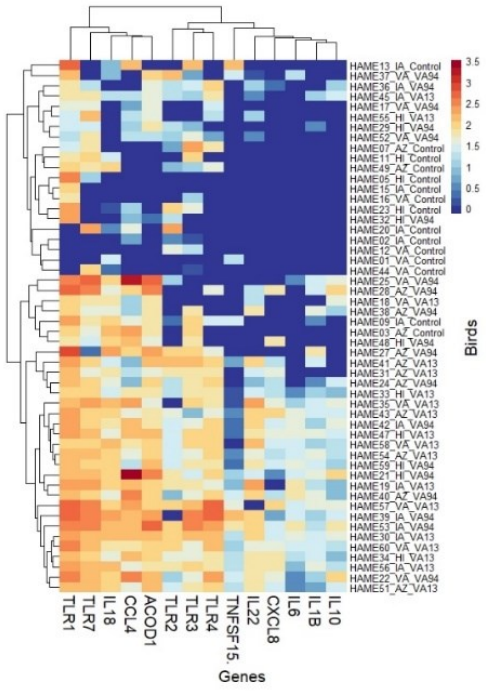

**Figure 11: Heatmap showing relative gene expression changes in inflammation-regulating genes (cytokines and receptors) in conjunctiva across house finches from four different populations belonging to two types of *Mycoplasma gallisepticum* (MG)-infected treatments (VA1994 and VA2013) and controls.** The y-axis provides information on individual birds (including population name and treatment group); the x-axis shows the gene names; the colour indicates normalised read count from no change (blue) to high fold-change (red). **(Courtesy:** [181]

**In our paper 6,** 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 [124, 181]. 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. However, a previous study on house finches mentioned that the pathogen load and the immune-related gene expressions are not directly related to house finch populations [182]. Our previous research [181], also identified the possible immunomodulating role of *BCL10* in the emergence of tolerance in MG. Thus we conclude that the IA populations' relatively strong pro-inflammatory response upon the MG infection, could be reduced later by the *BCL10* downregulation, weakening the overall inflammatory response that harms the host health. 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.

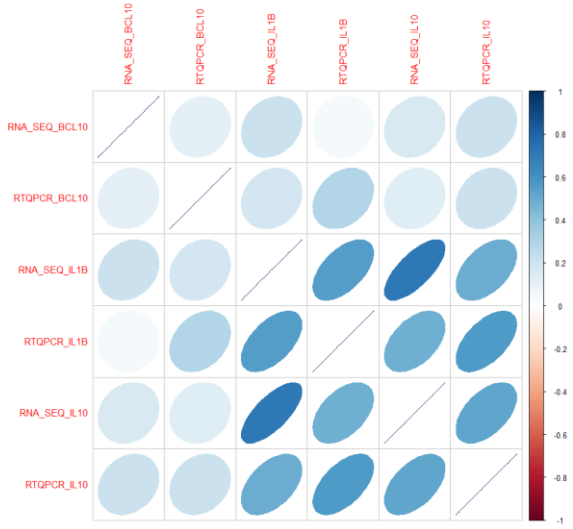


**Figure 12**: **House finch Transcriptomics vs RT-qPCR.** The gene-pairs with positive correlation are depicted with positive slopes and blue colour and genes with negative correlation are depicted with negative slopes and red colour. The intensity of the colour and cloud shape indicates the size of the correlation coefficient.

## 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 in birds. 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 peripheral treatment induced by DSS and LPS treatments in parrots demonstrated that both treatments significantly influenced neuroinflammation. However, the LPS had a more noticeable effect on neuroinflammation compared to the DSS treatment. In our zebra finch transcriptomics study by comparing the two different transcriptomics methods, we identified that the QuantSeq method can act as a cost-effective alternative method to the classical RNA-Seq method in identifying key markers of inflammation-related genes.

In the 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, it suggests 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 the *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. Though there was no correlation in the *BCL10* gene expression from our transcriptomics and QuantSeq study, its comparable expression pattern with the pro-inflammatory cytokines suggests a pivotal role for this gene in the pro-inflammatory cytokine regulation in the house finch population during mycoplasmal infection. Our future investigation will concentrate on clarifying the role of various immune cell subsets in the immune response to MG along side

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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# Appendix