Charles University

Faculty of Science

Department of Zoology

Study programe: Zoology

Mgr. Martin Těšický

Host-microbiota, pro-inflammatory immunity and physiological senescence in wild birds

Mikrobiota hostitele, zánětlivá imunitní odpověď a fyziologická senescence u volně žijících ptáků

Doctoral thesis

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

Co-supervisor: prof. Mgr. Tomáš Albrecht, Ph.D.

Co-supervisor: Mgr. Hana Velová, Ph.D.

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Title

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Title in Czech

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Author

Martin Těšický

Department of Zoology

Faculty of Science

Charles University

Czech Republic

Study programme: Zoology

Branch of study: Zoology

Supervisor

doc. Mgr. Michal Vinkler, Ph.D.

Associate professor

Department of Zoology

Faculty of Science

Charles University

Czech Republic

Co-supervisor

prof. Mgr. Tomáš Albrecht, Ph.D.

Full professor

Department of Zoology

Faculty of Science

Charles University

Czech Republic

Institute of Vertebrate Biology

The Czech Academy of Sciences

Czech Republic

Co-supervisor

Mgr. Hana Velová, Ph.D.

Department of Zoology

Faculty of Science

Charles University

Czech Republic

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Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Tuto práci jsem zpracoval samostatně a uvedl všechny použité informační zdroje a literaturu.

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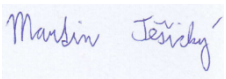

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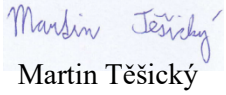

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STATEMENT OF CONTRIBUTION

This thesis is a cumulative work consisting of 15 manuscripts (11 published and 4 submitted). I am the first and corresponding author of 4 papers and the second author of 2 papers. The Journal Impact Factor Quartile (JIFQ) is from the Web of Science, using the highest quartile from all categories that include a particular journal. Journal metrics are valid for the year of publication. Citation reports are valid on the 1st of January 2023. My contributions to each manuscript comply with the *CRediT authorship statement* (Brand et al. 2015).

Kropáčková, L., **Těšický, M.**, Albrecht, T., Kubovčiak, J., Čížková, D., Tomášek, O., Martin, J-F., Bobek, L., Králová, T., Procházka, P. & Kreisinger, J. (2017). Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence. *Molecular Ecology*, *26*(19), 5292–5304. doi: 10.1111/mec.14144

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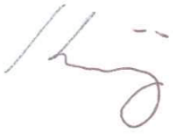

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Contribution of Martin Těšický: Formal analysis, Investigation, Data curation, Writing – Review & Editing

doc. RNDr. Michal Vinkler, Ph.D.

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Citations (Google Scholar|Web of Knowledge) = 0|0; IF_{2022} = 3.23; JIFQ = Q1

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doc. RNDr. Michal Vinkler, Ph.D.

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doc. RNDr. Michal Vinkler, Ph.D.

Bauerová, P., Krajzingrová, T., **Těšický, M**., Velová, H., Hraníček, J., Musil, S., Svobodová, J., Albrecht, T. & Vinkler, M. (2020). Longitudinally monitored lifetime changes in blood heavy metal concentrations and their health effects in urban birds. *Science of the Total Environment*, *723*. doi: 10.1016/j.scitotenv.2020.138002

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Contribution of Martin Těšický: Investigation, Writing – Review & Editing

doc. RNDr. Michal Vinkler, Ph.D.

Preface

Modern science is highly interdisciplinary and this work would be difficult to achieve without the involvement of many experts from different fields such as evolutionary immunology and genetics, ornithology, microbial ecology, biostatistics, bioinformatics and also analytical chemistry.

First of all, I am extremely grateful to my supervisor Michal Vinkler for his wide-ranging support. Michal opened the door to the fascinating world of evolutionary and ecological immunology for me. He was always a supportive mentor, open to discussions on various scientific and non-scientific topics, providing the necessary criticism and enough funding for our research, taking me to many congresses and networking me with others. I am very grateful to Tomáš Albrecht for his idea to study the physiological mechanisms of senescence and for his manifold help with the statistical analysis. I would like to express my sincere gratitude to Hana Velová, to whom I am indebted for her initial help and guidance in molecular genetic methods and bioinformatics. I am very grateful to Wieslaw Babik, who kindly hosted me in his Genomics and Experimental Evolution Group in Krakow, not only ones during my long Erasmus internships. He inspired my evolutionary biology thinking and became a great mentor who introduced me to computational bioinformatics for me. My great thanks also go to Jakub Kreisinger and Lucie Schmiedová, who invited me to participate in their projects on the evolution of the host microbiota, who always kindly helped me multiple times with my projects, including wet lab, and who provided me with bioinformatic and statistical analysis of sequencing microbial data. I would like to also well appreciate Marián Novotný for his help with the three-dimensional modelling of protein structures. Analysis of blood antioxidants would hardly have been possible without the great help of Oldřich Tomášek.

From the other collaborators, I am grateful to Kamila Syslová for performing oxidative damage marker analysis, Jakub Hraníček and Stanislav Musil for performing the heavy metal analysis, Anna Bryjová and Dagmar Čížková from Studenec for their kind help in the wet lab and Vladimír Beneš and Boris Tichý for sequencing our samples. I am also grateful to our external collaborators Daniel Elleder and Piotr Minias who invited me to participate in various immunogenetics projects.

I must also mention the current and former members of our Evolutionary and Ecological Immunology Laboratory, who created a pleasant working environment and provided me with numerous pieces of advice: Zuzana Swiederská and Barbora Bílková for measuring the oxidative burst, Tereza Krajzingrová, who performed the haematological analysis of the relative white blood cell counts, Mercedes Gomez for her help with the qPCR analysis, and further Dan Divín, Baleraj Melepat, Nithya Kuttiyarthu Veetil, Eleni Voukali, Katka Černá and Tao Li. I would like to thank all the other people in the Department of Zoology for the inspiring, creative, friendly and supportive environment, including our secretariat who helped in the smooth completion of many tasks. Many thanks also belong to the team members of Wieslaw's Genomics and Experimental Evolution Group not only for many useful suggestions but also for nice spare time in Krakow and its surroundings, namely Bernardo Flores Antunes, Tomek Gaczorek, Magdalena Migalska, Gemma Palomar, Piotr Zieliński and Anastasiia Mykhailenko!

Almost a decade of field great tit research could be hardly achieved without the involvement of many field collaborators including Jana Svobodová, Petra Bauerová, Hana Pinkasová, Jitka Vinklerová, Jiří Eliáš (also

providing me with plumage ornament measurements and absolute white blood cell count), Tomáš Vrkoslav (who also helped me with the ptilochronological analysis), Sylvie Dlugošová, Julie Vacková, Petra Blahutová, Petra Špatenková, Lenka Pelikánová, Barbora Stolínová, Monika Dvořáková, and Martin Rychlý. I am grateful to Steven Fiddaman, who kindly devoted much of his time to linguistic correction and helpful comments on the final draft, and to Karel Kodejš for the beautiful illustrations.

Last but not least, I would like to thank a lot to my family, my wife Klára and my friends for all their great care and wide-ranging support.

Looking back, I think that we came up with some important novel findings in the field of microbial ecology, evolutionary immunology and biogerontology and some of them even open new research directions. At the same time, there are more samples to process and data to be analysed (especially those the most integrative ones that would better crosslink various topics). This, together with great topic diversity and also some gaps in my understanding of the investigated systems studied despite my continuos effort, made it impossible for me to summarise the content of this work in a single logical and comprehensive chapter. Nevertheless, I hope that this does not diminish the importance of this work. I also hope that it will be possible to finish most of the work that has been started in the future. Perhaps I should mention that I extended my doctoral studies by two and a half years, partly because of involvement in several research projects, two long internships abroad that went far beyond the original scope of the doctoral thesis, COVID-related issues but also because I tried to find bacteria in nearly sterile samples and other scientific issues 😊. As a consequence, the topic of my dissertation has growth up a bit from the initial "*Biology of ageing and immunosenescence in the great tit (Parus major)*" to the current range…

Martin Těšický

Prague, 3rd January 2023

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List of content

Summary

Triggered by microbial ligands, inflammation serves as a "double-edged sword" to fight infections on the one hand, but on the other hand causing tissue damage due to oxidative stress if it is dysregulated. For example, chronic inflammation can contribute to *inflammaging*, which is now widely regarded as one of the causes of ageing. In my interdisciplinary dissertation, my colleagues and I investigated three interrelated aspects of inflammation, using an evolutionary framework and various free-living birds as models: (1) ecological and evolutionary determinants of gut microbiota (GM) composition and diversity, a driver of wild bird immunity, (2) diversity in immune genes affecting inflammatory responses in wild birds and (3) inflammation-related physiological senescence in a free-living passerine bird, the great tit (*Parus major*). Firstly, using *16S rRNA* gene metabarcoding, we revealed high intra- and interspecific variation in passerine gut microbiota (GM) dominated by the major phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Although in mammals GM depends strongly on host phylogeny and diet, in birds we found only moderate effects of phylogeny and very limited effects of host geography and ecology on GM composition. While microbiota diverged between the upper and lower gastrointestinal tracts (GIT), the microbiota of the adjacent tissues in the lower GIT was very similar, which is consistent with the relatively homogeneous GIT morphology in passerines and parrots. To understand the initial recruitment of GM and the mechanisms behind it in birds, we further investigated the microbiome of avian egg content and developing embryos in the great tit. We found that bird eggs were nearly sterile before hatching, suggesting that GM must predominantly form only after hatching in passerines. All this shows that GM is different in passerines compared to mammals and highlights that results derived from mammalian GM studies cannot be generally translated to passerines. Secondly, we developed a broadly applicable methodological pipeline to detect adaptive variation in protein-coding genes based on structural evolutionary bioinformatics, positive selection and adaptive convergence testing. Adopting this pipeline, we revealed that receptors of innate immunity, such as Toll-like (*TLRs*) and RIG-like receptors (*RLRs*) were highly variable in their ligand-binding regions in birds and much of their variation evolved adaptively. For the first time, we detected multiple gene losses in viral-sensing *RLRs*, retinoic acid-inducible gene I (*RIG-I*) and melanoma differentiation-associated protein 5 (*MDA5*) across the avian phylogeny. We further discovered the intriguing gene loss of cannabinoid receptor 2 (*CNR2*) in parrots, which negatively regulates inflammation and whose loss led to increased brain neuroinflammation. Thirdly, using longitudinally monitored great tits, we demonstrated senescence in multiple physiological traits that differ in their lifetime trajectories Chronic inflammation, which was positively associated with general oxidative stress damage, increased with ageing, documenting for the first time *inflammaging* in birds. Conversely, induced cellular inflammatory responses underwent bell-curved trajectories, consistent with *immunosenescence*. The same polynomial age-related trend in male plasma testosterone documented *hormonal senescence*. In contrast, levels of heavy metals in blood were largely independent of age, showing that bird blood can be used to monitor current heavy metal exposure even if the age is not known. All this suggests that small passerines undergo similar age-related changes as mammals.

Souhrn

Mikrobiálními ligandy vyvolaný zánět je klíčový imunologický proces, který na jedné straně zajišťuje obranyschopnost proti infekčním onemocněním, ale jeho dysregulace může vést také k oxidačnímu poškození tkání. K tomu dochází např. během chronického zánětu, který může způsobovat tzv. *inflammaeging*, o němž se nyní uvažuje jako o možné příčině stárnutí. Ve své multidisciplinární disertaci jsem za využití evolučně-komparativních přístupů a ptáků jako modelové skupiny zkoumal tři vzájemně propojené aspekty zánětu: (1) evoluční a ekologické determinanty vnitro- a mezidruhové variability střevní mikrobioty (SM), které ovlivňují evoluci ptačí imunity, (2) genetickou diverzitu imunitních genů ovlivňující zánětlivou imunitní odpověď u ptáků a (3) stárnutí ve fyziologických znacích spojených s tzv. *inflammagingem* na modelu volně žijícího pěvce, sýkory koňadry (*Parus major*). (1) Pomocí sekvenování bakteriální *16S rRNA* jsme zjistili značnou vnitro- i mezidruhovou diverzitu ve složení SM u pěvců a dominanci taxononů z kmenů Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. V porovnání se savčí SM, které je do velké míry determinována fylogenezí hostitele a jeho stravou, naše výsledky u pěvců ukazují pouze na středně silný vliv fylogeneze hostitele a velmi slabý vliv ekologických faktorů (potrava a geografie). Zatímco složení mikrobioty mezi dolním a horním trávicím traktem bylo značně divergované, SM se mezi různými úseky střeva příliš nelišila, což také dobře koresponduje s málo morfologicky diverzifikovaným dolním trávícím traktem létavých ptáků (u pěvců a papoušků). Sekvenování mikrobiomu ptačího vejce u sýkory koňadry odhalilo, že jejich vejce je *téměř sterilní* a že ke kolonizaci ptačího střeva mikrobiotou dochází u pěvců až po vylíhnutí. Naše výsledky tak ukazují, že složení SM se výrazně liší mezi savci a pěvci a že výsledky získané studiem savčí mikrobioty nejsou univerzálně přenositelné na ptáky. (2) Abychom detekovali adaptivní variabilitu v imunitních protein-kódujících genech, vyvinuli jsme univerzální metodologický postup využívající detekci pozitivní selekce, adaptivní konvergence a dalších evolučně-bioinformatických metod. Vazebná místa receptorů vrozené imunity, konkrétně Toll-like receptorů (*TLRs*) a RIG-like receptorů (*RLRs*) byla u ptáků velmi variabilní a většina této variability byla predikovaná jako adaptivní. Jako první jsme u ptáků detekovali mnohočetné ztráty v RLRs, konkrétně v genech *RIG-I* (*retinoic acid-inducible gene I*) a *MDA5* (*melanoma differentiation-associated protein 5*). Podobně také gen pro kanabinoidní receptor 2 (*CNR2*) byl opakovaně ztracen u papoušků, což by mohlo vysvětlit, proč papoušci častěji trpí neurozánětem v mozku a různými neurodegenerativními onemocněními. (3) Naše výsledky u sýkory koňadry ukázaly na fyziologickou senescenci v mnoha sledovaných znacích, jejichž trajektorie se nicméně během stárnutí značně lišila. Zatímco chronický zánět spolu s oxidační poškozením tkáním postupně lineárně narůstal s věkem, což poprvé u ptáků jednoznačně ukazuje na vliv *inflamagingu* během stárnutí, experimentálně vyvolaná buněčně-zánětlivá odpověď měla polynomickou závislost na věku, což ukazuje na *immunosenescenci* ve funkční imunitní odpovědi. Podobnou polynomickou závislost na věku měla také hladina samčího testosteronu, což naznačuje na hormonální senescenci. Naopak hladina těžkých kovů v krvi prakticky narůstala během stárnutí u opakovaně odchycených jedinců, což naznačuje, že stanovení těžkých kovů v krvi se může využít pro biomonitoring environmentální kontaminace těžkými kovy i v situacích, neznáme-li přesně věk odchycených ptáků. Naše výsledky tak ukazují, že u malých pěvců dochází k podobným fyziologickým změnám během stárnutí jako u savců.

List of papers

Host microbiota in gastrointestinal tract

I. Kropáčková, L., **Těšický, M.**, Albrecht, T., Kubovčiak, J., Čížková, D., Tomášek, O., Martin, J-F., Bobek, L., Králová, T., Procházka, P. & Kreisinger, J. (2017). Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence. *Molecular Ecology*, *26*(19), 5292–5304. doi: 10.1111/mec.14144

II. Schmiedová, L., Kreisinger, J., Kubovčiak, J., **Těšický, M.**, Martin, F.-P.J., Tomášek, O., Kauzálová, T., Sedláček, O. & Albrecht, T. (2023). Gut microbiota variation between climatic zones and due to migration strategy in passerine birds. *Frontiers in Microbiology*, 1–13. doi.org/10.3389/fmicb.2023.1080017

III. Kubovčiak, J., Schmiedová, L., Albrecht, T., **Těšický, M.**, Tomášek, O., Kauzálová, T. & Kreisinger, J. (2022). Within-community variation of interspecific divergence patterns in passerine gut microbiota. *Ecology and Evolution*, 12:e9071. https://doi.org/10.1002/ece3.9071

IV. Kropáčková, L., Pechmanová, H., Vinkler, M., Svobodová, J., Velová, H., **Těšičký, M.**, Martin, J.-F. & Kreisinger, J. (2017). Variation between the oral and faecal microbiota in a free-living passerine bird, the great tit (*Parus major*). *PLoS ONE*, *12*(6). doi: 10.1371/journal.pone.01799450

V. Schmiedová, L., Černá, K., Li, T., **Těšický, M.**, Kreisinger, J. & Vinkler, M.: Bacterial communities along parrot digestive and respiratory tracts: the effects of sample type, species and time *(submitted to International Microbiology*)

VI. **Těšický, M.**, Schmiedová, L., Krajzingrová, T., Gómez Samblás, M.M., Bauerová, P., Kreisinger, J. & Vinkler, M.: Nearly (?) sterile avian egg (*submitted to FEMS Microbiology Ecology*)

Evolution of innate immunity receptor diversity

VII. Vinkler, M., Fiddaman, S.R., **Těšický, M.**, O'Connor, E.A., Savage A.E, Lenz, T.L., Smith, A.L., Kaufman, J., Bolnick, D., Davies, CH.L., Dedic, N., Flies, A.S., Gómez Samblás, M. M., Henschen, A., Novák, K., Palomar, G., Raven, N., Samake, K., Slade, J., Veetil, N. K., Voukali, E., Höglund, J., Richardson, D.S. & Westerdahl, H.: Understanding the evolution of immune genes in vertebrates (*submitted to Journal of Evolutionary Biology*)

VIII. **Těšický, M.**, Velová, H., Novotný, M., Kreisinger, J., Beneš, V. & Vinkler, M. (2020). Positive selection and convergent evolution shape molecular phenotypic traits of innate immunity receptors in tits (Paridae). *Molecular Ecology*, (April), 3056–3070. doi: 10.1111/mec.15547

IX. Włodarczyk, R., **Těšický, M.**, Vinkler M., Novotný, M., Remisiewicz, M., Janiszewski, T. & Minias, P.: Divergent evolution drives high toll-like receptor (TLR) diversity in passerine birds: buntings and finches (*submitted to Developmental and Comparative Immunology*)

X. Krchlíková, V., Hron, T., **Těšický, M**., Li, T., Hejnar, J., Vinkler, M., & Elleder, D. (2021). Repeated MDA5 gene loss in birds: An evolutionary perspective. *Viruses*, *13*(11), 1–12. doi: 10.3390/v13112131

XI. Krchlíková, V., Hron, T., **Těšický, M.**, Li, T., Ungrová, L., Hejnar, J., Vinkler, M. & Elleder, D. (2023). Dynamic evolution of avian RNA virus sensors: Repeated loss of *RIG-I* and *RIPLET*. *Viruses, (1)*15, 1-15. doi.org/10.3390/v15010003

XII. Divín, D., Goméz Samblas, M., Kuttiyarthu Veetil, N., Voukali, E., Świderská, Z., Krajzingrová, T., **Těšický, M.**, Beneš, V., Elleder, D., Bartoš, O. & Vinkler, M.: Cannabinoid receptor loss makes parrots susceptible to neuroinflammation. *Proceedings of the Royal Society B*, 289. doi: 10.1098/rspb.2022.1941

Physiological senescence

XIII. **Těšický, M.**, Krajzingrová, T., Eliáš, J., Velová, H., Svobodová, J., Bauerová, P., Albrecht, T. & Vinkler, M. (2022). Inter-annual repeatability and age-dependent changes in plasma testosterone levels in a longitudinally monitored free-living passerine bird. *Oecologia*, *198*(1), 53–66. doi: 10.1007/s00442-021-05077-5

XIV. **Těšický, M.**, Krajzingrová, T., Świderská, Z., Syslová, K., Bílková, B., Eliáš, J., Velová, H., Svobodová, J., Bauerová, P., Albrecht, T. & Vinkler, M. (2021). Longitudinal evidence for immunosenescence and inflammaging in free-living great tits. *Experimental Gerontology*, *154*, 111527. doi: https://doi.org/10.1016/j.exger.2021.111527

XV. Bauerová, P., Krajzingrová, T., **Těšický, M**., Velová, H., Hraníček, J., Musil, S., Svobodová, J., Albrecht, T. & Vinkler, M. (2020). Longitudinally monitored lifetime changes in blood heavy metal concentrations and their health effects in urban birds. *Science of the Total Environment*, *723*. doi: 10.1016/j.scitotenv.2020.138002

Other papers (not part of the thesis)

Palomar, G., Dudek, K., Wielstra, B., Jockusch, E. L., Vinkler, M., Arntzen, J. W., Ficetola, G.F., Matsunami, M., Waldman, B., **Těšický, M.**, Zieliński, P & Babik, W. (2021). Molecular Evolution of Antigen-Processing Genes in Salamanders: Do They Coevolve with MHC Class I Genes? *Genome Biology and Evolution*, *13*(2), 1–15. doi: 10.1093/gbe/evaa259

Veetil, N.K., Oliveira, H.C., Gómez Samblás, M.M., Divín, D., Melepat, B., Voukali, E., Świderska, Z., Krajzingrová, T., **Těšický, M.**, Beneš, V., Madsen, O. & Vinkler, M. Application of the 3' mRNA transcriptomic sequencing (QuantSeq) for identification of differential gene expression during neuroinflammation in the zebra finch (*submitted to Immune network*)

1. General introduction

Parasitism is one of the most common relationships on Earth. Virtually all living organisms are challenged by some parasites, such as bacteria and viruses. According to the common definition, parasites tend to reduce the fitness of the host by reducing its nutrition or causing pathogenesis (Schmid-Hempel 2021). Animal hosts have developed the immune system as a basic tool to protect themselves from parasite attacks. The proper functioning of the immune system has a paramount effect on individual reproduction and survival, i.e. on two essential features that determine the fitness of the organism. As the first line of defence, inflammation is the key immunological process in the fight against pathogens and affects other physiological systems, short- and long-term fitness and survival of the individual. Inflammation is a universal cellular effector immune response that occurs in all vertebrates (Sorci and Faivre 2009).

During inflammation, phagocytic cells encapsulate pathogens by phagocytosis into the phagosome, a membrane vesicle that further fuses with other vesicles containing highly reactive molecules. Here, the encapsulated bacteria are lysed by the production of toxic reactive oxygen species (ROS) or by granzymes in the reaction known as oxidative burst (Danilova 2006). However, its activation is costly and the strength and duration of inflammation must be tightly regulated, otherwise it leads to self-damaging immunopathology (Graham et al. 2005; Ashley et al. 2012). This is all orchestrated by various pro-inflammatory and anti-inflammatory molecules (Feghali and Wright 1997). While weak inflammation may not suppress the infection, leading to persistence (i.e. chronic inflammation), excessive inflammation can cause more tissue damage through oxidative stress than the pathogen itself (Sorci and Faivre 2009). During senescence, inflammation often has a self-reinforcing effect. It gradually increases and impairs various tissues, accelerating senescence and causing inflammaging, which is now widely regarded as one of the mechanisms of ageing (Pawelec et al. 2014).

Whether inflammation is triggered depends initially on the activation of specific innate immunity receptors on phagocytic cells, such as pattern recognition receptors (PRRs), which recognise specific pathogenic ligands as in the lock-and-key system (Mogensen 2009; Kawai and Akira 2011). In natural populations, *Reed Queen dynamics* predict great diversity in both the germ-encoded PRRs and their microbial ligands (Miller et al. 2005). While most microbial ligands originate from the commensal microbiota and normally only stimulate the immune system via PRRs to maintain intestinal homeostasis, some ligands originate from pathogens and can trigger a strong inflammatory response (Honda and Littman 2012; Eloe-Fadrosh and Rasko 2013; Ost and Round 2018). In the mammalian gut, for example, Firmicutes produce substances short-chain fatty acids (SCFAs) that generally prevent inflammation, while Bacteriodetes have more pro-inflammatory effects (Skillington et al. 2021). In young and healthy people, there is an equilibrium in the

abundance of the major bacterial phyla, but this can be shifted towards an increased proportion of Bacteroidetes to Firmicutes in older people, leading to a pro-inflammatory state (Claesson et al. 2011; Skillington et al. 2021). Although these mechanisms are postulated in biomedical studies, these studies often work with laboratory models that have reduced variability in both immune genes and microbiota, and where natural selection is not taken into account or where individuals are kept under stable conditions (Pedersen and Babayan 2011). To better understand the evolution of inflammation, we need to go into the wild and examine the host-pathogen co-evolution of both interacting counterparts (diversity of microbes and innate immunity receptors that recognise them) along with changes occurring in this relationship during ageing (i.e. inflammation-related senescence) in natural populations. In particular, wild birds, homeothermic amniotes evolving in parallel to the best-studied mammals and with their enormous ecological and evolutionary diversity, could serve as alternative models for such investigations. Currently, these mechanisms are poorly understood in avian systems and this motivated my colleagues and I to focus on wild birds. Besides the fundamental understanding of the host-parasite arms race or the causes of ageing, uncovering these mechanisms could also have practical implications, e.g. for animal breeding or understanding resistance to infectious diseases (zoonosis). This is in line with the idea of the One Health concept, which assumes that human health is closely linked to animal health and the state of the entire ecosystem (Prata et al. 2022).

1.1 Host microbiota in the gastrointestinal tract

The vertebrate gastrointestinal tract (GIT) is colonised by taxonomically diversified microbial communities, mainly commensal bacteria (Xu and Gordon 2003; Costello et al. 2009; Kreisinger et al. 2014). In the host gut, the number of bacterial cells is comparable to or even slightly higher than the number of somatic cells in the host, at around 100 trillion (Sender et al. 2016), but the total number of bacterial genes may be as much as two orders of magnitude higher (Qin et al. 2010). The host gut microbiota (GM) plays a prominent role in host physiology. It affects nutrient digestion, production of some vitamins such as B12 (Bäckhed et al. 2005), detoxification (Grond et al. 2018), GIT development (Ost and Round 2018) and gut-brain-axis signalling (Strandwitz 2018). The impact of GM on the development and balance of the immune system is particularly important. The gut forms the largest surface area exposed to microbes (e.g. human GIT mucosal surface area including the crypts and microvilli is about half of a badminton court ~ 30 m^2; Helander and Fändriks 2014) and is interspersed with gut-associated lymphoid tissue (GALT), the largest mass of lymphoid tissue in the body (Mörbe et al. 2021). For instance, a healthy, balanced commensal microbiota stimulates PRRs via their ligands, which help induce a tolerogenic immune response through regulatory lymphocytes (T_{reg}; via secretion of anti-inflammatory cytokines, IL-10 and TGF-β) and maintain intestinal homeostasis. This is characterized by the active innate mucosal

barrier, antimicrobial peptide and IgA secretion that all prevent pathogen colonisation. In contrast, dysregulated (pathogenic) microbiota can break the innate physical barrier mechanisms and lead to gut leakage and inflammation. In this environment, the epithelium can secrete pro-inflammatory cytokines, i.e. IL-1 and IL-6 in response to danger signals. Together with the dendritic cells, this triggers a potentially harmful pro-inflammatory immunity towards Th_1 and Th_{17} response (Ost and Round 2018). Furthermore, it has recently become clear that the composition of the microbiota can influence onset and/or progression of many diseases, including the inflammatory ones, such as colorectal cancer or inflammatory bowel disease (Assayag et al. 1997; Honda and Littman 2012).

Host GM has been studied for decades, but mostly in laboratory mammals and humans (Nguyen et al. 2015). The widespread use of next-generation sequencing (NGS) has revolutionised the field of microbial ecology in the past decade, opening it for studies in wild animals (Boughner and Singh 2016). These studies have revealed striking differences in GM composition not only between laboratory and wild living animals (Xenoulis et al. 2010; Kreisinger et al. 2014; McKenzie et al. 2017) but also after introducing wild individuals into captivity (Kohl and Dearing 2014; Kohl et al. 2014). This implies that to understand host-microbiota relationships and their evolution, we need to study free-living populations that are subject to natural selection and wild birds could be an alternative evolutionary model to the most studied mammals. Despite the great diversity and ecological importance of birds (Oliveros et al. 2019), the study of GM in birds has lagged behind that of mammals (Sun et al. 2022). Publications on the GM of mammals outnumber those of birds by 10:1 and are dominated by studies on domestic poultry (Grond et al. 2018) which mostly ignore genetic variation between individuals. In poultry, research is mostly commercially motivated and focuses on the role of GM in meat production, the effects of diet, antibiotics and probiotics on GM using commercial inbred lines (Černá 2022). While there are only two major phyla in mammals, Firmicutes and Bacteroidetes in GM, there are also other equally abundant major phyla in birds, Actinobacteria and Proteobacteria, which can even dominate GIT and form well-diversified microbial communities at both intra- and interspecific levels (Hird et al. 2015; Garcia-Mazcorro et al. 2017; Grond et al. 2018). The GIT of birds differs also in physiology and morphology from mammals. Birds have ingluvies where fermentation of non-easy degraded products can take place. They have two stomachs with well-separated chemical (proventriculus) and mechanical food digestion (gizzard; Grond et al. 2018). The cloaca of birds is the single posterior opening for GIT, the reproductive tract and the urinary tract, which means that the microbiota mixes to some extent between the different compartments. Also, some differences exist in food digestion and nutrient uptake in the avian gut (Caviedes-Vidal et al. 2007; Mcwhorter et al. 2009). This is evident especially in passerine and parrot birds, where the lower intestine GIT is even less compartmentalised due to their active flight (Grond et al. 2018). The caecum, which normally allows the fermentation of non-digestible products (mainly cellulose fibres), is greatly reduced and

the colon is shortened, meaning ingested food passes through relatively quickly (Price et al. 2015). All this indicates that GM can be regulated differently in birds than in mammals.

Wild bird GM can be driven by many extrinsic and intrinsic factors, but these are still relatively poorly understood compared to mammals and this was particularly evident at the time we initiated our studies. Therefore, my colleagues and I decided to focus on understanding the intra- and interspecific differences in GM in passerines and identify the key ecological, life-history and environmental factors that drive GM. For practical reasons, we investigated GM mostly by non-invasively sampling the faecal microbiota, as it is a good proxy for GM (Videvall et al. 2018; Berlow et al. 2020). In our studies, we tested the effects of host phylogeny, ecological factors (such as diet or habitat), climatic conditions, seasonality, and migration and other factors on microbial composition using large comparative interspecific passerine datasets. Further, we evaluated how microbiota differs across GIT gradients by sampling multiple tissue sites of the same individual. Last, but not least, we tried to address the question of whether bird eggs are colonised with bacteria prior to hatching.

1.2 Evolution of innate immunity receptor diversity

Immune genes undergo more dynamic evolution than most other gene types due to their engagement in host-pathogen arms races (Shultz and Sackton 2019). This is especially hypothesized for immune genes that directly interact with microbes, such as surface pattern-recognition receptors binding their pathogens- and microbial-derived ligands as in the key-lock system (Vinkler et. al., *under rev.*). The high structural diversity of microbial ligands can in turn generate high diversity of receptors that recognise them. Furthermore, their binding repertoire can even be expanded by multiple post-translational modifications in the ligand-binding region (LBR; Leifer and Medvedev 2016; Ricci-Azevedo et al. 2017) or by their conformational plasticity (Wieczorek et al. 2017). Yet, the LBR conformational space is to a certain extent functionally constrained, resulting also in the maintaining of some time-proven allelic variants (Těšický and Vinkler 2015) or to the reinvention of the same functional variants across different populations, species and times (Storz 2016). On the macroevolutionary scale, pathogen-mediated selection forces can also lead to gene gain (e.g. by gene duplication, leading to the creation of multiprotein families) or gene loss (Nei and Rooney 2005; Grueber et al. 2015). The fluid evolutionary dynamics are best illustrated in adaptive immunity genes of the major histocompatibility complex (*MHC*) genes in passerine birds, which exhibit both the most extreme diversity and highest copy number variation in immune genes ever found (Minias et al. 2019). For instance, in the *MHC-I* of the sedge warbler (*Acrocephalus schoenobaenus*), 3566 nucleotide alleles translated into 2760 protein variants were detected in 863 individuals. Interestingly, the maximum number of alleles per individual was 65, corresponding to

up to 33 functional *MHC-I* loci (Biedrzycka et al. 2017). Diversity in host immune genes increases through repeated episodes of pathogen-driven selective sweeps (as part of a reciprocal coevolutionary arms race) when the direction of positive selection differs between species, between and within populations, and between generations (Vinkler et. al., *under rev.*). Within populations, the generated polymorphism can subsequently be maintained in the long term by balancing selection, through three mutually non-exclusive forms: Heterozygote advantage, negative frequency-dependent selection, or temporally or spatially fluctuating selection (Woolhouse et al. 2002; Spurgin and Richardson 2010; Minias and Vinkler 2022). If the balancing selection pressure is strong enough, some identical alleles or allelic lineages may even persist through speciation events as the balanced trans-species polymorphism (TSP) and even exist in descendant species for tens of millions of years (Těšický and Vinkler 2015). Other evolutionary phenomena that may increase the diversity of immunity genes include concerted evolution (Nei and Rooney 2005; Balasubramaniam et al. 2016; Pavlovich et al. 2018), gene conversion (Högstrand and Böhme 1999; Huang 2011) or adaptive introgression due to hybridisation (Hedrick 2013; Grossen et al. 2014; Fijarczyk et al. 2018). Due to common selection pressure, some identical immune variants can also evolve independently in unrelated species without a direct common ancestor as convergent evolution (Li et al. 2011; Storz 2016). Such recurrently evolving variation in different species or populations may be of particular importance, preferentially targeting functional sites such as enzymatic cleavage sites or LBRs, but its identification is challenging.

The microbiota interacts with the immune system mainly through innate immunity pattern recognition receptors (PRRs; e.g. Toll-like receptors, *TLRs*) and adaptive immunity (T-cell and B-cell receptor signalling, *BCR* and *TCR*, respectively, and *MHC*). While the generation of enormous somatic variability in *BCRs* and *TCRs* and also extreme *MHC* polymorphism in birds are relatively well known (Härtle et al. 2022; Kaufman 2022; Smith and Göbel 2022), genetic variability of innate immunity receptors in birds is much less understood, but see, e.g., Alcaide and Edwards (2011); Vinkler et al. (2014). This is because germ-line encoded innate immunity genes were long thought to be invariant or to have little variability. However, we now know that adaptive and innate immunity do not form two distinct arms, but rather are interlinked layers of regulatory and effector mechanisms (Vinkler et. al., *under review*). Innate immunity genes include evolutionarily and functionally unrelated molecules, ranging from PRRs, regulatory cytokines, and effector antimicrobial peptides to transcriptional factors, their regulators and many others (Buchmann 2014). In my dissertation, I narrowed my scope to promising innate immunity candidates, GM-recognising and inflammation-triggering receptors of innate immunity (PRRs) and their regulators (cannabinoid receptors, *CNRs*), all of which have been shown to influence host fitness and disease susceptibility (Leveque et al. 2003; Magor et al. 2013; Tschirren et al. 2013; de Jong et al. 2016).

Germline-encoded PRRs are the first line of innate immunity defence and sense potential harmful stimuli, such as pathogenic structures (i.e. pathogen-associated molecular patterns, PAMPs; whose expression cannot be avoided) or self-molecules from dead or stressed cells, or tissue damage (damage-associated molecular patterns, DAMPs), initiate the inflammation and also modulate immune response in later phases (Chang 2010; Takeuchi and Akira 2010). In birds, each host is equipped with multiple PRRs of four basic types which have different but also partly overlapping functions: *TLRs*, retinoic acid-inducible gene I (RIG-I)-like receptors (*RLRs*), nucleotide oligomerization domain (NOD)-like receptors (*NLRs*), C-type lectin receptors (*CLRs*). Among, them the best known are *TLRs*. Their discoveries the first in insects (1995) and then in humans (1997) have revolutionized our view on innate immunity and received much attention mainly in biomedical and immunological studies (O'Neill et al. 2013). Since their first description in passerines more than a decade ago (Vinkler and Albrecht 2009; Vinkler et al. 2009), they have also attracted the attention of evolutionary biologists and have become one of the most studied genes beyond *MHC* in wild bird immunogenetics. In birds, ten *TLRs* have been described, located either on the extracellular side of the cytoplasmic membrane (*TLR10*[*1A*], *TLR1*[*1B*], *TLR2A*, *TLR2B*, *TLR4*, *TLR5*, and *TLR15*) or in various cellular vesicles, such as in the endosome (*TLR3*, *TLR7*, and *TLR21*; Roach et al. 2005; Brownlie and Allan 2011). TLRs are well conserved in their 3D structure; consisting of N-terminal leucine-rich repeat (LRR) ectodomain with the central ligand-binding region (LBRs), the transmembrane domain, and intracellular toll-interleukin-1 receptor (TIR) signalling domain (Botos et al. 2011). TLRs recognize very structurally diverse microbial ligands ranging from viral (e.g. TLR3: dsRNA), bacterial (TLR4: LPS, TLR5: flagellin), or protozoan molecules (e.g. GPI: TLR4) but greatly differ in their ligand specificity (Kumar et al. 2009; Kawai and Akira 2011). Avian *TLRs* also evolve with duplications (i.e. *TLR7*) or pseudogenisations (e.g. *TLR5*, Bainova et al. 2014) that can even be cryptic without disrupting the open-reading frame, as recently reported for *TLR15* in penguins (Fiddaman et al. 2022). Positive selection operates predominantly only specific residues within LBRs (Králová et al. 2018; Świderská et al. 2018; Minias and Vinkler 2022) but most protein structure is functionally constrained by strong purifying selection (Raven et al. 2017). Among positively selected sites (PSS), selection often targets residues that alter the electrostatic surface charge distribution at or near functional sites (i.e. ligand-binding or dimerisation site), as most ligands are only non-covalently bound via electrostatic interactions. This can lead to substantial differences in the surface charge distribution of the TLR molecular phenotype within and between species (Vinkler et al. 2014a; Králová et al. 2018). On the macroevolutionary scale, avian bacterial-sensing *TLRs* are subject to stronger diversifying selection than viral-sensing *TLRs*, probably reflecting the higher structural variability of bacterial ligands than viral nucleic acids (Velová et al. 2018). Although authors in multiple studies have attempted to identify adaptive variation in immune genes, including *TLRs* (e.g. Alcaide and Edwards 2011; Grueber et al. 2014; Minias et al. 2021b), using only the codon-based *dN*/*dS* scans of positive

selection scans, these methods may suffer from identifying a high number of false positives (Nozawa et al. 2009) and we need to have a more reliable approach to detect functionally important single nucleotide variation (SNVs).

In contrast to *TLRs*, which recognise many different ligands, *RLRs* are a small helicase family of three intracellular receptors specialised only for viral sensing: retinoic acid-inducible gene I (*RIG-I* or *DDX58*), melanoma differentiation-associated protein 5 (*MDA5*) and laboratory of genetics and physiology 2 (*LGP2*; Yoneyama et al. 2005; Rehwinkel and Gack 2020). Being well conserved in their 3D structures, they all consist of a central helicase domain and a carboxy-terminal domain (CTD). *RIG-I* and *MDA5* also possess two N-terminal caspase activation and recruitment domains (CARD). The helicase and CTD domains directly participate in RNA binding, while the CARD domains are responsible for downstream signalling. Since *LPG2* lacks the CARD domains, it is thought to downregulate the function of *MDA5* and *RIG-I* (Rodriguez et al. 2014; Brisse and Ly 2019). Although both *RIG-I* and *MDA5* recognise exogenous RNAs, their function is not redundant (Kato et al. 2006). *RIG-I* binds shorter single- or double-stranded RNA (ssRNA and dsRNA, respectively) with a cap structure or single phosphate (Pichlmair et al. 2006; Kato et al. 2008). In contrast, *MDA5* senses longer dsRNA and their internal duplex structures (Kato et al. 2008; Wu et al. 2013). Interestingly, *RIG-I* was reported pseudogenised in chickens, which has been linked to increased susceptibility to avian influenza (Barber et al. 2010; Magor et al. 2013). Given their involvement in pathogen recognition, one would expect them to be subject to relatively strong positive selection in their LBRs, but little is known about how dynamically these receptors evolve in birds.

The evolution of cannabinoid receptors, which negatively regulate inflammation at the borders of the immune and nervous systems in vertebrates, also suffers from a lack of understanding. CNRs are membrane-bound G-coupled proteins that bind endogenous endocannabinoids (Mackie 2008). Vertebrates are equipped with two *CNRs* with clearly distinguished functions (Elphick 2012). *CNR1* is mainly expressed in the brain and cells of the nervous system, where it is involved in the regulation of emotion, memory, neuropeptide synthesis and bird singing. *CNR1* is also secreted in the gut, where it regulates gut motility and secretion (Cristino et al. 2014; Cani et al. 2016). In contrast, *CNR2* is mainly expressed in immune cells in immune organs such as the tonsils or spleen. *CNR2* generally has immunosuppressive effects, reduces pain and the expression of pro-inflammatory cytokines, and contributes to negative feedback regulation (Vincent *et al.* 2016).

But which other immune genes should be targeted in eco-evolutionary research in birds? In my thesis I first reviewed current knowledge on the evolution of immune gene diversity in vertebrates. Then I attempted to develop a new evolutionary bioinformatic predictive approach to (i) estimate the functional significance of the identified PSS and (ii) to test for evolutionary convergence in PSS

and surface electrostatic charge, assuming that molecular and phenotypic traits popped up multiple times in evolutionary history independently in non-related species are more likely to be functional. Then using the comparative interspecies perspective and various evolutionary bioinformatics predictions, I attempted to better understand the evolution of the adaptive diversity in these innate immunity receptors (*TLRs*, *RLRs* and *CNRs*) and the mechanisms that give rise to them (such as positive selection, convergence or pseudogenization, etc.) in birds. As we also reported gene losses of several receptors in *RLRs* and *CNRs* we also tried to understand the evolutionary and functional consequences of such gene losses in birds.

1.3 Physiological senescence

Senescence (ageing) is a persistent decline in physiological functions with age, leading to an increased risk of mortality but a decline in reproductive success, as the inevitable extrinsic mortality reduces the strength of selection against poor performance with age (Ricklefs 2008). While the clinical symptoms of ageing are relatively well known, especially in humans and laboratory rodents (Holtze et al. 2021), the actual causes of senescence remain puzzling despite decades of research. Although natural selection should favour long-lived species, as they have more opportunities to reproduce than short-lived species, there is enormous diversity not only in the life span between species across the tree of life (Monaghan et al. 2008), but also between individuals of the same species (Selman et al. 2012). Moreover, recent comparative animal studies are disproving the classical biomedical view that senescence should inevitably lead to an increase in mortality and a decline in fertility. Interestingly, it has been shown, for instance, that fertility in the long-lived alpine swift (*Alpus melba*) actually increases during ageing and is the highest in the oldest cohorts (Jones et al. 2014). This suggests that senescence is a much more complex process than we thought before (Jones et al. 2014; Holtze et al. 2021) and that using non-traditional ageing vertebrate models is necessary to better understand the causes of ageing (Harper and Holmes 2021; Holtze et al. 2021).

In particular, small free-living passerines may be an ideal group for biomedical studies of longevity and senescence, as they are relatively long-lived and exposed to natural selection and various trade-offs (Lavoie 2005). Although birds have higher basal glucose level and metabolic rate than mammals of similar size, they also have high fertility even in old individuals and live ca. 2-3 years longer (Travin and Feniouk 2016). They also have some adaptations for longevity and coping with oxidative stress, such as relatively long telomeres, higher proportion of unsaturated fatty acid in their membranes, higher antioxidant levels or proteins preventing DNA damage and are more resistant to damages from glycosylation and glycoxidation (Holmes and Ottinger 2003; Travin and Feniouk 2016). Unfortunately, age-related physiological changes in free-living passerine birds have not been sufficiently researched due to technical difficulties in repeatedly sampling the same

individuals (Bouwhuis and Vedder 2017). Anecdotally, it should also be noted that just a few decades ago, small passerines were believed to "simply have no time to age" due to high extrinsic mortality from predation and avian senescence has been longed overlooked (Vleck et al. 2007; Bouwhuis and Vedder 2017). From the perspective of avian immunity, a major cell type performing potentially self-damaging oxidative burst is the heterophil, a functional counterpart of the neutrophil in mammals. Compared to neutrophils, bird heterophils lack one of the enzymes (myeloperoxidase) producing tissue-damaging highly reactive compounds, and as consequence, they have lower ROS production during oxidative burst response than mammals. Yet, avian oxidative burst is still very efficient in microbial killing (Genovese et al. 2013). We may assume that birds less suffer from inflammaeging-related tissue damage, but virtually nothing is known about the role of inflammaging in avian senescence. All this motivated my colleagues and I to investigate how ageing affects inflammation-related physiological traits in wild passerines. In this chapter, I will first introduce the general evolutionary theories and proximal mechanisms of ageing, and then explain how ageing affects the immune system and vice versa.

Three main mutually non-exclusive evolutionary hypotheses have been proposed to explain the evolutionary causation of senescence (Kirkwood and Austad 2000; Hughes and Reynolds 2005). Briefly, (i) *Mutation accumulation theory*; Medawar, 1952) assumes that mutations accumulate during life, but with increasing age, deleterious mutations are not effectively removed from a population because natural selection weakens in old cohorts. So deleterious mutations are in the "selection shadow" and can be passed on to the next generation. Similarly, (ii) *Antagonistic pleiotropy theory* (Williams, 1957) predicts that some mutations bringing benefits in early life, such as better reproductive performance or physical attractiveness (i.e. that is why they are selected for), will have detrimental effects later. The currently most popular (iii) *Disposable soma theory (DST*; Kirkwood, 1977) also assumes a weakening of selection in older cohorts combined with a trade-off in the allocation of energy resources between the energetically costly self-maintenance processes (such as antioxidant defence, immune system, and tissue repair) and reproduction. Individuals should, therefore, weigh their investment in actual reproduction to maximise their lifelong fitness. According to *DST*, young individuals should invest more in self-maintenance activities while older individuals should invest more in reproduction (Ricklefs 1998; Costantini 2014a). The *DST* has been confirmed by several studies in birds, where young individuals that invested more in reproduction had earlier onset of senescence than those that invested less or even skipped it (Bouwhuis et al. 2010; Kim et al. 2011; Hammers et al. 2013).

At the mechanistic level, there are many other mutually non-exclusive hypotheses. For instance, the *Cell senescence telomere hypothesis* (Weinert and Timiras 2003) assumes cellular senescence and chromosome instability after reaching the critical number of cell divisions which is accompanied by the shortening of telomeres (Hayflick Limit). The *Oxidative stress hypothesis of*

ageing (also termed the *Free radical theory of ageing*; Gilca et al. 2007; Monaghan et al. 2008) assumes that free radicals (ROS, and reactive nitrogen species, RNS) that are mainly generated in mitochondria in the respiratory chain during oxidative phosphorylation cause oxidative damage of various biologically relevant molecules, resulting in functional physiological decline and senescence. In addition to energy metabolism, ROS are also produced by the immune system, especially during the oxidative burst reaction (Danilova 2006 and see also above) and in high doses they may cause oxidative damage if not scavenged (Gilca, Stoian, Atanasiu, & Virgolici, 2007). To ensure redox balance in the organism and prevent damage from oxidative stress, organisms have evolved various antioxidant molecules that eliminate ROS by converting them into less-reactive compounds. Antioxidants are generally well conserved in vertebrates, including birds (Costantini 2008, 2014b) and traditionally consist of antioxidant enzymes, non-enzymatic and non-dietary antioxidants. They often differ in their substrate specificity and affinity and work synergically with other enzymes. Superoxide dismutase (SOD) catalyses the dismutation of highly reactive superoxide anion into less reactive hydrogen peroxide and oxygen (Surai et al. 2019). Subsequently, hydrogen peroxide is synergically broken down by catalase (CAT) and glutathione peroxidase (GPX). While CAT reduces hydrogen peroxide with low affinity to water and oxygen, GPX breaks down peroxides with much higher affinity to water, so that it is continuously removed while it is normally produced (Pisoschi and Pop 2015). Non-enzymatic antioxidants (such as glutathione) possess sulfhydryl group (i.e. -SH) on their cysteine residue and exist in either oxidised or reduced form. Only in their reduced form they are capable neutralizing ROS, after that they are oxidised and must be recycled by specific enzymes, such as glutathione reductase. Dietary non-enzymatic antioxidants, such as vitamin E, carotenoids, or polyphenols, are highly condition-specific and can also contribute to maintaining redox balance (Edge et al. 1997; Vinkler and Albrecht 2010).

Current studies have shown that ROS-induced oxidative stress tissue damage may provide a currency to quantify physiological costs in *DST* that influence growth, reproduction, or senescence (Costantini 2014a). Besides, ROS can also target guanine in telomeres, further accelerating telomere shortening (Von Zglinicki 2002). The trade-off in energy expenditure is regulated by steroid hormones. Among them, testosterone serves as the key endocrine mediator, controlling the balance between investment in reproduction on the one hand and self-maintenance on the other, and influencing many condition-dependent traits (Fusani 2008; Kempenaers et al. 2008; Hau and Goymann 2015). Specifically, testosterone promotes current reproductive success at the cost of future reproduction (Ketterson 1992), increases ornamentation (e.g. the *Immunocompetence hypothesis*, Folstad and Karter 1992 or the *Oxidation handicap hypothesis*; Alonso-Alvarez et al. 2008), aggressivity and social rank dominance (Hau and Goymann 2015) but has generally immunosuppressive effect (Foo et al. 2017). In birds, enormous variations in testosterone levels exist between different individuals, sexes, and seasons. For example, in the blue tit (*Cyanistes*

caeruleus) a ca. 200-fold difference was recorded in testosterone levels between different males of the same population (Kempenaers et al. 2008). However, the evidence of whether testosterone levels undergo any age-related changes in birds is mixed (e.g. Peters et al. 2002; Smith et al. 2005; Madsen et al. 2007; Wilcoxen et al. 2013) and it is not known whether birds also experience endocrinological senescence in testosterone levels as seen in mammals (Harman et al. 2001; Wolf et al. 2018).

The function and state of the immune system changes during life, depending not only on genetic factors (chapter 1.2) but also on the interactions with microbes (chapter 1.1). *Immunosenescence* is generally defined as a decline in immune functions with age (Pawelec 2018). The signs of *immunosenescence* are well documented in humans and laboratory models and include, for example, increased risk of infection, poorer response to vaccination or reduced ability to fight cancer, and persistence of chronic inflammation (Gruver et al. 2007; Kovacs et al. 2009; Wang et al. 2022). During ageing, inflammatory status often shifts towards augmented pro-inflammatory reactivity, which in turn can lead to increased oxidative stress damage (*inflammaging*, Franceschi *et al.* 2007). Nowadays, the role of *inflammaging* in ageing and in causing inflammatory numerous age-related diseases is becoming more and more recognized (Pawelec et al. 2014; Chen et al. 2014). As mounting inflammation is costly in terms of resource allocation and it can be shifted with the altered antioxidant capacities (Costantini and Møller 2009), natural selection should favour fast mounting immune response and its termination (Costantini 2014c). Despite this elaborated theoretical framework, the role of inflammation in ageing of wild birds that are exposed to a wide range of pathogens but selected to regulate their immune responses to avoid immunopathology is still poorly understood (Pedersen and Babayan 2011) and we do not know whether *inflammaging* is an active mechanistic process that drives ageing in wild birds.

Although initially overlooked, *immunosenescence* has recently been confirmed in wild birds and many other vertebrates (Peters et al. 2019). In birds, this includes documented declines in adaptive immunity, such as humoral immune response (Cichoń et al. 2003; Saino et al. 2003; Lavoie 2005; Lavoie et al. 2007), lymphocyte proliferation (Palacios et al. 2007; Vleck et al. 2011; Counihan and Hollmén 2018) and much less in innate immunity but see Peters et al. (2019) for a recent meta-analysis. Functional decline appears to be consistently greater in adaptive immunity than in innate immunity, and is universal in vertebrates, including birds (Peters et al. 2019). To date, age-related changes in avian pro-inflammatory immunity have only been observed in very heterogeneous traits, such as the frequency of different leucocyte types (Norte et al. 2009), complement and haptoglobin levels (Vermeulen et al. 2017) and response to phytohaemagglutinin (PHA; Noreen, Bourgeon, & Bech, 2011; see also Peters et al. 2019 for a recent metanalysis) that do not represent a general inflammatory immune response. Furthermore, these analyses almost exclusively used cohort design and can be biased by population-wide effects, such as selective appearance or selective

disappearance and we clearly need to assess lifetime trajectories of immune traits from longitudinal data (Zhang et al. 2015; Bouwhuis and Vedder 2017). Moreover, the inflammatory response encompasses both acute and long-term inflammation, which differ in their potential physiological costs but little is known about their short- and long-term effect of inflammation on the individual condition. Although some physiological costs of inflammation have been shown in wild birds, such as effects on telomere shortening (Asghar et al. 2015), shifts in redox balance or increased oxidative damage (Costantini 2022 for meta-analysis), little is known about how are these relationships mediated during senescence.

Heavy metals can induce ROS and RONS formation that causes oxidative stress damage either directly (Stohs and Bagchi 1995; Flora et al. 2008; Jaishankar et al. 2014) or indirectly via induction of inflammation (Li et al. 2017; Seremelis et al. 2019; Anka et al. 2022). As consequence, heavy metals can pose a series threat to human and animal health (WHO 1995, 2007; Järup 2003), including birds (Eeva et al. 2005; Bauerová et al. 2017). This occurs particularly in polluted urban or industrial areas where they can accumulate in much higher concentrations in various animal tissues and become toxic (Scheifler et al. 2006; Roux and Marra 2007; Sharma 2014; Celik et al. 2021). While some studies in birds have found changes in heavy metal concentrations between sexes, no study in birds has examined whether they accumulate in the blood over time. This is of particular importance because non-destructive heavy metal determination in the blood of wild birds has been recently proposed as a good indicator of environmental heavy metal exposure (Coeurdassier et al. 2012; Bailly et al. 2017; Bauerová et al. 2017). However, potential age-related accumulation of heavy metals in blood would limit their usage since the determination of age in wild birds is not easily possible.

Within this chapter, I have attempted to examine senescence in mutliple physiological traits of a free-living population of the great tit longitudinally monitored for nearly a decade. Among these traits, I tested for *hormonal senescence* in testosterone levels, *immunosenescence* and *inflammaeging* using different types of pro-inflammatory immune responses, including chronic and acute inflammation, and accumulation of certain heavy metals in the blood. To understand the health consequences of these age-related changes, we also linked their trait values to the individual condition (such haematological markers, oxidative stress damage, antioxidants or plumage colouration).

2. Research questions and aims

In my dissertation, I explored three interrelated topics: (1) ecological and evolutionary determinants of microbiota composition and diversity, a driver of wild bird immunity, (2) diversity in immune genes affecting inflammatory responses in wild birds, and (3) inflammation-related physiological senescence in a passerine bird model, the great tit (*Parus major*). My general research questions for each topic and the specific objectives for each manuscript are as follows:

(1) What is the intra- and interspecific variation in the host gastrointestinal tract microbiota of wild birds? Which evolutionary and ecological factors drive microbial variation? How is the gastrointestinal tract microbiota formed during avian embryogenesis?

- To describe GM variation in passerines and ecological and evolutionary factors that determine it using large comparative interspecies passerine datasets (**papers I – III**)
- To describe variation of microbiota across different GIT tissues using selected passerine and parrot species (**papers IV – V**)
- To describe microbial communities in a bird egg shortly after laying and before the hatching in the great tit testing different mechanisms of bacterial colonisation (**paper VI**)

(2) How does genetic variation in innate immunity receptors between individuals and species contribute to differences in resistance to infectious diseases in wild birds? How does this variation evolve? Can we predict the adaptive functional effects of this variability using evolutionary bioinformatics tools?

- To review the current state of knowledge of immune gene diversity evolution in vertebrates and to propose new hypotheses to be tested (**paper VII**)
- To develop a methodological pipeline that identifies adaptive variation based on identifying convergence in any protein-coding genes (**paper VIII**)
- To describe overall and adaptive intra- and interspecific variation of selected *TLRs* and the evolutionary mechanisms that generate it in tits (**paper VIII**) and finches and buntings (**paper IX**)
- To describe evolution of *RLRs* (*RIG-I* and *MDA5*) in birds (**papers X – XI**)
- To describe evolution of cannabinoid receptors (*CNRs*) in birds and the functional consequences of *CNR2* gene loss (**paper XII**)

(3) Does senescence occur in various physiological and pro-inflammatory immune traits in a free-living passerine bird, the great tit? Do these traits follow the same, or different lifetime trajectories? What are the functional consequences of the observed age-related changes?

- To investigate whether plasma testosterone levels undergo age-related changes and whether testosterone levels affect various condition-related traits in the great tit (**paper XIII**)
- To test for age-related changes in inflammatory response and whether these are linked with changes in antioxidant/ oxidative stress marker levels in the great tit (**paper XIV**)
- To examine whether heavy metals accumulate in the blood over time and whether they have significant effects on individual health in the great tit (**paper XV**)

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3. General methods

In this chapter, I try to give a rather general overview of our approaches, advocate their use or highlight their specificities (if any), rather than describe in detail the methods that can be easily found in the individual manuscripts.

3.1 Study species

In my dissertation, I used various bird species from different taxonomical levels, ranging from whole birds, through passerine and parrot clades, families of tits and finches and buntings to the great tit.

- **Passerines and parrots**

Passerine birds are the evolutionarily and ecologically most diverse avian crown order with ca. 6000 current species, making them an excellent group to test various evolutionary hypotheses (Oliveros et al. 2019). Furthermore, many wild species are migratory or live in contact with pets and commercial poultry, which increases their potential for zoonotic disease transmission (Reed et al. 2003; Boseret et al. 2013) and requires attention from the perspective of One Health Concept (Prata et al. 2022). Passerines were captured in mist nests and sampled in different parts of the Czech Republic (with my involvement), Poland and Cameroon or their genetic samples were obtained from genetics banks. Parrots are a sister group of passerines; they are also reasonably diversified and are mostly easily bred in captivity and popular among hobby breeders, making them suitable for evolutionary research.

- **Great tit (*Parus major*)**

The great tit (Linnaeus, 1758) is a small (12.5-14 cm), sexually dichromatic passerine belonging to the Paridae family (Cramp and Perrins 1993). The great tit has a black head and neck, prominent white cheeks, olive upperparts and yellow underparts, although variations exist amongst the numerous subspecies (Cramp and Perrins 1993). Both sexes have melanin-pigmented black breast stripes and carotenoid-based plumage colouration, with sexual selection playing a role (Senar and Quesada 2006; Hegyi et al. 2007). Males have wider breast stripes and more vivid yellow colouration. The great tit is a common, generalist species widely distributed predominantly in the temperate continental zone of the Palearctic region (Cramp and Perrins 1993). The diet of the great tit consists mainly of insects and spiders preyed upon by foliage gleaning in summer and increasingly of various seeds and berries in winter (Cramp and Perrins 1993).

Being an abundant and widespread natural tree cavity nester, the great tit also successfully occupies man-made nest boxes, tolerates stress during capture and is a popular avian model for eco-

evolutionary research (Cramp and Perrins 1993). For longitudinal studies, it is particularly important to have high breeding site fidelity, low dispersal and mortality rates. In homogeneous habitats, most individuals (67 %) do not change their territory between years, and those that do change do not move more than 200 metres. Females disperse more than males, and the tendency to disperse decreases with age (Andreu and Barba 2006). Brood mortality is usually low, but can vary considerably locally and seasonally (e.g. in a deciduous forest in England, about 23 % of nests were predated). Annual survival was about 15-20 % for juveniles and about 50 % (range 27-65 %) for adults (≥ 1 year), with slightly lower survival for females (Payevsky 2006). Life expectancy is approximately 11-23 months for individuals that have survived the first winter (Payevsky 2006), and the maximum recorded age in the wild is 15.4 years (Fransson et al. 2017). Without ringing records, age can only be determined by the difference in colouration of the primary and secondary coverts, but only as young or adult individuals (Jeni and Winkler 2020).

All studies on great tits were conducted on a free-living population in an urban forest on the outskirts of Prague (Ďáblický and Čimický háj, Czech Republic; 50°8'10.591 "N, 14°27'51.144 "E, ~ 315-360 m above sea level, total area ~ 0.9 km^2). The habitat is a temperate deciduous forest (~ 60-120 years old), partially isolated by urban and agricultural areas. In 2010, 244 nest boxes were installed in both forests in a regular grid of 50 × 50 m about 2-3 m above the ground. In total, ca. 60-80 nest boxes are occupied by great tits each year. The fieldwork was carried out from 2011 to 2018 during the pre-breeding and breeding season of the great tits (mostly from April to early June). I have participated in fieldwork since 2012 and coordinated field sampling from 2015 to 2018.

Figure 1: Great tit male and study site, Čimický háj forest (Photo: M. Těšický)

3.2 Microbial genotyping

Since next-generation sequencing (NGS) technologies can reveal enormous bacterial diversity previously hidden in culture-based studies (Boughner and Singh 2016), we also used culture-independent NGS *16S rRNA* gene metabarcoding in our studies. Bacterial DNA was isolated from various sample types stored deep-frozen in ethanol (faeces, oral cavity swabs, or different tissues) with the microbial extraction kit. We then prepared the sequencing libraries by amplifying the V3-V4 region of the *16S rRNA* gene in a two-step PCR and sequenced the resulting amplicons using Illumina Miseq paired-end sequencing. This metabarcoding approach allows us to distinguish microbial taxa mainly at the level of genera rather than at lower levels, e.g. individual strains. Bearing in mind that potential environmental and between-sample contamination is a critical issue for samples with low bacterial biomass (Salter et al. 2014), we have developed a methodologically advanced protocol that includes several measures: working strictly in a clean environment, using multiple negative control types, separate DNA extraction and amplification of low and high bacterial biomass samples, performing PCR amplification in technical duplicates, and further taking only consensually present amplicon sequence variants (ASVs) in both duplicates, and statistical filtering of potentially contaminating ASVs, etc. Finally, the levels of a few candidate taxa were verified by more accurate taxa-specific probe-based *16S rRNA* gene (DNA) qPCR assays, as given PCR stochasticity and amplification bias, quantification of taxa from sequencing data can be inaccurate (Grond et al. 2017).

3.3 Assessing adaptive variation in immune genes

To describe the intra- and interspecific variation of immune genes in birds, we obtained sequences from different sources: (i) for genome-wide scans by downloading sequences from publicly available databases (ENSEMBL or NCBI), (ii) by targeted amplicon-based sequencing of individual LBR of genes (Illumina MiSeq and Sanger method) from genomic DNA extracted from blood and other tissues, and (iii) by extracting sequences from the sequenced transcriptome of ileum tissue (mRNA-Seq by Illumina NextSeq 500).

To detect adaptively evolving convergent adaptations in immune genes, we developed a new innovative pipeline that uses combinations of several advanced evolutionary bioinformatic approaches. This highly flexible pipeline consists of several filtering steps and decision points (schematically shown in Figure 2) that minimise the risk of final single nucleotide variants (SNVs) being false positives and can be universally applied to all protein-coding genes. Briefly, it predicts either positively selected sites (PSS) identified by dN/dS-based methods or post-translation modifications, both of which use multiple methods. Protein three-dimensional (3D) structures are predicted using protein homology modelling and the identified PSS are visualised in their 3D

models. The only PSS identified by multiple methods (to avoid false positive identification), with physicochemical non-conservative substitutions (likely to alter the residue function), located on the protein surface (allowing their interaction with ligands) and located close to the functionally relevant residues (known from the literature search) are further considered. The electrostatic surface charge distribution is calculated for each 3D protein model. Evolutionary convergence is then assessed (i) in individual amino acid sites (e.g. positively selected or post-translationally modified) based on the maximum parsimony ancestral state reconstruction and (ii) in the electrostatic surface charge as mismatches between the electrostatic surface charge dendrograms and the species phylogeny.

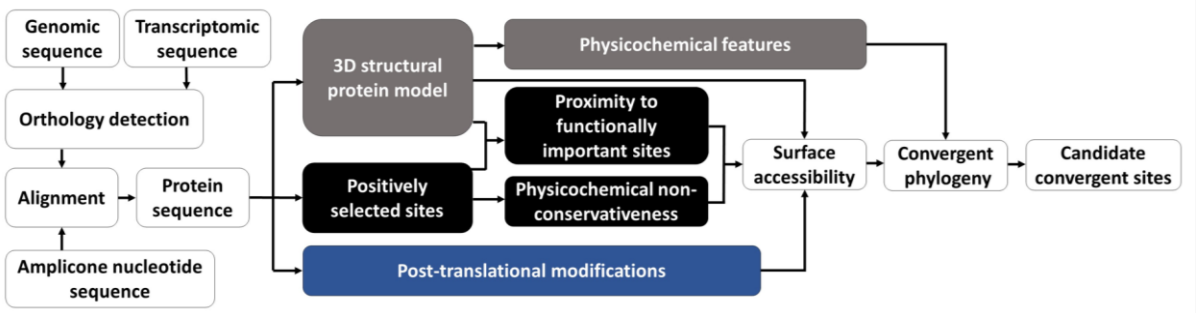


Figure 2: Scheme of the pipeline for identifying functionally relevant variation based on the detection of molecular convergence. Adapted from Těšický et al. (2020), wherein also see for more details.

3.4 Differential gene expression analysis

In my thesis, I mainly focused on gene structure analysis, but in we also analysed differential gene expression (DGE) of the proinflammatory immune response. Interestingly, we first performed the novel QuantSeq 3'mRNA transcriptome sequencing (Illumina Hiseq 2500 platform). The biggest advantage of QuantSeq over classical full RNAseq is the lower price of sequencing, which allowed us to include more individuals in the analysis. This seems particularly important when working with non-model (outbreed) populations, where genetic heterogeneity of individuals may also cause spurious differences in DGE between control and treatment groups (Veetil et al., *submitted*). Second, because DGE assessment with RNA-seq alone can be inaccurate, we verified the DGE of some candidates with specific conventional RT-qPCR assays based on TaqMan probes.

3.5 Haematological methods

Various leucocyte and erythrocyte counts in the blood represent good proxies of health status and physiological stress in birds (Ots et al. 1998; Davis et al. 2008). However, since there are no well-optimised molecular surface markers for leucocytes in passerines that allow automatic differentiation by flow cytometry (e.g. using CD45 as in poultry; Bílková et al. 2017), we quantified leucocytes manually using standard light microscopy. From blood smears, we quantified the relative white blood cell count (RWBC) and immature erythrocyte count (IEC), and from blood diluted in Natt and Herrick solution we determined the absolute white blood cell count (TWBC) and total red blood cell count (TRBC).

3.6 Measuring inflammation

For background inflammation, we measured for the first time in birds the concentration of leukotriene B4 (LTB4; pro-inflammatory markers secreted by granulocytes) by high-performance liquid chromatography-electrospray ionisation-high-resolution mass spectrometry (HPLC-ESI1-HRMS) from fresh frozen blood. For acute inflammation, the oxidative burst of whole blood after *in vitro* LPS stimulation was measured using the Pholasin-based chemiluminescence method with a commercially available kit. The major advantage of this method is that the oxidative burst represents a general cellular effector pro-inflammatory response and its interpretation is straightforward compared to the various skin-swelling assays popular among ornithologists (e.g. phytohaemagglutinin, PHA; Vinkler et al. 2010, 2014b) and other heterogeneous approaches used in biogerontological studies (summarised in Peters et al. 2019). Moreover, the measurement of LPS-induced oxidative burst in birds has recently been optimised (Sild and Hõrak 2010; Sild et al. 2011; Sepp et al. 2012) and, unlike other oxidative burst assays using non-specific stimulants (e.g. Kukovetz et al. 1997), the molecular mechanism based on activation of *TLR4* by LPS on phagocytes is well known (Farnell et al. 2003; Remer et al. 2003).

3.7 Measuring oxidative stress damage and antioxidant markers

To measure the level of oxidative damage, there are many approaches based on assessing single molecules or total oxidative damage using commercial kits, such as the popular Reactive Oxygen Method (ROM; van de Crommenacker *et al.* 2011). However, relying only on the assessment of a single oxidative marker or only on total lipid and protein damage (e.g. ROM) and not distinguishing between different types of molecules can both be misleading and contribute significantly to heterogeneity in the results of different studies (Selman et al. 2012). To overcome these caveats, in our study we first identified a set of several novel markers whose levels are elevated in chronic age-

related diseases in humans from biomedical studies (Syslová et al. 2014) and then we measured oxidation products of nucleic acids (8-hydroxy-2'-deoxyguanosine, 8-OHdG; 8-hydroxyguanosine, 8-OHG), proteins (o-tyrosine, O-Tyr; 3-nitrotyrosine, 3-NOTyr) and lipids (8-isoprostane, 8-ISO) for the first time in a wild bird longitudinal study. Oxidative stress markers were analysed from deep-frozen fresh blood using high-performance liquid chromatography-electrospray-ionisation-high-resolution mass spectrometry (HPLC-ESI1-HRMS).

In contrast to previous studies in which total antioxidant capacity (e.g. TAC; Selman et al., 2012) was usually measured without distinguishing between the different antioxidant types, we measured the levels of free reactive thiols as well as the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) from deep-frozen whole blood separately, based on colourimetric absorbance changes measured with a microplate reader. To measure free reactive thiols in their reduced form (i.e. thiol molecules from low molecular weight to protein complexes with common SS-bonds), we used a modified microplate-based assay utilizing Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid; DNTB).

3.8 Ptiolochronological and plumage ornament analysis, testosterone and heavy metal level measurement

Feather growth rate (FGR) of tail feathers is a condition-dependent trait that provides information on individual nutritional status during moult (Grubb 2006). FGR was determined from the scanned tail feathers as the mean growth bar width in graphical software. Both melanin and carotenoid-based plumage ornaments are condition-dependent traits serving also as honest signals in sexual selection in birds (e.g. Albrecht et al. 2009; Svobodová et al. 2013; Guindre-Parker and Love 2014) including great tits (Quesada and Senar 2006; Hegyi et al. 2007). Their expression has been shown to be partially controlled by plasma testosterone levels (Alonso-Alvarez et al. 2009). To measure the melanin-pigmented black breast stripe, the ventral part of individuals was scanned in a dark tent and the ornamental size was later measured in a graphical programme. The carotenoids of the yellow breast plumage were analysed with a spectrophotometer using the carotenoid feathers fixed on a glass slide according to Senar and Quesada (2006). Although plumage can also be measured directly on the live bird, this method is preferred because it is easier to measure. We later calculated yellow chroma and total brightness from the spectral data (Montgomerie 2006). Plasma testosterone was measured with the testosterone ELISA kit from freshly frozen plasma obtained by centrifugation. Heavy metal contamination of blood by Pb, Cu, Zn and As was measured from whole blood samples stored in ethanol using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

4. Results and discussion

4.1 Host microbiota in the gastrointestinal tract

Especially in recent years, the identification of extrinsic and intrinsic factors influencing the GM (and following the publication of some of our papers) has received some attention in birds (reviewed in Grond et al. 2018; Matheen et al. 2022; Sun et al. 2022 and see Figure 3). Intrinsic factors included, for instance, host phylogeny between penguins (Dewar et al. 2013), different avian orders in Central America (Hird et al. 2015) or Central Africa (Capunitan et al. 2020); diet between different Darwinian finches (Loo et al. 2019b, a) or penguins (Dewar et al. 2014); social maternal transmission (Kreisinger et al. 2017); or ontogenic changes (Dewar et al. 2017). Extrinsic factors included, e.g., the effects of urbanisation-linked habitat differences on the GM profile in the white-crowned sparrow (*Zonotrichia leucophrys;* Berlow et al. 2021) or altitude, habitat and geography in Darwinian finches (Loo et al. 2019a, b).

Consistent with other studies in wild birds (Hird et al. 2015; Bodawatta et al. 2018; Grond et al. 2018, 2019), our results in adult GM confirmed the dominance of major phyla Proteobacteria, Firmicutes, Actinobacteria, Chlamydiae and Bacteroidetes, with similar proportions of major phyla as in other avian studies (**paper I – V**). We identified high intra- and interspecific variation in passerine GM, yet most of the observed variability remained unexplained. Out of various life-history and ecological traits, only host phylogeny was a moderate predictor of GM composition (**paper I**). Bacteria affecting immune and metabolic functions showed stronger host specificity than other groups (**paper III**). Contrary to our expectations, the overall GM diversity did not differ between temperate and tropical passerines but in the tropics the GM profile changed significantly between wet and dry seasons (**paper II**). When looking at microbiota across the gastrointestinal tract, in the great tit (**paper IV**), oral microbiota was relatively homogenous and very different in diversity and composition from faecal microbiota, suggesting that it is regulated by different mechanisms. **In paper V**, in parrots sampling across the GIT gradient revealed only a little difference between the different gut locations, showing that the morphologically poorly differentiated gut is inhabited by relatively homogeneous microbial communities. In **paper VI**, we revealed only negligible microbiota communities in great tit egg and developing embryonic gut. This suggests that the main colonisation of the gut with bacteria occurs after hatching.

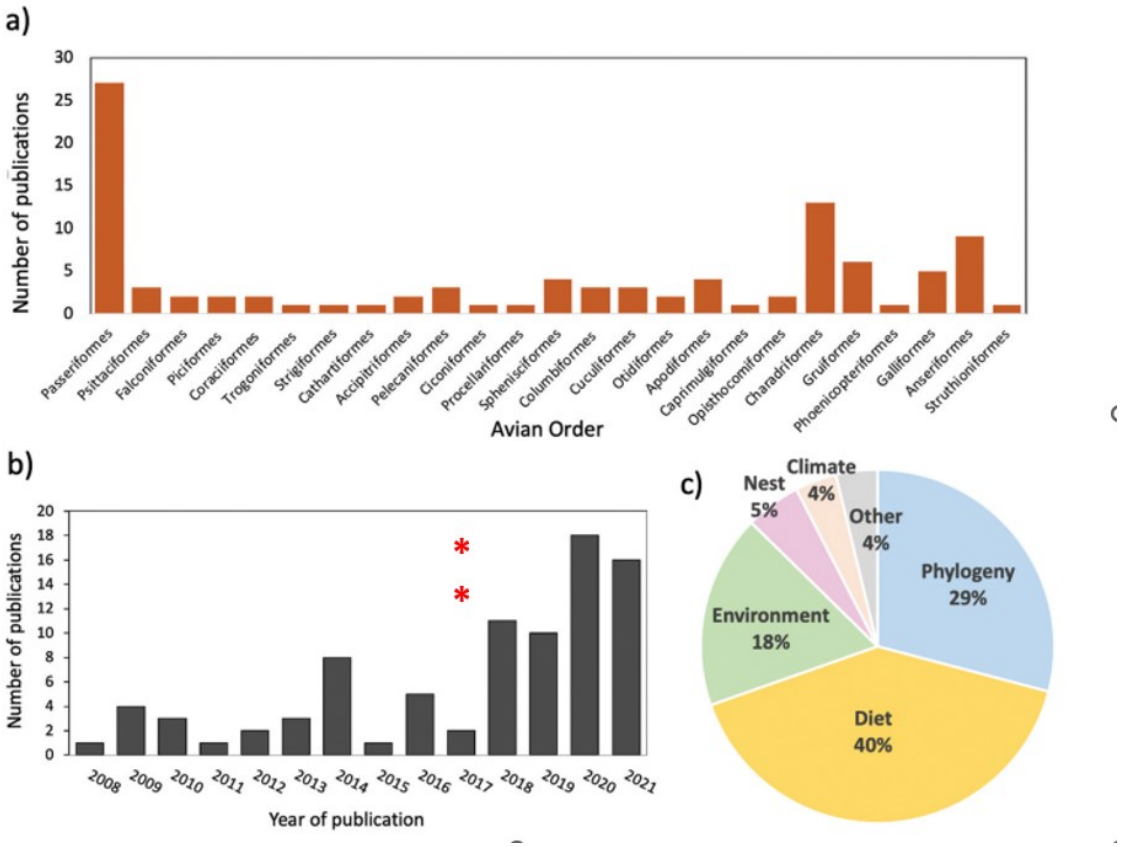


Figure 3: Number of studies showing the effect of various GM drivers in wild birds. Studies from 2008-2021 (N = 86) presented as (a) total number of studies across different avian orders, (b) comparison between years, and (c) proportion of studies that identified at least one dominant factor on GM diversity and composition (N = 79). The key factors can be divided into very broad categories: diet (N = 32 studies), phylogenetic (N = 23), external environment/ habitat (N = 14), nest environment (N = 4), climate (N = 3; e.g. seasonal changes) and 'other' (N = 3), including studies that identified migration behaviour, sex and life stage as drivers (adopted from Sun et al. 2022 wherein see for more details). Red asterisks indicate the date of publication of our **papers I** and **IV**, illustrating we were among the first to question these factors in birds. It should be mentioned, however, that much caution when interpreting and generalising results from different studies is needed as datasets differ greatly in terms of taxonomic scope and the number of individuals, as well as bioinformatic and statistical approaches.

In **paper I**, we provide the first comprehensive insight into interspecific and interindividual GM variation in 53 old-world passerine species. Although previous mammalian studies have shown relatively strong effects of various life-history traits on GM profile, such as diet (Ley et al. 2008; Muegge et al. 2011; Gomez et al. 2019), we surprisingly found only very limited effect of host ecology (diet, habitat, etc.) and geography but a stronger effect of phylogeny on microbiota composition. Similar results were also obtained by Hird et al. (2015) in neotropical birds among different avian orders in Central America (Hird et al. 2015) or Central Africa (Capunitan et al. 2020). The significant effect of phylogeny was also revealed between a few penguin species (Dewar

et al. 2013). Compared to mammals or non-flying birds, the passerine GIT underwent massive reductions due to active flight, especially in the parts involved in bacterial fermentation (e.g. colon and caecum; Mcwhorter et al. 2009; Price et al. 2015), and we think this may limit the adaptation of GM to the host ecology. The effect of phylogeny on GM could be explained by the neutral trans-generation transfer of bacteria suggesting that most bacteria in passerine GM are not adaptive. Alternatively, bacteria can co-diversify by interacting with some host selection mechanisms, such as immune genes. Although we cannot clearly distinguish between the trans-generation transfer and the selection mechanism based on our data, we might have better supported the selection scenario and speculate that GM in evolution is first recruited from the environment and then selectively filtered by immune genes. Although the effects of immune genes on GM diversity are largely unknown in wild birds, it has recently been suggested that *MHC* gene diversity affects GM in sticklebacks (Bolnick et al. 2014) and more recently in Seychellean warblers (Davies et al. 2022). Here, specific alleles of *MHC-I* and, to a lesser extent *MHC-II*, but not in *TLR3* impacted the GM profile in the Seychelles warblers. The influence of the immune system on GM could also be illustrated by the correlation between the strength of the pro-inflammatory immune response against phytohaemagglutinin (PHA) and some operational taxonomic units (OTUs) in the barn swallow (*Hirundo rustica;* Kreisinger et al. 2018). All this suggests that in future studies we should try to map co-divergence between host GM and some immune genes/ traits and e.g. bacterial-sensing PRRs could be suitable candidates.

Decreasing biotic diversity with increasing latitude is a widely known macroecological rule that has been documented for a broad range of taxa (Owen and Owen 1974; McCoy and Connor 1980; Guernier et al. 2004), but whether it is valid also for GM remains unresolved. Many ecological factors change with both latitude and altitude, such as temperature, UV radiation and humidity, and all of these factors can also affect the pool of available environmental bacteria and potentially also interact with the host's immune system. Furthermore, many temperate birds undertake long and energetically costly migrations to the tropics that temporally significantly reduce GIT mass (about 30 % of GIT weight; Battley et al. 2000). Also, conditions on wintering grounds can propagate as carry-over effects on their fitness at breeding sites (Norris et al. 2004; Legagneux et al. 2012) that could also affect GM. To investigate the effect of these potential drivers, in **paper II**, we directly compared the diversity and composition of GM for the first time in a large comparative dataset of 99 tropical and temperate passerines (with residents/short-distance migrants and long-distance migrants). Surprisingly, we identified no consistent differences in diversity and composition between tropical and temperate passerines. As **in paper I**, host phylogeny explained significant GM variation between species, and unlike temperate passerine birds, diet affected GM profile. Interestingly, much more profound differences were found between dry and wet seasons in tropical passerine birds when, in particular, the proportion of Firmicutes consistently increased during the

wet season. It remains to be determined whether such substantial changes are directly attributable to different environmental factors such as food availability or humidity, or to changes in physiological conditions linked with breeding (Tye 1992; Fotso 1996). In any case, seasonal changes of GM have been documented in a few temperate birds (Dong et al. 2019; Dietz et al. 2022) and diet also played some role between different Darwinian finches (Loo et al. 2019b, a) or penguins, in which the proportion of Fusobacteria increases sharply food starvation (Dewar et al. 2014). To address the effects of migration on GM, we first directly compared the GM of two trans-Sahara migrants captured at both their wintering and breeding sites: the garden warbler (*Sylvia borin*) and the willow warbler (*Phylloscopus trochilus*). Although one might expect substantial carry-over effects leading to a mixing of their GM from both sites, their GM converged well with the overall GM of other passerines at the sampling site. This suggests that GM is highly plastic, strongly influenced by the environment, and has limited OTU stability over time (see also Kreisinger et al. 2017; Risely et al. 2017, 2018 for further support). Second, we compared GM between temperate trans-Saharan migrants and short-distance migrants or resident birds. There were no differences in α- and β-diversity of GM between these groups, except for the increased abundance of three lactic acid bacteria (LAB; genera *Carnobacterium*, *Enterococcus* and *Lactococcus*) in trans-Saharan migrants. LABs prefer energy-rich substrates and ferment carbohydrates even under anoxic conditions (Kandler 1983). We hypothesise that they may increase the food efficiency (Abe et al. 1995) of long-distance migrants during their extremely energetically demanding migration, which is often associated with considerable food shortages and starvation.

Although whole GM correlated well with host phylogeny in previous avian GM studies (Waite and Taylor 2014; Hird et al. 2015), including ours (**papers I)**, this pattern can be caused by only a few OTUs or as a methodological artefact. Therefore, in **paper III**, we further assess the tightness of individual OTU associations with host phylogeny on a much finer scale by re-analysing the dataset from **paper I**. We revealed that the majority of OTUs were not well correlated with host phylogeny. We found that the majority of OTUs analysed showed significant variation in the distribution of OTUs between host species and that only relatively few OTUs were highly host-specific. This may suggest that the previously reported whole GM co-divergence was due to changes in the abundance of relatively unrelated bacteria between host species. Highly host-specific OTUs included bacteria that interact closely with their hosts, i.e. affecting immunity and metabolism, or potential pathogens. For example, *Candidatus Savagella* from Segmented Filamentous Bacteria (SFB) is known commensal that binds the intestinal wall (Thompson et al. 2012), stimulates immune system development (Hedblom et al. 2018) and prevents colonisation of the gut by pathogens (Shi et al. 2019). *Helicobacter* (with high specificity also found previously; Falush et al. 2003), *Yersinia*, *Ureaplasma*, *Mycoplasma*, *Clostridium* (*sensu stricto*), and *Escherichia/Shigella* are known potential pathogens. Other host-specific bacteria were LAB (*Enterococcus*, *Lactococcus,* or

Carnobacterium), which may bring many benefits to their host, from metabolising complex carbohydrates on the one hand to out-competing pathogens on the other (Cox and Dalloul 2015; Ajuwon 2016). This study may suggest that in future studies we should better focus on the analysis of individual OTUs rather than general descriptions of the entire GM communities in order to understand the role of the microbiome.

Mammalian studies suggested significant differences in microbiota communities between different GIT sections (Costello et al. 2009; Suzuki and Nachman 2016) that correlated with nutrient and oxygen availability. In mammals, the highest diversity is typically found in the large intestine and its caeca (Suzuki and Nachman 2016). In birds, our knowledge of microbiota in the different GIT is limited to domestic galloanserids with large caeca (Sekelja et al. 2012; Han et al. 2016; Xiao et al. 2017), and any information on microbiota profile of various GIT parts in wild birds was lacking. In **paper IV** we described for the first time in passerines the microbiota from two GIT sections, from non-invasively collected faecal samples and oral cavity swabs of the great tit. Diversity of the oral microbiota was much higher than in faeces, yet with a relatively homogeneous composition, suggesting that the oral microbiota is mostly recruited from the environment. In contrast, the less diverse faecal microbiota was much more inter-individually variable and did not correlate with the oral microbiota at the individual level. This may suggest that the faecal microbiota is more tightly regulated by some intrinsic mechanisms, such as the immune system. In the following **paper V**, we confirmed a clear divergence of microbial communities between the upper (dominance of Proteobacteria and Actinobacteria) and lower GIT (dominance of Actinobacteria) in invasively and non-invasively collected samples across the GIT gradient of six parrot species. In contrast, continuous sampling in different lower GIT tissues revealed relatively homogeneous microbial communities, which corresponds well to the relatively low morphological diversity of the parrot gut. Importantly, consistent with previous studies (Videvall et al. 2018; Berlow et al. 2020), our analysis confirmed that faecal samples, which are most commonly used in field GM studies, represent whole gut communities well (much better than cloacal swabs).

In birds, the GM microbiota is mostly studied in adult individuals and less in young individuals (Grond et al. 2018) and it remains unclear when the first bacteria colonise the avian GIT. For example in humans, the microbiota is mostly absent in the placenta and foetus under physiological conditions, but bacteria colonise the gut post-partum (de Goffau et al. 2019). Is the avian egg also initially sterile or do females deposit bacteria into the egg to control the initial microbiome of embryos and chicks? Do bacteria colonise eggs later in embryonic development? In birds, two mechanisms of egg colonisation by bacteria have been postulated theoretically: (1) *vertical transmission* from the oviduct of the mother and (2) *horizontal bacterial trans-shell migration* across the eggshell pores after egg laying, yet these mechanisms have received mixed empirical support. While only negligible microbiota have been revealed in the embryonic gut of two Arctic

shorebirds (Grond et al. 2017), fairly diversified microbiota have existed in egg contents and developing embryos of chickens (Ding et al. 2017, 2022; Lee et al. 2019; Akinyemi et al. 2020), suggesting that the first bacteria may colonise the eggs before hatching. However, all of these chicken studies suffer from significant methodological caveats, such as not seriously considering the problem of contamination. This is critically important in studies with low bacterial biomass (Eisenhofer *et al.* 2019; Salter *et al.* 2014) and limits the conclusions of the aforementioned studies. **In paper VI**, we came up with an innovative design to study the microbiome of avian eggs. For the first time in such a study, we used two *16S rRNA* DNA metabarcoding protocols with sample amplification in technical PCR duplicates, including multiple negative control types, combined various bioinformatic steps to remove potentially contaminating ASVs together with confirmatory candidate qPCR ASVs-targeting approach, and analysed the microbial profiles of freshly laid eggs, embryonic GIT (day 13 of embryonic development) and female maternal faces in great tits (*Parus major*) from a total of 240 samples and 57 nests. Contrary to some previous studies in birds (e.g. Ding et al., 2017; Trevelline et al., 2018), our results showed negligible and inconsistent microbiota communities in freshly laid eggs and with high proportion of potential contaminants. In embryonic GIT samples, there were more bacterial ASVs but still dominated by potential contaminants. Of the three potentially pathogenic ASVs further targeted by more accurate qPCR (*Corynebacterium*, Barbosa and Palacios 2009; Risely *et al.* 2018; *Clostridium*, Tsiodras et al. 2008; Benskin et al. 2009; and *Dietzia* (Koerner et al. 2009; Olowo-okere et al. 2022), our results indicated that only *Dietzia* could be passed from mother to egg by *vertical transfer*. Similarly, the very rare vertical transfer has been suggested for *Salmonella* in chickens (Keller et al. 1995; Gantois et al. 2009; Pedroso 2009). We find out that *bacterial trans-shell migration* seemed to contribute slightly more to the formation of simple and low-abundant bacterial communities in tit embryos than *vertical transmission*. Nevertheless, our data suggest that the GM in passerines predominantly forms after hatching. This is also supported by the high ontogenetic dynamics of passerine microbiota during the nestling period (e.g. Chen et al., 2020; Kreisinger et al., 2017; Teyssier et al., 2018). Further studies of egg and embryonic microbiota across avian phylogeny are needed to determine whether the described discrepancy in microbiota recruitment between passerines and chickens reflects differences in life-history traits (e.g. altricial vs. precocial nestlings) or rather methodological differences between studies.

4.2 Evolution of innate immunity receptor diversity

Identifying functional immune gene variations involved in pathogen resistance or tolerance is a major challenge in evolutionary immunology (Vinkler et al. 2022). However, this is complicated by the fact that more than one-fifth of all genes in vertebrate genomes are involved in immunity (Vinkler et. al., *submitted*). But even defining what the true immunity gene is is not straightforward.

Until recently, we mostly relied on the candidate gene approach when studying host-parasite coevolution, limiting ourselves to a few known candidates, such as the (Acevedo-Whitehouse and Cunningham 2006; Těšický and Vinkler 2015; Vinkler et. al., *submitted*). However, it is now becoming clear that studying *MHC* genes itself cannot explain the most variation adaptive variation against infectious diseases (Acevedo-Whitehouse and Cunningham 2006). Although most of our inferences about evolutionary immunology come from the *MHC*, given its complex and rapid evolution, the *MHC* is not a convenient model for an immune system (Vinkler et. al., submitted). The massive democratisation of transcriptomics and genomics platforms has opened up unprecedented opportunities for finding new immune gene candidates. But how do we find the needle in the haystack among thousands of immune genes? In our initial two papers, we first outlined the ways how to search for such candidate genes, theoretically in **paper VII** from animal genomes/transcriptomes, and then practically in **paper VIII** by developing an evolutionary bioinformatics pipeline for predicting candidate putative functionally evolving SNVs. We have demonstrated the high sequence variation and strong pattern of positive selection on TLR LBRs in tits, finches and buntings (**papers VIII** and **VIX**, respectively) and on RLR LBRs in most bird clades (**papers X** and **XI**). Importantly, our evolutionary bioinformatic prediction of the TLR and RLR molecular phenotypes (**papers VII-XI**) showed that some of the SNVs detected could be putatively functional and thus candidates for further *in vitro* mutagenesis testing. Intriguingly, we demonstrated that both *MDA5* and *RIG-I* underwent multiple losses in bird evolution (**papers X** and **XI**). In **paper XII**, we also showed that loss of *CNR2* due chromosomal re-arrangments in parrots is associated with increased neuroinflammation in the brain.

In **paper VII**, to stimulate comparative research in evolutionary immunology beyond the *MHC* genes, we proposed a new hierarchical classification of immune genes: (i) the *core immunome*: genes whose primary (and in many cases, only) physiological function is to recognise, and/or respond to, pathogens; (ii) the *peripheral immunome*: genes with a clear immunological role, but which also contribute to non-immune physiological functions; and (iii) non-immune resistance genes (NIRGs; which could be called the *accessory immunome* or even the *resistome*) that primarily only confer resistance to pathogens and have no immune function. Second, we advised on how to retrieve the comprehensive core immune gene list from various gene databases using a standardised vocabulary, and reviewed the current state-of-the-art approaches to study the molecular evolution of immune genes. Third, we proposed a new set of stimulating evolutionary questions for further research. For example, it should be clarified whether the strength of parasite-mediated selection on immunity is the same for different types of immunity genes or whether their different cellular localisations and functions play a role.

Although PRRs (*TLRs*) have been studied in multiple bird studies, these studies mostly focus on a simple description of their variability and detection of positively selected sites (e.g. Alcaide and

Edwards 2011; Grueber et al. 2015; Minias et al. 2021), but only rarely attempted to investigate whether the observed variation is putatively functional, but see Levy et al. (2020) and Fiddaman et al. (2022). This could best be achieved by testing candidate SNVs using *in vitro* expressional assays. However, such an approach is laborious and costly and we first need to obtain the short-list fined-tuned candidate SNVs. In **paper VIII** adopting the state-of-the-art predictive structural bioinformatics, we first outlined a new broadly applicable methodological approach to identify candidate SNVs with high potential adaptive value. Our approach first utilizes the identification of PSS or post-translationally modified sites based on the consensus of multiple methods combined with 3D structural protein modeling, their various annotations, and the evaluation of whether they evolve in a convergent manner (Figure 2). Second, applying this pipeline, we revealed that bacterial-sensing *TLR4* and *TLR5* in tits are well diversified at the interspecific levels in their LBRs, as shown by their relatively high nucleotide diversity as well the proportions of PSS. Tit TLR variability was on similar levels as indicated in other bird and rodent TLR studies (e.g. Alcaide and Edwards 2011; Fornůsková et al. 2013; Králová et al. 2018; Velová et al. 2018), but much lower than in *MHC* genes (Alcaide et al. 2007; O'Connor et al. 2016; Minias et al. 2021b, c). Interestingly, we identified a widespread pattern of convergence in *TLRs* on multiple levels. First, most of the PSSs we identified also evolved convergently and met our criteria (Figure 2), suggesting that convergence frequently targets sites involved in or close to ligand binding sites. Second, we identified convergence in post-translational modifications as an independent gain or loss of N-glycosylation and phosphorylation motifs. This is an interesting finding as both alterations can affect the binding properties of amino acids in TLRs, either facilitating or preventing the formation of bonds with pathogenic ligands (Ricci-Azevedo et al. 2017). In immune genes, convergence has so far been tested mainly in *MHC* genes using *MHC*-specific approaches developed based on mismatches of PBR- vs non-PBR-based trees or synonymous vs. non-synonymous trees (Yeager and Hughes 1999; Kriener et al. 2000; Xu et al. 2008; Li et al. 2011), but this limits their applicability beyond *MHC*. In our study, we came up with the idea to also test molecular convergence using the discrepancy between the surface charge distribution phenogram and the species phylogeny, making this method universally applicable to all protein-coding data. Third, convergence was found to be common in overall TLR4 and TLR5 LBRs surface charge distribution involved in ligand binding. In the last step, we attempted to explain the observed convergence in surface charge by some general ecological traits (such as habitat, distribution or diet that could serve as a proxy for the GM profile), since no metabarcoding GM data were available. However, contrary to our expectations, our analysis did not reveal any links between the ecological traits and surface charge, suggesting that information on GM may be needed to explain TLR evolution.

Following this paper, as most TLR research in non-model bird species focused on bottlenecked populations with depleted genetic variation (e.g. Grueber et al. 2013; Gonzalez-Quevedo et al.

2015; Vlček et al. 2022), in **paper IX**, we investigated intra- and interspecific variation in three *TLR* genes (*TLR1A*, *TLR3*, *TLR4*) in eleven species of buntings and finches with large effective population sizes. Eight individuals were sampled per species. We found extraordinary variability in finch and bunting *TLRs*, yet with high differences between loci and species. For instance, the maximum allelic richness was in the common redpoll (*Acanthis flammea*) where all 15 *TLR1A* nucleotide alleles were translated into unique protein haplotypes. In contrast, in the hawfinch (*Coccothraustes coccothraustes*) there were only four nucleotide alleles translated into two TLR1A protein variants. Consistent with previous findings (Velová et al. 2018), viral-sensing *TLR3* was the most conserved *TLR* locus. We identified a relatively high number of PSS (especially in *TLR1A* and *TLR4*) and most of them were putatively functional, including several amino acid substitutions that alter surface distribution in LBR. Using the innovative *in silico* mutagenesis approach, we have shown that even non-synonymous substitutions in PSS buried in the protein backbone structure can strongly affect the electrostatic potential through the polarization of a few amino acids in their neighbourhood. Despite the relatively recent species divergence, no identical nucleotide *TLR* alleles were shared between species, suggesting rapid TLR evolution. In fact, only one allelic lineage was shared between two closely related *Fringilla* species, the common finch and the brambling, that diverged ca. 8 Mya (Fjeldsa et al. 2020), suggesting there is only little trans-species polymorphism in *TLRs* (TSP; Klein et al. 2007). All this corresponds well with our unpublished findings in tits, where identical alleles of *TLR4* and *TLR5* are shared only between closely related species (no longer than 4 Mya; Těšický et.al., *in prep.*). In contrast, in *MHC* genes, identical or nearly identical alleles are commonly shared and maintained between species as balanced TSP over tens of millions of years (reviewed in Těšický and Vinkler 2015).

In **papers X** and **XI**, we found the widespread positive selection in *MDA5* and *RIG-I* in birds spanning several functional domains (mainly in helicase and CTD domains). Using genome-wide *in silico* scans, we also demonstrated that both *MDA5* (twice) and *RIG-I* (up to sixteen times) were lost in avian evolution, indicating dynamic evolution of avian *RLRs*. Interestingly, *RIG-I* deactivation also involved the whole Galliform clade wherein we have detected gradual inactivation in several species. Recently, the similar pseudogenisation in *RIG-I* has been described in mammals in tree shrews (Xu et al. 2016) and also in *MDA5* in pangolins (Fischer et al. 2020). However, the actual causes and consequences of such events remain unresolved. In our studies, we revealed that avian orders lacking one of these receptors always lost only one receptor (not a single species lost both receptors simultaneously), still indicating their functional necessity for RNA recognition. This led us to hypothesise that the loss of one receptor might be compensated by the function of the other. Therefore, we tested whether adaptive compensatory evolution occurs in the molecular phenotype of *MDA5* in species where *RIG-I* had been pseudogenised and vice versa. While we did not detect specific compensatory adaptive evolution in *MDA5*-deficient species, we find some

evidence for compensatory evolution in *MDA5* after *RIG-I* loss in Galliformes, based on the overall surface charge clustering and specific PSSs likely affecting dsRNA binding. How can species benefit from such pseudogenisation, assuming that it is primarily not detrimental? (i) Loss of an infection sensor might be accompanied by an acquired tolerance to a particular pathogen. (ii) Given the redundancy of the immune system, the function of the missing sensor may be replaced by another related gene. For instance, in the tree shrew *RIG-I* absence was compensated by the *MDA5* sensor at least for infection with Sendai virus by *MDA5* sensor (Xu et al. 2016). In contrast, intensive research has not revealed functional compensation in chicken MDA5 following *RIG-I* loss (Barber et al. 2010; Karpala et al. 2011; Lee et al. 2020). (iii) Loss might prevent the occurrence of autoimmune diseases, as in humans some mutations in both *MDA5* and *RIG-I* have been associated with undesired binding of self RNA (Dias Junior et al. 2019; Lei et al. 2022). The numerous *RIG-I* losses in birds are particularly intriguing, suggesting a long-term continuing tendency for *RLR* losses during avian (similarly as *TLR5* in avian evolution; Bainova et al. 2014) and it opens new avenues for experimental work.

In paper XII we found out that *CNRs* binding primarily self-origin endocannabinoids had only a negligible pattern of positive selection in all tetrapods. This supports the hypothesis that immune receptors recognising self-ligands are under weaker selection than those binding exogenous ligands (Vinkler et. al, *under rev*.). We demonstrated that pseudogenisation of *CNR2* in parrots occurred via two independent chromosomal re-arrangements. Since we did not find any molecular adaptations in *CNR2* that would compensate for the neuroimmunomodulatory effect of *CNR1*, we designed *in vitro* inflammation experiment to test the functional effect of such gene loss using transcriptomic data. Specifically, we experimentally induced peripheral sterile inflammation in six CNR2-deficient parrots and one passerine with intact *CNR2*, the zebra finch (*Taeniopygia guttata*). According to our predictions, we revealed that the experimentally induced peripheral inflammation propagated to the brain in parrots but not in the zebra finch. In parrots, it caused neuroinflammation in the brain characterised by the upregulation of proinflammatory cytokines (including interleukin 1 beta, *IL1B* and *IL6*). Loss of the *CNR2* gene is an interesting finding which might explain why parrots are more susceptible to neuroimmune diseases (Rinder et al. 2009; Staeheli et al. 2010) and infection-triggered behavioural disorders (Rubinstein and Lightfoot 2012; Speer 2014).

4.3 Physiological senescence

Since birds represent an analogous homeothermic lineage of amniotes with various adaptations for longevity, understanding molecular and physiological age-related changes may shed new light on the causes of senescence. Until recently, senescence in molecular physiological traits has been poorly documented in longitudinal studies of free-living birds (see reviews Stier et al. 2015 and

Bouwhuis and Vedder 2017) but see (e.g. Bize et al. 2014). Moreover, much of the current gerontological research in birds has been devoted to telomere shortening (e.g. Hammers et al. 2015; Bichet et al. 2020; Sheldon et al. 2021; Kauzálová et al. 2022). Although these studies documented that telomere shortening could serve as a good proxy of the biological age of the organism, there have also been significant methodological difficulties in measuring telomere length, leading to a substantial bias in the prevailing results (Foote et al. 2013; Kärkkäinen et al. 2021). Our studies in great tits are thus some of the first in wild passerine birds to use a longitudinal dataset to reveal age-related trajectories in simultaneously measured multiple physiological traits. In **paper XIII** we reported *endocrinological senescence* of plasma testosterone levels in males but not in females, suggesting that testosterone in males has similar dynamics to mammals. In **paper XIV**, long-term inflammation was found to increase progressively with age, indicating for the first time in birds *inflammaging*. Consistent with this *inflammaging* hypothesis, we found that the higher the background inflammation, the greater the tissue damage due to oxidative stress. In contrast, *in vitro* LPS-induced acute inflammation had a polynomial dependence on age, with an immunosenescent decline in older individuals. **In paper XV**, contrary to our expectations, of the four heavy metals measured in blood (Zn, Pb, As, Cd), only Pb changed with age and had non-linear dependence with the highest levels in very young and very old individuals. These results suggest that bird blood can be used to monitor actual heavy metal concentrations, even if the precise age of the individuals is not known.

In **paper XIII** we assessed inter-annual stability and age-related changes in plasma testosterone levels in 49 repeatedly captured great tits. As variation in testosterone levels reflect the social ranks of individuals in the population as well as investment in plumage ornaments (Hau and Goymann 2015), we expected high inter-annual stability of testosterone levels. Surprisingly, we revealed high inter-annual repeatability in plasma testosterone only in females but much lower inter-annual repeatability in males. The finding probably resulted from its higher ontogenetic dynamics in males compared to females. In males, testosterone levels had polynomic dependence on age, peaking in mid-life and followed by a steady decline. Such a trajectory in which testosterone levels co-vary with the drop in reproductive performance (Ottinger 1996) was expected in birds but previous studies (e.g. Peters et al. 2002; Smith et al. 2005; Madsen et al. 2007) have brought much controversy in testosterone lifetime dynamics. However, none of these studies were longitudinal and their results may be biased by the selective disappearance (Zhang et al. 2015). Likewise, as the similar bell-curved lifetime trajectory in male testosterone levels exist also in humans (Vermeulen et al. 1972; Harman et al. 2001), our data thus show similar *endocrinological senescence* of testosterone levels in birds and mammals. Surprisingly, plasma testosterone levels in females remained stable throughout life, in contrast to those in males. This also contrasts with the observed age-related decline in female plasma testosterone levels recently demonstrated in two passerine

birds (Moreno et al. 2014; Adámková et al. 2019) albeit in cross-sectional data. Further longitudinal studies are needed to clarify whether ontogenetic parallels between mammals and birds exist also in female testosterone levels. Finally, as testosterone regulates multiple condition-related traits, including ornamental expression (Duckworth et al. 2004; Galván et al. 2010; Vinkler and Albrecht 2010), we also tested for its association with sexually selected yellow breast ornamentation and melanin black stripe (Senar and Quesada 2006; Hegyi et al. 2007), heterophil:lymphocyte ratio (H/L ratio, the indication of physiological stress, Davis et al. 2008) and FGR indicating nutritional status (Grubb 2006). Only the yellow brightness of breast ornament in males was positively correlated with plasma testosterone levels. This is an interesting finding as this ornament serves as a quality indicator in tits (Senar et al. 2002) and may play role in sexual selection.

In our **paper XIV** we compared lifetime patterns in different pro-inflammatory traits: (i) chronic inflammation measured as leukotriene B4 levels (LTB4) and (ii) acute inflammation measured as cellular oxidative burst after *in vitro* LPS challenge in 54 repeatedly captured great tits. For the first time, we revealed a linear increase in LTB4 with age in birds. LTB4 is a pro-inflammatory lipid regulator that drives leucocyte adhesion to the endothelium, their recruitment to sites of infection, and further RONS production by granulocytes and its level is chronically elevated in various human inflammatory diseases (He et al. 2020). Our finding thus proves for the first time that birds can suffer from *inflammaeging* as humans (Franceschi et al. 2000, 2007). In contrast, cellular oxidative burst response showed a polynomial dependence on age, with the highest peak in middle-aged individuals followed by a steady decline. This clearly illustrates the functional *immunosenescence* in general effector cellular inflammatory response in old individuals that was not functionally compensated by the increased number of absolute granulocytes. Our study is the first longitudinal vertebrate study that showed such a bell-curve trend in oxidative burst, although the age-related decline has already been observed in other studies in birds (Sild and Hõrak 2010), fish (Stosik et al. 2002) and in some studies in humans (Fulop et al. 2004; Moroni et al. 2005). As oxidative burst response seems to be worse during ageing in a variety of taxa, future studies should focus on a detailed investigation of the molecular and cellular processes underlying these age-related changes. Antioxidants such as GPX, SOD or thiols play a crucial role in maintaining oxide-redox balance in the organism during the oxidative burst (Forman and Torres 2002). We found a negative link between GPX activity and LTB4 levels, suggesting that chronic inflammation depletes the antioxidant availability for free-radical clearance, which may induce further oxidative stress and promote further inflammation (Pisoschi and Pop 2015). In fact, overall oxidative damage correlated positively and strongly with LTB4 levels while individuals with elevated LTB4 levels had further reduced GPX activity, further supporting *inflammaging* hypothesis.

Although the *Oxidative stress hypothesis of ageing* is theoretically well established, there is mixed evidence across taxa, tissues and markers used (reviewed in Costantini 2019), and current evidence

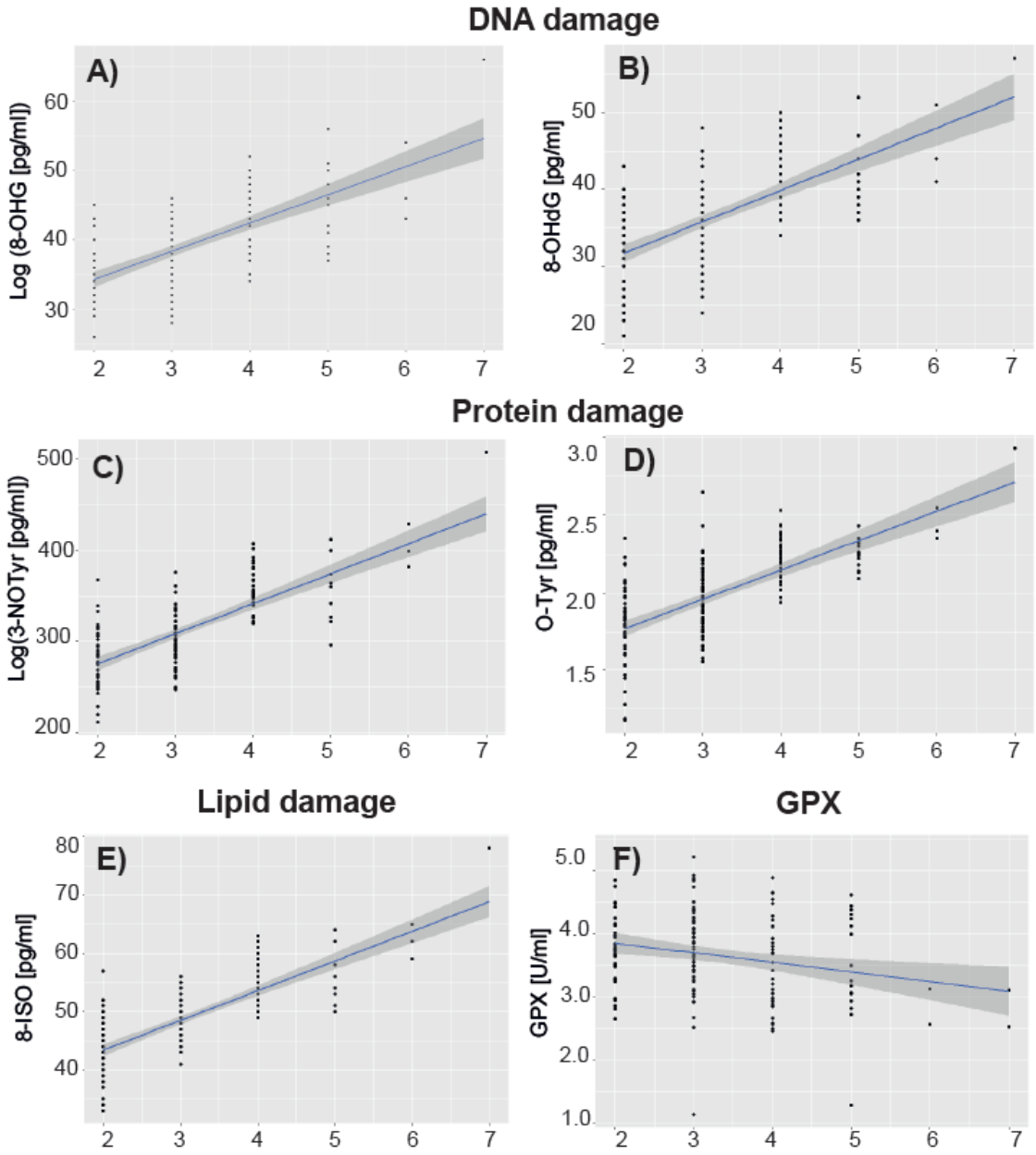
in birds is only limited to cross-cohort captive and laboratory studies. For instance, mitochondrial ROS production increased during ageing in pigeons and mice (Sasaki et al. 2008, 2010) and also in zebra finches (Salmón et al. 2022), but this was not compensated by increased AOX levels, resulting in age-related accumulation of various oxidative stress damages (Sasaki et al. 2010). In captive zebra finches, various oxidative stress damages increased during ageing, but the rate was modulated by environmental conditions (Marasco et al. 2017) suggesting that the pattern of senescence can be different in wild birds that are more likely cope with various trade-offs. Interestingly, in our unpublished results using the same longitudinal dataset, we found that oxidative damage progressively accumulated with age in the great tit and the observed trends were highly consistent across all nucleic acid, protein and lipid markers (Figure 4 and Těšický et al., *in prep*.).

Antioxidant levels can adopt various trajectories during ageing, e.g. becoming upregulated to compensate for increased oxidative stress (Stau et al. 2018), decreasing as a consequence of increased oxidative stress (Berglund et al. 2014) or remaining unchanged (Sasaki et al. 2008), but their dynamics are often tissue-specific or context-dependent (Costantini 2019). In our study, the accumulation of general oxidative stress damage during ageing was not compensated by antioxidant upregulation. While GPX activity decreased with age, free reactive thiol levels and SOD activity remained unchanged during ageing (Figure 4 and Těšický et al., *in prep.*). Our longitudinal data thus clearly demonstrate senescence in multiple physiological traits adopting often contrasting trajectories in great tits. Whether this is the observed age-propagating oxidative stress damage mainly caused by increased mitochondrial ROS production during ageing (Salmón et al. 2022) or by *inflammaging* (Franceschi et al. 2007) cannot be distinguished from our correlation data and require further investigation. However, the strong positive correlation between oxidative stress damage and chronic inflammation suggests an important role of *inflammaging* in avian senescence.

In **paper XV** we evaluated longitudinal accumulation of four heavy metals (Zn, Pb, Cd, As) and their possible effects on health-related haematological traits in 185 recaptured great tits. Contrary to our predictions, we found weak and non-linear age-related changes in blood only for Pb, with the highest levels occuring in nestlings and the oldest senescent individuals. No age-related changes were detected for Cd and Zn (As was excluded from the analysis due to low concentration below the detection limit). This bimodal pattern for Pb may be explained on the one hand by the feeding on metal-rich food (such as caterpillars, spiders or beetles (Heikens et al. 2001; Fritsch et al. 2012) in nestlings (Eeva et al. 2005; Anssens et al. 2020) and on the other hand by the impaired detoxification mechanism in older individuals (Berglund et al. 2014; and see also **paper XIV**). It should be noted that the blood primarily serves as a transport medium, with generally short half-life dynamics of various heavy metals (ranging only from units to tens of hours for As; Lehmann et al. 2001; up to one month for Pb), and that these heavy metals terminally accumulate in other tissues, such as bones (Pb; Järup 2003) or kidneys (Cd; Faroon et al. 2012). But even from such

terminal sites they can be released again and the blood can mirror both current and long-term levels (WHO 1995). This is also confirmed by our findings that Pb may be decontaminated from the blood of young individuals, while it can later accumulate in the body (possibly in the bones) and then increasingly re-enter the bloodstream of the very senescent individuals. In contrast to previous studies (Bauerová et al. 2017), we did not find any association between heavy metals and anaemia-like conditions. This is probably due to sampling in relatively low-pollution habitats. Nevertheless, we found that total white blood cell counts (TWBC) were positively correlated with all three heavy metal levels assessed, suggesting that their toxicity may increase leucocyte proliferation (Dumonceaux and Harrison 1994; Jones 2015). Our results demonstrate that bird blood can be used for actual heavy metal monitoring, even if the age is not known.

Figure 4: Age-dependent changes in oxidative damage marker and antioxidant levels in repeatedly captured great tits (N_{ind} = 54, N_{obs} = 116).
Oxidative damage of nucleic acids: (A) 8-hydroxy-2'-deoxyguanosine (8-OHdG), (B) 8-hydroxyguanosine (8-OHG); proteins: (C) 3-nitrotyrosine (3-NOTyr), (D) o-tyrosine (O-Tyr) and lipids: (E) 8-isoprostane (8- ISO) are shown. Out of three antioxidants measured (glutathione peroxidase, GPX; superoxide dismutase, SOD and free reactive thiols), only GPX levels with the only detected age-related changes are shown. Oxidative damage marker/antioxidant levels are plotted against minimal estimated age of birds using the same statistical approach as in **paper XIV** (adopted from Těšický et al., *in prep.*).



5. General conclusion and future perspectives

In my work, I have provided some novel insights into the evolution of inflammation and antimicrobial resistance in birds. By studying (1) ecological and evolutionary determinants of GM composition and diversity that activate and modulate inflammation, (2) diversity of immune genes that influence inflammatory responses, and (3) inflammation-induced physiological senescence in wild birds, I have contributed to expanding our knowledge of host-pathogen co-evolution in birds, paving the way for future research in microbial ecology, evolutionary immunology and biogerontology (not only) in birds.

In **papers I – III** using our large comparative passerine datasets we revealed high intra- and interspecific variation in passerine GM. Host phylogeny and, to a very limited extent, also host ecology shaped interspecific variation of GM in passerines, yet their overall contribution was much smaller than in similar mammalian studies and most of the variation remained unexplained (**papers I – II**). Our study, in which we for the first time directly compared GM between tropical and passerine birds, but also during the rainy and dry seasons, showed that GM is significantly influenced by environmental conditions and is very plastic (**paper II**). Our deeper analysis of GM co-divergence with host phylogeny on a finer scale revealed that only relatively few OTUs were highly host-specific (mostly OTUs with a clear physiological role, such as lactic acid bacteria or potential pathogens; **paper III**). The lower GIT harboured relatively homogenous microbial communities at different sites (**paper V**). This corresponds well with the relatively poorly differentiated gut in parrots and passerines as the adaptation to active flight (e.g. substantial reduction of the ceacum and colon that might be compensated by more effective nutrient absorption; Caviedes-Vidal *et al.* 2007). Surprisingly, analogous morphological and physiological gut adaptions also convergently evolved in bats, making bat GM more similar to passerine GM than most other mammals (Song et al. 2020). It has been hypothesised that the high unexplained variability and limited stability of most OTUs in passerine GM may be due to the fact that a large proportion of GM rapidly passes through the passerine gut and does not colonise it (Schmiedová 2022), which is also supported by the high proportion of insect microbial symbionts in passerine GM. This does not mean at all that GM is "less important" in passerines than in other vertebrates, yet we needed to clearly distinguish true gut colonisers physiologically interacting with their host from transient food-derived OTUs. Since most avian studies have been so far focused on GM in adult birds, overlooking the importance of GM during the earliest ontogenetic development, **paper VI** aimed to clarify how and when the first bacteria colonise the avian egg. Initially, this lead us to develop a methodologically advanced metabarcoding protocol to account for environmental bacterial contaminations, as this had been severely neglected in previous low-bacterial biomass studies in birds. Our results have shown for the first time in passerines that bird eggs are nearly sterile, suggesting that GM predominantly forms only after hatching. This contrasts with all studies

in chickens that, however, suffer from considerable methodological caveats. In the future, studies across avian phylogeny and with the proper methodology are needed to clarify the timing and mechanism of the earliest bacterial colonisation of bird egg. Elucidating the mechanisms of GIT microbiota establishment in early ontogeny can improve our understanding of parental effects and contribute to basic knowledge of wildlife evolutionary ecology and zoohygienic and veterinary applications that minimise the risks of disease transmission. In summary, all our microbial results indicate that bird GM including their drivers is significantly different from that of mammals and that the results of studies on mammals cannot generally be applied to birds. Our results also indicate that from the pioneering research describing major patterns in GM diversity across species and tissues, future research should clearly shift more towards understanding the functional role of specific candidate OTUs (e.g. temporally stable or highly host-specific OTUs) and attempt to link them to the host phenotype and genotype. For instance, the association of candidate OTUs with the genetic diversity of immune genes and their molecular phenotypes (such as surface electrostatic charge distribution which influences microbial recognition) is highly desirable via the candidate gene approach (e.g. with PRRs) or via the microbiome-genome-wide association study (mGWAS). Alternatively, it can also be helpful to link an individual's health status, which may be modulated in large part by encoded variability in immune genes, to the GM. Advances in modern -omics methods (metagenomics, metatranscriptomics and metabolomics) will also allow investigation of the functional role of bacteria in birds in the future. It is tempting to speculate that perhaps the stability of overall functional metabolic pathways as a whole is more important than the stability of individual OTUs. In this scenario, low temporal OTU stability could be compensated for by the analogous function of other OTUs, so that functional metabolic pathways could remain stable.

The conceptual framework developed for categorising immune genes (**paper VII**), together with our newly outlined *state-of-the-art* methodological pipeline identifying putative functional variation based on adaptive convergence (**paper VIII**), can be applied to systematically search for functional SNVs in antiparasitic resistance in immune genes across genomes far beyond the best-studied *MHC*. We have illustrated that predicting molecular phenotype by employing structural 3D protein modelling (now widely available thanks to AlphaFold) in combination with electrostatic surface charge analysis and testing for adaptive convergence is key to revealing functionally important immunological adaptations. This can also have multiple practical implications, e.g. in animal breeding, in predicting the zoonotic potential of infectious diseases, or in conservation genetics, where decisions about which populations to prioritise conservationally can be better made on assessing adaptive variability encoded in immune genes rather than by neutral markers alone. Although RLRs are core immune genes recognising viral ligands, we discovered striking multiple gene losses of *RIG-I* and *MDA5*, occurring at least 15 times and 2 times, respectively, in avian history (**papers X** and **XI**). This opened the avenue for a functional understanding of the benefits

or costs of such events. Future studies should investigate whether species lacking these receptors are more susceptible to infection by highly pathogenic avian influenza or whether the receptor function is compensated by another immune receptor. In parrots, we not only discovered two independent gene losses of *CNR2* gene that negatively regulate inflammation, but also experimentally demonstrated that inflammation induced in the periphery causes neuroinflammation in their brains. However, this was not true for sister passerines with intact *CNR2* (**paper XII**). Such a finding could explain why parrots are more prone to various psychological and neuroimmune disorders. And more importantly, given the advanced cognitive abilities of parrots, this might suggest that parrots in captivity could serve as a promising biomedical model for studying various inflammation-related behavioural syndromes.

Biogerontological studies in the wild can provide a unique inside into the mechanisms of ageing. But only in a more demanding longitudinal design, they allow to truly disentangle the within-individual trends from population-wide effects that might bias the observed trajectories. Here, I benefited from a nearly decade-long longitudinal monitoring of a population of free-living great tits and provided robust evidence for physiological senescence and *immunosenescence* (**papers XIII – XV** and Těšický et. al, *in prep.*). However, lifetime trajectories of the various markers greatly differed. Using a novel set of biomarkers in wild research, we found out that individuals linearly accumulated various oxidative tissue damage to nucleic acids, proteins and lipids, supporting the *Oxidative stress hypothesis of ageing* (Těšický et. al, *in prep.*). Our finding that chronic inflammation also linearly increases during ageing, provides the first evidence that *inflammaging* plays a role in avian ageing. In contrast, the LPS-induced acute inflammatory response (cellular oxidative burst) peaked in midlife, demonstrating functional *immunosenescence* (**paper XIV**). Despite much controversy in previous studies of lifetime testosterone dynamics in birds, our study on plasma testosterone in great tits (**paper XIII**) provided clear evidence of *hormonal senescence* in males, with the bell-curve trend also corresponding with the best individual condition at midlife (Bouwhuis et al. 2009). In a nutshell, despite their different adaptations for longevity, small passerine birds appear to age similarly to humans and laboratory mammals in the investigated traits. Future studies should reveal the costs of such trajectories, e.g. through the effects on lifetime fitness. While the observed senescence in some traits may be a passive consequence of physiological decline without functional constraints, its decline in other traits may be driven by excessive costs to maintain them, followed by active diversion from energetically costly traits (Letters 2018; Gaillard and Lemaître 2020). Future studies should reveal whether this novel set of oxidative stress damage markers can indicate the biological age of organisms, e.g. through their linking with telomere shortening and other biological age indicators (Barrett et al. 2013; Jylhävä et al. 2017; Vaiserman and Krasnienkov 2021).

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Appendix: papers