Charles University

Faculty of Science

Study programme: Zoology

Branch of study: Zoology

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Variability of the domestic chicken breeds in selected immunological traits of hen and egg

Variabilita plemen kura domácího ve vybraných imunologických znacích slepice a vejce

Ph.D. thesis

Supervisor: RNDr. Michal Vinkler Ph.D.

Prague, 2018

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V Praze dne 28. 5. 2018

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Acknowledgement

I would first like to thank my supervisor Michal Vinkler. His guidance, valuable suggestions and consultations always helped me in research and in writing of this thesis.

I would also like to appreciate all co-authors of presented papers, namely to Jozef Janda, Lukáš Zita, Denis Laloe, Mathieu Charles, Vladimir Beneš and Pavel Stopka for efficient cooperation and knowledge sharing during our research. Especially I must accent contribution of Zuzana Świderská, my main co-worker on chicken-related topics. At this point I would like to acknowledge Ivana Gardiánová and Anežka Fabiánová, who provided me with exceptional assistance with chicken breeding and Andrea Rau, who provided me with advice in the field of biostatistics. For linguistic help I am gratefully indepted to my dearest friend Kateřina Šollarová. For graphical design of cover, I thank to Martin Vašátko.

I also must mention all the members and former members of Laboratory for Evolutionary and Ecological Immunology - Hana Velová, Tereza Kraisingerová, Sylvie Dlugošová, Adéla Šmídová, Lucie Buchtová, Martin Těšický and Petra Bauerová, who provided me help with everyday work challenges and shared valuable opinions and advice with me.

I am especially grateful to my parents for moral and financial support throughout my years of studies. A very special gratitude goes out to my second family Parta TEĎka and Sl.Sl. They supplied me with enormous amount of humour, continuous encouragement, energy and unlimited positivity, so needed during long journey towards this thesis.

The last and main thanks belong to my life partner Marek Sýkora, who has been with me through good times and bad, believed in me infinitely and never given up on me.

Abstract

The avian immune system is a complex system of defence mechanisms that protect bird hosts against threats from ubiquitous pathogens. According to the co-evolutionary models, variability in immune traits of hosts is the key component providing ability to adapt and enhance their defence mechanisms in presence of constant selective pathogen pressure. Domestic chicken (*Gallus gallus* f. *domestica*) is used as a model organism in avian biology and also is one of the most important food-producing animals, not only for their meat but also for the egg production. Unfortunately, in research usually only inbred chicken lines are used and modern poultry husbandry is tight with unilateral breeding towards highly productive breeds. Those approaches decrease intra-population polymorphism in chickens. However, especially in case of farm animals, searching and extending the pool of immune variability and enhancing pathogen resistance is crucial for sustaining healthy and biologically secure populations and their products. Morphologically highly distinct traditional chicken breeds, which have evolved for hundreds years under different selective pressures, may represent this desirable immunological variability.

In my thesis I described variability in chosen immunological traits, haematological parameters and proteomic composition of defence proteins in an egg white, in five traditional chicken breeds: Araucana, Booted Bantam, Czech Golden Pencilled, Minorca and Rosecomb Bantam. At first, using Phytohaemagglutinin skin-swelling test my collaborators and I highlighted large effect of blood cellular composition on the course of pro-inflammatory response, showing importance of the haematological variance in birds. As a next step, by novel and modified method of flow cytometry with fluorescently labelled antibodies we showed immunologically relevant variability in a blood cellular composition between chicken breeds. We also described a complete chicken egg white proteome using a tandem mass spectrometry analysis and revealed differences in amounts of egg antimicrobial defence proteins, pointing towards variance in egg-protective capacity of the chosen breeds. Egg white properties could be largely influenced by maternal organism as it is formed in a part of hen oviduct called magnum. Utilizing next generation Illumina sequencing, we were the first to sequence whole magnum transcriptome and compare it with the complete egg white proteome. Although mean protein amount in egg white and mean mRNA expression are strongly correlated, in mRNA expression we did not find the same pattern of inter-breed variability as in proteome. We presuppose that the observed variation in egg white composition probably results from a post-transcriptional regulation creating a discrepancy between proteomic and transcriptomic data. Taken together, results of my thesis show great variability in immune-phenotype of chicken breeds with potential effect on their parasite resistance and biosecurity of their eggs.

Abstrakt v českém jazyce

Imunitní systém ptáků je komplexním systémem obranných mechanismů zamířených proti širokému spektru patogenních nákaz. Dle koevolučních modelů je variabilita v imunitním systému hostitele naprosto zásadním parametrem poskytujícím schopnost adaptace a zlepšování těchto obranných mechanismů při neustálém selekčním tlaku ze strany patogenů. Kur domácí (*Gallus gallus* f. *domestica*) je často využívaným modelovým druhem v biologii ale zároveň také jedním z nejdůležitějších hospodářských zvířat, jak pro produkci masa, tak i vajec. Pro výzkumné účely jsou ale bohužel ve většině případů používány inbrední linie kura domácího a moderní drůbeží produkce je spojena s jednostranným šlechtěním směrem k vysoké produktivitě plemen. To vede ke ztrátám vnitro-populačního polymorfismu slepic. Ovšem právě v případě hospodářských zvířat je hledání a rozšiřování zdrojů imunologické variability pro účely zesílení resistence proti patogenům naprosto zásadní z hlediska udržení zdravé populace drůbeže a biologické nezávadnosti jejích produktů. Požadovanou imunologickou variabilitu můžeme hledat u morfologicky vysoce rozrůzněných tradičních plemen kura domácího, která se po staletí vyvíjela pod vlivem nejrůznějších selekčních tlaků.

V mojí dizertační práci jsem popsala variabilitu ve vybraných imunologických znacích, konkrétně v hematologických parametrech a v proteomickém složení vaječného bílku u pěti tradičních plemen slepic: Araukana, Rousná zakrslá, Česká zlatá kropenatá, Minorka a Bantamka. Nejprve jsme s mými spolupracovníky s využitím otokového kožního testu potvrdili efekt buněčného složení krve na průběh prozánětlivé reakce a poukázali tak na význam variability hematologických znaků u ptáků. V dalším kroku jsme pomocí moderní metody průtokové cytometrie s fluorescenčně značenými protilátkami objevili imunologicky relevantní variabilitu ve složení periferní krve mezi vybranými plemeny. Metoda tandemové hmotnostní spektrometrie nám umožnila popsat kompletní proteom bílku a odhalit v něm rozdíly v množství antimikrobiálních proteinů, které ukazují na odlišnosti v obranyschopnosti vajec u různých plemen. Vzhledem k tomu, že se vaječný bílek formuje v oviduktu slepice, v části zvané magnum, jeho vlastnosti mohou být z velké části ovlivněny mateřským organismem. S využitím sekvenace nové generace na platformě Illumina jsme jako první popsali transkriptom slepičího magna a porovnali ho s proteomem vaječného bílku. Přestože dle našich dat existuje silná korelace mezi průměrnou expresí mRNA a průměrným množstvím daného proteinu v bílku, v případě exprese mRNA nelze mezi plemeny pozorovat variabilitu nalezenou v proteomu. Předpokládáme proto, že rozdíly ve složení vaječného bílku jsou výsledkem posttranskripčních regulací, které vytváří nesoulad mezi výsledky proteomické a transkriptomické analýzy. Výsledky mojí práce ukazují významnou variabilitu v imuno-fenotypu plemen kura domácího s potenciálním efektem na jejich parazitární resistenci a biologickou nezávadnost vajec.

Contents

List of included publications

PAPER I

Bilkova Barbora, Vinklerova Jitka & Vinkler Michal (2015) The Relationship Between Health and Cell-Mediated Immunity Measured in Ecology: Phytohaemagglutinin Skin-Swelling Test Mirrors Blood Cellular Composition. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology, 323, 767-777.

PAPER II

Bilkova Barbora, Bainova Zuzana, Janda Josef, Zita Lukaš & Vinkler, Michal (2017) Different breeds, different blood: Cytometric analysis of whole blood cellular composition in chicken breeds. Veterinary Immunology and Immunopathology, 188, 71-77.

PAPER III

Bilkova Barbora, Swiderska Zuzana, Zita Lukas, Laloe Denis, Charles Mathieu, Benes Vladimir, Stopka Pavel & Vinkler, Michal (2018) Domestic fowl breed variation in egg white protein expression: application of proteomics and transcriptomics. Submitted into Journal of Proteomic Research

General Introduction

Since parasitism has been believed to be the most widespread life-history strategy, all organisms are constantly exposed to attacks of a wide range of parasites (Windsor 1998). Parasites exploit sources originating from the host organisms, limit their fitness and as a result create huge selective pressure on host population (Schmid-Hempel 2011). To be able to resist constant threat of the parasitic attacks, hosts have continuously evolved durable defence mechanisms. It in return enhances the selection in parasites, forcing them to create new arms to invade their hosts (Thompson 1994). As a result of this ongoing co-evolutionary process, often referred as arms race (Woolhouse et al. 2002, Dawkins and Krebs 1979), hosts evolved robust immune system capable of pathogen recognition and elimination.

According to the Red Queen hypothesis of the host-pathogen co-evolution, variability in an immune system is the key component to this never-ending fight of hosts and their parasites (Carius, Little and Ebert 2001, Decaestecker et al. 2007). As parasitic organisms have usually much more rapid rate of evolution, maintenance of a pool of genetic variance could provide flexibility for counteraction to host population (Haldane 1992, Hughes et al. 2005, Chapman et al. 2016). High levels of genetic variability may not allow parasites to adapt equally well to all individuals of the host population, while this heterogeneity also provides the host population with a tool to recognise a broader spectrum of pathogens than would be possible by one specific host genotype (Tellier and Brown 2007b). Genetic polymorphism in a host population is facilitated by constant oscillations of allele frequencies by negative frequency dependent selection. For parasite, it became advantageous to adapt to most common host variant, which increases fitness of rare host alleles leading to their spread in population and subsequent loss of their advantage caused by parasite counter adaptations (Ferrer-Admetlla et al. 2008, Tellier and Brown 2007a, Tellier and Brown 2007b). It has been shown on many examples from both vertebrates and invertebrates, that host-parasite coevolution and parasite-mediated selection promote genetic polymorphism in immune genes (Bernatchez and Landry 2003, Horin et al. 2004, Turner et al. 2012, Croze et al. 2016). Despite most attention is paid to diversity of genes involved in adaptive immunity (Schou et al. 2007, Schou et al. 2010, Smith et al. 2011, Brennan et al. 2012, Abduriyim et al. 2017, Schwensow et al. 2017), evidence of genetic polymorphism with effects on host resistance to infectious diseases is now well documented also for genes involved in innate immunity (Ferrer-Admetlla et al. 2008,

Tschirren, Raberg and Westerdahl 2011, Grueber, Wallis and Jamieson 2013, Tschirren et al. 2013, Dalton et al. 2016, Chapman et al. 2016, Cadwell et al. 2017). It all applies that sustaining of immunological variability is absolutely crucial for ability of host population to resist pathogens.

With production of over 73 billion animals worldwide, domestic chicken (*Gallus gallus* f. *domestica*) is considered to be one of the most important food-producing animals (Food and agriculture organisation, FAO 2016). Chicken pathogen resistance is therefore highly interesting not only from immunological and evolutionary perspective, but also from agricultural and economic point of view. Breeding of livestock is mainly focused on high productivity of these animals, including fast grow, low feed intake, high weigh in broilers and high egg production in laying chicken breeds (Emmerson 1997, Yuan et al. 2015, Emrani et al. 2017, Sell-Kubiak et al. 2017). Moreover, morphological homogeneity of animals belongs to desirable objectives in commercial breeding, as it assures easier, effective and therefore more economical animal handling, housing and overall breeding management (Romero et al. 2009). Those one sided breeding approaches can lead to genetic uniformity and loss of so important immunological variability, contributing to higher susceptibility of chicken population and loss of natural resistance (Notter 1999, Muir et al. 2008, Schachner et al. 2018, Schilling et al. 2018).

Unfortunately, unilateral selection towards highly specialized chicken breeds also seems to have baleful effect on other physiological traits (Rauw et al. 1998, Institute of medicine and national research council 2004). Selection on high body mass and increased grow performance has in many cases showed deleterious effects on immune system of poultry and other domestic animals (Miller, Siegel and Dunnington 1992, Qureshi and Havenstein 1994, Nestor et al. 1996, van der Most et al. 2011). One example of this suggested genetic trade-off between levels of immunity and high production effectivity can be found in the negative correlation between egg weight and strength and natural antibody levels in White-leghorn chickens (van der Klein et al. 2015). Taken together, unpremeditated one sided approaches in poultry breeding could lead to deleterious consequences concerning pathogen resistance of chickens.

As multiple diseases could be easily spread from livestock and their products to humans, maintenance of healthy, pathogen-free poultry is essential for maintenance of human health and food security. Moreover sickness of animals is associated with financial loss because infected birds have usually higher feed intake or lower weight or egg production (Murillo et al. 2016).

So far, the pathogen control in livestock and their products had been ensured mainly by medication, vaccination and presence of high sanitary standards (Pasquali et al. 2011, Uotani et al. 2017, Umar et al. 2017, Aidara-Kane et al. 2018). In developing countries that are largely depended on local small-scale farming where the hygienic standards are low and risk of food contamination is often enormous the pharmaceutical treatment of animals is usually unavailable (Bagust 2013, Mahoro et al. 2017, Schilling et al. 2018). What is more, global extensive antibiotic usage in animal production is fraught with the risk of rise of microbial antibiotic-resistance (Seal et al. 2013, Aidara-Kane et al. 2018). Theoretically, in eggs the major source of foodborne diseases (Majowicz et al. 2010), alternative mechanical treatments towards microbial sterility are possible. Unfortunately there has been concern that washing of an egg surface leads to higher level of contamination by causing damage to the protective outer cuticle of the egg (Favier et al. 2000, Gole et al. 2014) and pasteurisation could to some extent the egg properties (Alamprese et al. 2005, Lechevalier et al. 2017). Naturally, neither of the treatments can be performed on fertilised eggs intended for breeding purposes.

It all stresses the importance of alternative ways of enhancing disease resistance in the population of domestic chickens. It has been long suggested that poultry research should be directed towards more sustainable strategy of systematic breeding for immunologically relevant traits to improve natural disease resistance (Gavora and Spencer 1983, Kramer, Malek and Lamont 2003, Dunn 2004, Fulton 2004, Bagust 2013, Berghof et al. 2015, Schilling et al. 2018). The essence of proposed approach lays in describing and conserving immunological variability of chickens (Beaumont et al. 2003, Institute of medicine and national research council 2004). Non-commercial and indigenous chicken breeds are considered to represent a valuable source of morphologically, genetically and potentially also immunologically important diversity for maintaining future breeding options (Notter 1999, Toro, Fernandez and Caballero 2009, Groeneveld et al. 2010).

Chicken breeds were formed by selective breeding of individuals exhibiting required traits, leading to artificial selection for exaggeration of those specific traits. Each breed is defined mainly by its exterior phenotypic appearance allowing to distinguish other breeds of the same species (Jull 1932). Different breeds were formed under specific selective pressures in distinct environments and under various external influences generating variability in other than morphological traits as well. There has been a growing evidence of differences in immune responses against pathogens in non-commercial chicken breeds. For example, in comparison with commercial lines,

both stronger antibacterial and antiviral immune response were observed in Egyptian, Libyan or Vietnamese chicken breeds (Redmond et al. 2009, Schou et al. 2010, El-Safty 2012, Zhang et al. 2018). Moreover, in multiple cases high level of genetic polymorphism in receptors of innate and acquired immunity was described in Chinese, Taiwanese or Finnish indigenous chickens (Ruan and Zheng 2011, Izadi, Ritland and Cheng 2011, Ruan, An and Wu 2015, Kannaki et al. 2017). All those reports support the significance of native chicken breeds in further improvements of chicken health and parasite resistance.

There is a long history of breeding phenotypically highly polymorphic traditional chicken breeds also in central Europe, but similarly as in other chicken breeds their immune variability has been so far poorly studied. The general origin of these traditional breeds is mapped to more than 200 years back. These breeds have been conserved to recent days mainly thanks to small hobby breeders. For the purpose of this thesis we selected five traditional layer chicken breeds of different morphology and origin: Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam. For the first time, we described immunologically relevant phenotypic variability between those chicken breeds and their eggs. This thesis is revealing variability in a blood cellular composition between chicken breeds, with potential role in immune responsiveness. We also described whole egg-white proteome of this chicken breeds and its significant between-breed variability, especially in antimicrobial egg-defence proteins. Finally, in this thesis we were able to link a complete proteome of the egg to complete transcriptome of oviduct of maternal organism. Those findings could possibly contribute in the future to further search for variability usable in chicken breeding of chicken with aim to increase disease resistance.

Aims of the Thesis

The general aim of my doctoral thesis is to describe variation in selected immunological traits of hens and their eggs across traditional domestic chicken breeds. For this purpose, we selected five breeds differing in their geographical origin and external phenotype: Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam.

At first, we explored the functional effect of haematological parameters and its variability on immune responsiveness in birds. Next aim was to describe natural variability between breeds in their haematological traits. Since eggs belong to key products of the domestic fowl that may differ across the breeds in quality, another goal was to describe proteomic composition of egg white. We focused mainly on between breed differences in the expression of proteins with antimicrobial activity. Given that the egg is a product that is formed in hen oviduct, the last aim of this study was to describe variability of mRNA expression in oviduct of the maternal organism and assess its relationship to egg proteome.

The partial aims are:

1) To describe the effect of variation in blood cellular composition on avian inflammatory responsiveness

paper I

2) To characterise inter-breed variability in selected haematological traits

paper II

3) To define, if there are between-breed differences in proteomic composition of the egg white

paper III

4) To link maternal oviduct gene expression to egg white proteome

paper III

Study Organism

The domestic chicken (*Gallus gallus* f. *domestica*) is agriculturally important animal serving for meat and egg production. In 2016 there were 73.7 billion chickens and 1.4 trillion eggs produced worldwide (FAO 2016). What is more, *G. gallus is* also used as model organism in bird immunology (Stern 2005, Burt 2007). Modern domestic chicken arouse from crossbreeding of four different subspecies of red junglefowl (*Gallus gallus gallus, G. g. spadiceus*, *G. g. jaboullei* and *G. g. murgha*; Hillel et al. 2003, Liu et al. 2006, Kanginakudru et al. 2008, Miao et al. 2013) in multiple domestication events (Liu et al. 2006, Kanginakudru et al. 2008). It is also speculated, that grey junglefowl (*Gallus sonnerati*) contributed to the genome of the modern domestic chickens (Eriksson et al. 2008).

Figure 1 Rosecomb Bantam (photo Hana Velová)

Unfortunately, in both immunological and veterinary research, typically only laboratory inbred lines and commercial strains of chickens are used. In my thesis, instead, we focused on so called traditional chicken breeds, which represent large phenotypic, genetic and potentially also immunological variability (Sharma et al. 2001, Berthouly et al. 2008). Origin of these traditional breeds dates more than 200 years back, and they have been evolving under different natural conditions and pathogenic pressures occurring in the different regions where these chickens have been bred (Pavel and Tuláček 2006, Scrivener 2008). Therefore, traditional chicken breeds are potentially a valuable source of immunological variation that is highly important for further improvements in commercial chicken health and biosecurity.

The five breeds used in my research were Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam. All of them can be considered as layer breeds, with relatively high laying capacity (Table 1). The Booted Bantam and Rosecomb Bantam also belong to dwarf fancy breeds that usually weight less than one kilogram and they are mostly breeded for their appearance rather than for the egg production *per se*. The first documented references on hens similar to Booted Bantams dates back to ancient Rome (Vašák 2008). Modern Booted Bantam breed were established in the first half of 20th century. They are described as cold hardy, vital and good flyers (Scrivener 2008, Vašák 2008). Most significant traits are long leg- and food-feathering and lyre-like body silhouette. Common colour varieties are millefleurs, porcelain, white and black (Scrivener 2008). The other bantam breed in this study, the Rosecomb Bantam was imported to Europe from Indonesia during 19th century (Vašák 2008). Rosecomb Bantams have calm character, but they are good flyers with strong loud crow. Distinctive features are rose comb, white ear lobes and rounded tail feathers (Scrivener 2008; Figure 1). Most popular variety is the dark black one with green reflection. Araucanas are a South-American breed that was brought to Europe only recently, in second half of the 20th century (Pavel and Tuláček 2006). They are well known for their green-coloured eggshells and extraordinary appearance (Figure 2). Araucanas have large frontal part of the body, feathered ear tufts and beards, green legs and no tail feathers (Scrivener 2008).

Figure 2 Araucana (photo Hana Velová)

On the contrary, Czech Golden Brindled is a traditional European breed that is specifically adapted to an inhospitable winter climate (Pavel and Tuláček 2006). In the 19th century, Czech Golden Brindleds were the most common breed in Czech countries, but they were slowly replaced by other chicken breeds. Nowadays they are again more common, but really rare outside Czech Republic (Vašák 2008). They are timid by nature, extremely good flyers and usually require large living space. Dominant colours on plumage are golden, brown-yellow, grey and black with green reflections (Scrivener 2008). Minorcas are vivid breed with large white eggs. Ancestors of Minorcas probably came from the Spanish island Menorca, and they were known in the Iberian Peninsula as early as in the first millennium of our century (Vašák 2008). The modern version of the breed was established in England (Scrivener 2008, Pavel and Tuláček 2006). Minorcas are very vivid, good flyers, but vulnerable to long periods of freezing weather. Most common is the ancestral black colour with green reflection. Long white ear lobes belong to their specific traits (Vašák 2008).

Table 1 Characteristics of chicken breeds used in this thesis. Origin - presupposed place of breed origin. Laying capacity - mean number of eggs laid per season. Weight - mean body weight of adult hen in kilograms. Data taken from Pavel and Tuláček (2006), Scrivener (2008) and Vašák (2008).

Breed	Origin	Laying capacity [eggs/ season]	weight [kg]
Araucana	South America	160-180	1.7 - 2.1
Booted Bantam	Europe	100-120	0.5 - 0.7
Czech Golden Brindled	Czech republic	160-190	2.0 - 2.5
Minorca	Spain (Menorca)	170-190	2.6 - 3.3
Rosecomb Bantam	Indonesia (Java)	60-90	0.4 - 0.6

For practical and technical reasons we used zebra finch (*Taeniopygia guttata*) as a model organism for experiment described in **paper I**. Similar to chickens, zebra finches are one of the classical model organisms in avian biology (Swaddle 2010, Griffith and Buchanan 2010, Balakrishnan, Edwards and Clayton 2010, Patterson and Fee 2015). It is a small passerine bird from family the Estrildidae, naturally inhabiting grasslands and forests of central Australia and also islands of Lesser Sundas and Timor. Zebra finches are

also maintained in captivity as popular domestic pet birds. In our research we used captive animals purchased from hobby breeders.

General Methods

Breeding of Domestic Chickens

As health-state, haematological parameters and protein expression can be largely influenced by external conditions (Dikshit et al. 2015, Gasparino et al. 2015, Matur et al. 2016, Olanrewaju et al. 2018), we needed to assure strictly standardised conditions for all the animals used for the purpose of this research project. Therefore, we bred all chickens from eggs to maturity under standardised conditions of a certified breeding facility. Working with commercially unavailable breeds, eggs were kindly provided to us by small non-commercial breeders located in the Czech Republic. The eggs had been incubated till hatching in an automatic egg incubator and hatched within period of one month, to assure approximately the same age of all animals. After hatching all hens were labelled with durable wing-marks and housed under standardised conditions at the animal facility of the Czech University of Life Sciences till maturity (to the age of about 224-244 days, estimated by egg production). Eggs from hens were collected for proteomic analysis of egg white. The hens were then euthanized by cervical dislocation. After rapid dissection the samples of the magnum tissue (the segment of the oviduct were egg white is formed) were immediately conserved in RNAlater reagent (Quiagen, Hilden, Germany), kept overnight at +4°C and afterwards stored at -80°C until the following transcriptomic analysis.

PHA Skin-swelling Test and Histological Analysis

The skin-swelling test is one of the most common methods to measure *in vivo* immune responsiveness in birds by non-specific stimulation of immune cells, in this case with plant lectin Phytohaemagglutinin (PHA). In the skin swelling test, PHA is injected to the subcutaneous tissue where it stimulates white blood cells (leukocytes), the effective cells of immune system. This triggeres immune response, during which leukocytes from blood stream are attracted inside the injected tissue, which manifests itself externally by tissue swelling. This response was described because T-cell dependent, as PHA binds to T-cell receptors subunits (Felsted et al. 1977, Powell 1980). However major migration of heterophils and monocytes into the tissue was also observed (Maxwell and Robertson 1998, Turmelle et al. 2010, Vinkler, Bainova and Albrecht 2010a, Brown, Shilton and Shine 2011, Vinkler et al. 2012, Finger et al. 2013, Salaberria et al. 2013). For *in vivo* studies is most commonly used the molecule of PHA (PHA-P). PHA-P is composed from

lymphoproliferative subunit PHA-L and haemagglutinative subunit PHA-E (Leavitt, Felsted and Bachur 1977). It was shown, that although PHA-E does not have the ability to stimulate T-lymphocytes, it also triggers the skin swelling response (Vinkler et al. 2010a). To provide clarity and simplicity to the test, we used PHA-L instead of PHA-P, as it non-specifically stimulates only T-lymphocytes without inducing agglutination of erythrocytes (Miller et al. 1975, Felsted et al. 1977).

We performed the PHA-skin swelling test as previously described in Vinkler et al. (2014). Before treatment, we measured the wing web (patagium) thickness of the birds, and then we injected this tissue with the PHA-L solution. After 24 hours, we measured the magnitude of the swelling response by repeated measurements of the patagium thickness. Twenty-four hours is the most commonly used period in eco-immunological studies, as by this time the oedema is large and should be dominated by the PHA-sensitive T-lymphocytes (Stadecker et al. 1977, Goto et al. 1978, McCorkle, Olah and Glick 1980, Martin et al. 2006). Index of the immune responsiveness was calculated as the difference between tissue thickness before and 24 hours after the treatment. Immediately after the metrical measurement, biopsy of the swollen tissue was punched and fixed with formalin for later histological analysis of the composition of infiltrated leukocytes. Size of the taken biopsy was minimal not to cause any significant harm to experimental animals. The histological analysis of patagium biopsies was performed according to Vinkler et al. (2010b). For visualisation and recognition of the tissue-infiltrated blood cells, common histological haematoxylin and eosin staining was used (Fischer et al. 2008). In stained tissue samples we were able to distinguish following main categories of blood cells: lymphocytes, monocytes, heterophils, basophils, erythrocytes and thrombocytes according to Lucas and Jamroz (1961).

Haematological Analysis

It has been long recognised that haematological parameters, mainly differential and absolute counts discussed in my thesis, are great proxy for avian physiological and health status (Ferrer 1990, Haile and Chanie 2014). To determine differential leukocyte counts in **paper I**, we used blood smear analysis, well established method for evaluating blood cellular composition suitable for field and non-laboratory condition (Campbell and Ellis 2007, Haile and Chanie 2014). Samples of a peripheral blood were taken into sterile heparinised syringes and afterwards small blood drops (approximately 2µl) were applied in

a thin layer on to the surfaces of glass slides. Then, the blood samples were dried and stained, in our case with Wright-Giemsa modified stain, revealing the differences in nuclear and cytoplasmic structures of different cell types. The individual cell types (lymphocytes, heterophils, monocytes, basophils and eosinophils) were visually recognised under light microscope according to Lucas and Jamroz (1961), providing relative cell count from approximately 150 cells per sample. This approach was optimal for our experiment, as it is technically undiscerning and allows easy storing of the blood smear samples till evaluation.

A much more precise and cutting-edge method of the haematological analysis is flow cytometry with fluorescently labelled antibodies against specific antigens expressed on blood cell surface (O'Connor et al. 2001, Mizrahi et al. 2018). Flow cytometry allows counting more than centuple blood cells compared to microscopic analysis in similar or shorter time. Unfortunately, whole blood cytometry in birds is challenging due to the presence of nucleated avian red blood cells (erythrocytes), which are similar in size and shape to leukocytes (Campbell and Ellis 2007). Erythrocytes are the most incident cells in blood (usually hundredfold more than leukocytes) and therefore their signal could easily block out signal from other cell types, making them unrecognisable (Beaufrere, Ammersbach and Tully 2013). In most of the previous studies, leukocytes were separated for the analysis (Bridle et al.,2006; De Boever et al., 2010; Fair et al., 2008; Chen et al., 2012), though this approach leads to cell-loss during the isolation process, or erythrocytes were lysed with buffer with unknown impact on other cells (De Boever et al., 2010). Also for most of the bird species, including zebra finches, specific antibodies against blood cell markers are not presently available. Flow-cytometric analysis of the whole blood also requires usage of fresh blood or specific fixation of samples (Paredes et al. 2015, Seliger et al. 2012), making this method inaccessible in field and non-laboratory conditions. Therefore it was not technically possible to use method of flow cytometry for haematological examination in **paper I**.

In 2012, Seliger et al. described novel method of whole-blood flow cytometry with fluorescently labelled antibodies in chickens. With slight modifications, this method allows us to measure both erythrocytes and leukocytes from chicken blood, without lysis or cell-separation. We used three different specific fluorescently labelled anti-chicken antibodies: Anti-CD45-PE for all leukocytes and thrombocytes (Schultz and Magor 2014), Anti-CD4-Alexa 700 for $CD4^+$ T-cells recognition (Chan et al. 1988) and Anti-macrophages-FITC (KUL01; Staines et al. 2014). For thrombocyte identification we used anti-human antibody

Anti-CD51/61-Alexa 647, as cross-reactivity on chicken cells was previously confirmed by Viertlboeck and Gobel (2007). Erythrocytes were distinguishes as CD45 negative cells. Based on forward and side scatter we identified lymphocytes as cohesive population with similar forward and side scatter to $CD4^+$ T-cells and heterophils as large cells with most complex structure of cytoplasm. Similarly as in analysis of blood smear, flow cytometry typically provides only information on relative numbers of the blood cells, as only fraction of a sample is measured. For assessment of absolute cell counts in blood, quantification beads in known density are usually added to sample (Barnett et al. 1999, Schnizlein-Bick et al. 2000, Pattanapanyasat et al. 2004). In **paper II**, we used a flow cytometer CytoFLEX (Beckman Coulter Inc., Brea, California, USA) with a volumetric sample injection module which is able to measure the exact volume of liquid used during analysis, allowing us to measure also absolute cell counts without the necessity to use any quantification beads.

Proteomic Analysis

We collected fresh eggs from individually caged hens for subsequent proteomic analysis. To minimise the effects of egg storage, we separated egg whites from egg yolks and other egg structures within the period of maximum two days after egg laying and kept them at -20°C until the proteomic analysis. So far, differences of protein abundances in egg white samples have been studied mainly by the method of two dimensional electrophoresis coupled with mass spectrometry protein identification (Omana et al. 2011, Wang et al. 2012, Soares et al. 2012, Guyot et al. 2016a). This method is based on comparison of sizes of the protein spots on the electrophoretic gel. Generally the differences in spots are detected by computer image software, but user intervention is usually still necessary, which makes the method time consuming and enlarge its impreciseness (Penque 2009). To identify and also quantify proteins and their abundance in egg white samples, we performed more up-to-date analysis of nano-reverse phase chromatography coupled with tandem mass spectrometry on Thermo Orbitrap Fusion mass spectrometer (Q-OT-qIT,157 Thermo), as previously described by Cerna et al. (2017). The provided MS/MS spectra were searched against the Uniprot database to identity the individual proteins in egg white. For quantification of relative protein abundances based on MS spectra, we applied label-free algorithm described by Cox et al. (2014).

Transcriptomic Analysis

We used RNA isolated from hen magnum to describe whole transcriptome. In the library preparation, selection of RNA with 3' polyadenylated (poly(A)) tails was used to separate mRNA from rRNA (de Moor and Richter 2001, Wu et al. 2008). Next, the mRNA molecules were reverse transcribed into cDNA and specific barcode sequences were added to cDNA samples from each individual for their later identification. Then all samples were pooled together. The library preparation and actual transcriptome sequencing were performed at the European Molecular Biology Laboratory (EMBL), Heidelberg using platform NextSeq 500 Illumina sequencer. Bi-directional sequencing on this particular platform generates 85-bases-long reads, providing approximately 25 million sequence pairs for each library. The sequencing results were submitted to the NCBI Sequence Read Archive (SRA Acc. No. SRP126816). For the following bioinformatics mainly the analysis software Trim Galore! Software (Babraham Bioinformatics, Braham Institute, Cambridge) and SICKLE (Joshi and Fass 2011) were used. Obtained sequences were aligned to the *G. gallus* reference genome assembly Gallus_gallus-5.0 (GCA_000002315.3) in the STAR software (Dobin et al. 2013).

Ethical Note

All research included in this study was approved by the Ethical Committee of the Faculty of Science, Charles University, Prague.

Results of Studies in Broader Context

Effect of Haematological Parameters on the Course of Immune Response

Avian blood serves as a main transport medium trough whole organism. It carries oxygen, nutrients and large variety of other biochemical substances from places of their origin to the effective sites. Also in the immune system of birds, blood plays an important role as it transports immunologically active white blood cells (leukocytes). Leukocytes could be considered main effective cells of an immune system, recognising and eliminating pathogenic agents. Leukocytes originate within the bone marrow, afterwards they migrate to primary and secondary lymphatic tissues where they differentiate and gain their effector function (Cyster 1999; Schat *et al.* 2014). There are five main white blood cell types described in immune system of birds: lymphocytes, monocytes, heterophils, basophils and eosinophils (Campbell & Ellis 2007; Schat *et al.* 2014).

Lymphocytes are mainly associated with acquired immune responses as they are responsible for adaptive antigen recognition. They are able to regulate many aspects of immune response, producing broad spectra of cytokines and also directly interacting with other cells of immune system. Lymphocytes are part of both a cell-mediated immunity and an antibody production (Tizard 2002; Schat *et al.* 2014). Monocytes are precursors of tissue macrophages, with the ability to phagocyte both autochthonous and allochthonous particles and afterwards they present the ingested antigens to lymphocytes (Lynch *et al.* 2018). Heterophils (avian homologues of the neutrophils present in other vertebrates) are also phagocytic cells containing cytotoxic granules with the ability to kill microscopic parasites such as bacteria (Maxwell & Robertson 1998). Basophils and eosinophils also have granules in their cytoplasm, but they mainly participate on eradication of multicellular parasites (Voehringer 2009, 2011; Bertellotti *et al.* 2016). Given the effector role of leukocytes in defence of birds, stimulation of the immune system with pathogenic infections or vaccination often results in changes in a blood cellular composition (Iseri & Klasing 2013; Fratto *et al.* 2014; Gottstein *et al.* 2015; Soria *et al.* 2015; Clark *et al.* 2016). Considering this, haematological parameters, mainly relative and absolute blood cell counts are commonly used as diagnostic markers of health-state and physiological state of

an individual (Talebi *et al.* 2005; Davis *et al.* 2008; Pickler *et al.* 2013; Lentfer *et al.* 2015; Neveling *et al.* 2017; Kar *et al.* 2018).

As different white blood cell types have different functions in immune system, cellular composition of peripheral blood can be also crucial in determining the course and manifestation of immune responses. In **paper I**, our aim was to investigate functional effects of the variation in haematological parameters on inflammatory immune response of the experimental individuals.

For this purpose, we chose the method of Phytohaemagglutinin (PHA) skin swelling test as an experimental immune challenge. Skin swelling test is the most common method of measuring capacity of an *in vivo* immune response used in both free-living and domestic birds (Smits *et al.* 1999; Martin *et al.* 2006; Vinkler *et al.* 2010; Goliomytis *et al.* 2015; Josserand *et al.* 2015; Kundu *et al.* 2016). This test is based on experimental stimulation of an immune response in skin by injecting a substance, which non-specifically activate and attracts large number of leukocytes (Williams & Benacerr 1972; Stadecker & Leskowit 1974; Ando *et al.* 2014).

The most commonly used pro-inflammatory stimulant in birds is PHA, bioactive lectin isolated from red kidney bean *Phaseolus vulgaris* (Licastro *et al.* 1993). In the skin-swelling test PHA is injected subcutaneously into tissue (usually into the wing patagium of birds, toe web of amphibians and reptiles or paw of small mammals; Martin *et al.* 2006; de Bellocq *et al.* 2007; Brown *et al.* 2011; Finger *et al.* 2013; Josserand *et al.* 2015; **Bilkova** *et al.* 2016). PHA treatment evoke an immune response which is afterwards externally manifested as tissue swelling. Difference between tissue thickens before and after PHA treatment is used as an index of the immune responsiveness (Smits *et al.* 1999). Usually it is presupposed, that the greater is the swelling responsiveness the greater is the individual's cell-mediated immune system capability (Sullivan & Erf 2017). Unfortunately, there is a large unclarity in interpretation of this index of responsiveness, as only visible outcome i.e. swelling is typically the measured variable, without proper understanding of immune mechanisms standing behind whole PHA induced immunological process.

Originally, PHA was described as a T-cell mitogen (Felsted *et al.* 1977; Leavitt *et al.* 1977). On molecular level, PHA triggers immune cellular response by binding to α, ß and γ chains of the T-cell receptors (PHA-L subunit; Licastro *et al.* 1993) and also to erythrocyte membrane compounds (PHA-E subunit; Felsted *et al.* 1977; Powell 1980). However, in swelling test T-cell grow factor interleukin-2 (IL-2) is expressed only in low levels (Vinkler *et al.* 2014) and swelling manifest too fast (usually six-hours after PHA

treatment; Martin *et al.* 2006; Turmelle *et al.* 2010; Xu & Wang 2010) for involvement of acquired T-cell mediated immunity. Although Sullivan & Erf (2017) observed dominance of lymphocytes, with majority of $CD4^+$ cells, during whole PHA stimulated response, multiple histological studies have showen that other leukocytes, marginally heterophils and monocytes, are the most common compound of the inflamed tissue (Maxwell & Robertson 1998; Turmelle *et al.* 2010; Vinkler *et al.* 2010; Brown *et al.* 2011; Vinkler *et al.* 2012; Finger *et al.* 2013; Salaberria *et al.* 2013). It has also been shown that application of erythroagglutinating sub-unit PHA-E stimulates stronger responses than injection of lymphoproliferative PHA-L (Vinkler et al., 2010a). These results suggest that PHA induce non-specific inflammation rather than acquired T-cellular response.

In our experiment, twenty four hours after treatment, there was massive cellular infiltration in PHA-L stimulated tissue dominated by heterophils, which is in consistence with some previous studies (Maxwell & Robertson 1998; Turmelle *et al.* 2010; Vinkler *et al.* 2010; Brown *et al.* 2011; Vinkler *et al.* 2012; Finger *et al.* 2013; Salaberria *et al.* 2013) and points towards already mentioned activation of non-specific innate immunity in PHA-induced inflammation.

Inflammation belongs to fundamental and the most important mechanisms of immune system, which is responsible for pathogen elimination in the host organism. It is first-line innate immune defence providing fast and effective elimination of any allochthones stimulus (Medzhitov 2008; Ashley *et al.* 2012). After recognition of danger signals by receptors of innate immunity, whole cascade of processes is initiated, leading to enhanced permeability of blood vessels and consequently to infiltration of white blood cells to site of inflammation (Medzhitov 2008; Ashley *et al.* 2012; Geering *et al.* 2013). Inappropriate regulation of inflammatory response triggers too week or too strong responsiveness, which both is considered to be potentially pathological (Lawrence & Gilroy 2007; Bian *et al.* 2012; Janssen & Henson 2012). It all leads to conclusion that inflammatory processes could largely affect fitness of individual and associated individual variability could therefore play role in natural and artificial selection (Ashley *et al.* 2012).

Our study shows that blood cellular composition prior PHA treatment largely affects the quality as well as the quantity of the inflammatory immune response, i.e. both the cellular infiltration and swelling response (**paper I**). Most straightforward relationship could be observed in lymphocyte counts, where their higher frequency in blood leads to their higher infiltration into the inflamed tissue. Together with basophils, they also positively affect magnitude of the skin swelling. On the contrary we did not find any

relationship between tissue cellular composition and thickness of the swelling response (external indication of the response). Similarly to our study, absence of relationship between number of cells in tissue and magnitude of swelling was described in other species (Turmelle *et al.* 2010; Merlo *et al.* 2014; **Bilkova** *et al.* 2016), indicating that the swelling index does not mirror underlying cellular processes in the tissue. We showed in **paper I** that blood cellular composition has straight functional effects on immuno-responsiveness as it is strongly related to the general pro-inflammatory capacity of an individual. Large effect of haematological composition on course of inflammation, affecting the health state of animals was also affirmed in pathogen induced inflammatory disease of chickens. Zekarias *et al.* (2000) showed, that two commercial hybrid lines have different ability to resist reactive amyloidosis induced by infection with bacteria *Enterococcus faecalis* and this difference is directly connected to variability in their haematological parameters. In conclusion, all our data suggest great role of haematological variability on inflammatory response and highlight the importance of haematological investigation in birds.

Variability in Haematological Parameters between Chicken Breeds

As the composition of leukocytes, the main effective cells of immunity, remarkably influences manifestation of responses to pathogens (**paper I**), description of inter-breed haematological variability is crucial for proper interpretation and evaluation of those diagnostic values (Olayemi & Oyewale 2002; Fair *et al.* 2008; Sample *et al.* 2015).

Haematological parameters in birds are often affected by multiple parameters as the sex (Oznurlu *et al.* 2012; Gryzinska *et al.* 2013), age (Islam *et al.* 2004; Gryzinska *et al.* 2013), laying period (Hrabcakova *et al.* 2014b), stress (Bedanova *et al.* 2007; Frigerio *et al.* 2017) and other external factors which could influence physiological state and well-being of the animal, as for example the diet (Cengiz & Kucukersan 2010; Waheed *et al.* 2017) or the housing conditions (Moe *et al.* 2010; Lentfer *et al.* 2015; Matur *et al.* 2016). For purpose of this thesis, it was thus necessary to provide identical standardised condition for all experimental animals used in our study (see general methods).

Variation in haematological parameters between breeds were already described in few other species of domestic animals [for example in goats (Arfuso *et al.* 2016), dogs (Lawrence *et al.* 2013) or cats (Paltrinieri *et al.* 2014)], but in most cases comprehensive data are missing. Unfortunately, there is insufficient information on blood cellular

composition also in the case of domestic chicken breeds. There is only little evidence of differences in haematological parameters between few African and Asian chicken breeds (Islam *et al.* 2004; Peters *et al.* 2011; Adenaike *et al.* 2016), and what is more, all these studies use only basic light microscopy analysis, which can provide only relatively unprecise estimates of the blood parameters.

For those reasons, we decided to perform haematological analysis of blood cellular composition using ninety-nine individuals from five selected chicken breeds: Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam (**paper II**). To obtain as valid data as possible, we optimised whole blood no-lyse flow cytometry method previously described by Seliger *et al.* (2012) using fluorescently labelled antibodies against the key leukocyte molecular markers determining the major leukocyte subsets. Although previously used in the domestic fowl (Seliger *et al.* 2012; Braukmann *et al.* 2015), whole blood flow cytometry still does not belong to well established methods in bird immunology, therefore we firstly had to confirm its results by their comparison with the data obtained using traditional methods of light microscopy examination of blood smears (Campbell & Ellis 2007). We confirmed significant correlation of frequencies of lymphocytes ($r = 0.65$) and heterophils ($r = 0.59$) between these two techniques. Naturally, we expect higher levels of preciseness in cytometric analysis, as it allows us to analyse more than centuple of blood cells compared to microscopic methods. Higher repeatability of cytometric measurements compared to microscopic analysis was shown also by Seliger *et al.* (2012). On the other hand, due to unavailability of specific antibodies, this method was not capable of distinguishing basophilic and eosinophilic granulocytes. We do not consider this deficiency as major disadvantage, as according to blood smear microscopic analysis both eosinophils and basophils are rare in chicken blood, with mean proportion of 2.60 % and 1.53 % respectively.

Cytometric analysis clearly shows significant differences in blood composition between different breeds (**paper II**). The lowest erythrocyte count was observed in Booted bantam breed. Main function of the red blood cells is the transport of oxygen into tissues. It was previously described in local breed of a Tibetan chicken that red blood cell count could be a sign of physiological adaptations to atmospheric pressure in different altitudes (Zhang *et al.* 2007). Nevertheless, contribution of multiple selective pressures is possible in Booted Bantam and without further testing we could only speculate about the associations of erythrocyte counts with particular physiology-affecting environmental factors.

Significant differences could be observed also in differential leukocyte count. We were able to find between- breed variability in the ratio of heterophils to lymphocytes (H/L) and also in $CD4^+$ T-lymphocyte and monocyte frequencies (**paper II**). The most outstanding values in our dataset were observed in breeds Czech Golden Brindled and Araucanas. Czech Golden Brindled is a traditional European breed, claimed to be adapted to harsh cold environment, while Araucanas have origin in South America (Pavel & Tuláček 2006; Scrivener 2008). Compared to other breeds, Czech Golden Brindled has lowest H/L ratio and highest proportion of $CD4^+$ T-lymphocytes. Araucanas, on the other hand, have highest H/L ratio and high monocyte frequency (**paper II**). $CD4^+$ cells play major role in B-cellular immunity and an antibody production, thus it is not surprising that increase of this cellular type was observed in vaccinated birds (Bridle *et al.* 2006; Calefi *et al.* 2016; Zhu *et al.* 2016) and also in birds with grater antibody production (Parmentier *et al.* 1995; Dalgaard *et al.* 2010). Therefore, breed-specific variation in frequencies of $CD4^+$ lymphocyte might indicate different capacities of humoral immune responses.

Increase in both heterophil and monocyte frequencies, as seen in Araucanas, is usually related to impaired health of an individual (Dehnhard *et al.* 2011). Higher heterophil proportions were described for example after infection with *Plasmodium* sp., *Staphylococcus aureus, Mycoplasma gallisepticum* or filarial nematodes infection (Andreasen *et al.* 1991; Edler *et al.* 2004; Fratto *et al.* 2014; Clark *et al.* 2016) and also in flea infested birds (Boughton *et al.* 2006). As all animals in our study were kept in the same standardized conditions and none of them displayed any signs of illness, we do not expect the differences to originate from parasitic infection. Also in chickens as in other birds, H/L ratio is frequently used as indicator of long term stress (Al-Murrani *et al.* 2002; Davis *et al.* 2008), though it could arise from many external factors (Al-Murrani *et al.* 2002; Lentfer *et al.* 2015; Lindholm *et al.* 2018). On the other hand, higher heterophil frequency could be sign of the ability to trigger a stronger innate immune response and pre-adaptation towards fighting bacterial pathogens. To sum up, we were able to describe differences in chicken breeds that are probably linked to their ability of the immune response. We speculate that the Czech Golden Brindled might show high stress-resistance or adaptation to infections requiring rapid humoral or helper-T-cell mediated responses. In contrast, Araucanas might be more vulnerable to stress or possess immunological adaptations leading to rapid innate immune responsiveness.

These described haematological differences could also influence non-immune physiological traits, as it was shown that selection for a high H/L ratio results in reduced body weight, reduced egg production and lowered egg hatchability (Al-Murrani *et al.* 2006), suggesting cost of maintaing hight heterophile frequencies. Simmilarly higher lymphocyte count was measured in chickens selected to lower mortality and higher egg production (Cheng *et al.* 2001). Increase of lymphocyte count was also detecable in time of highest lying capacity during the laying period (Hrabcakova *et al.* 2014a). The ability of mounting an immune response is considered costly and thus it could impact investments into other traits such as reproduction and egg laying of the hens (Iseri & Klasing 2014). The distinct immunological adaptations of hens from different breeds suggested by our results might potentially affect the egg production and thus influence egg composition and also the ability of its protection against parasites.

Between-breed Variability in Egg White Composition

From both biological and economical point of view, egg belongs to essential domestic fowl products. Primarily, chicken egg serves for protection and nourishment of chicken embryo during their development, but in the same time it is also a valuable food source for human population. According to FAO (2016), over one and a quarter billion of eggs is annually produced worldwide.

Chicken egg is successively produced by differentiated parts of the hen ovary and oviduct. The whole process takes from 18 to 24 hours (Nys & Guyot 2011). After its release from the ovary, egg yolk is captured by infundibulum, where the potential insemination takes place. Then it is passed to magnum, where egg white is secreted. Afterwards the whole structure moves to isthmus, where egg white is covered with eggshell membranes, and finally the eggshell is deposited on the egg surface by the shell gland i.e. uterus (McLelland 1990; Figure 3).

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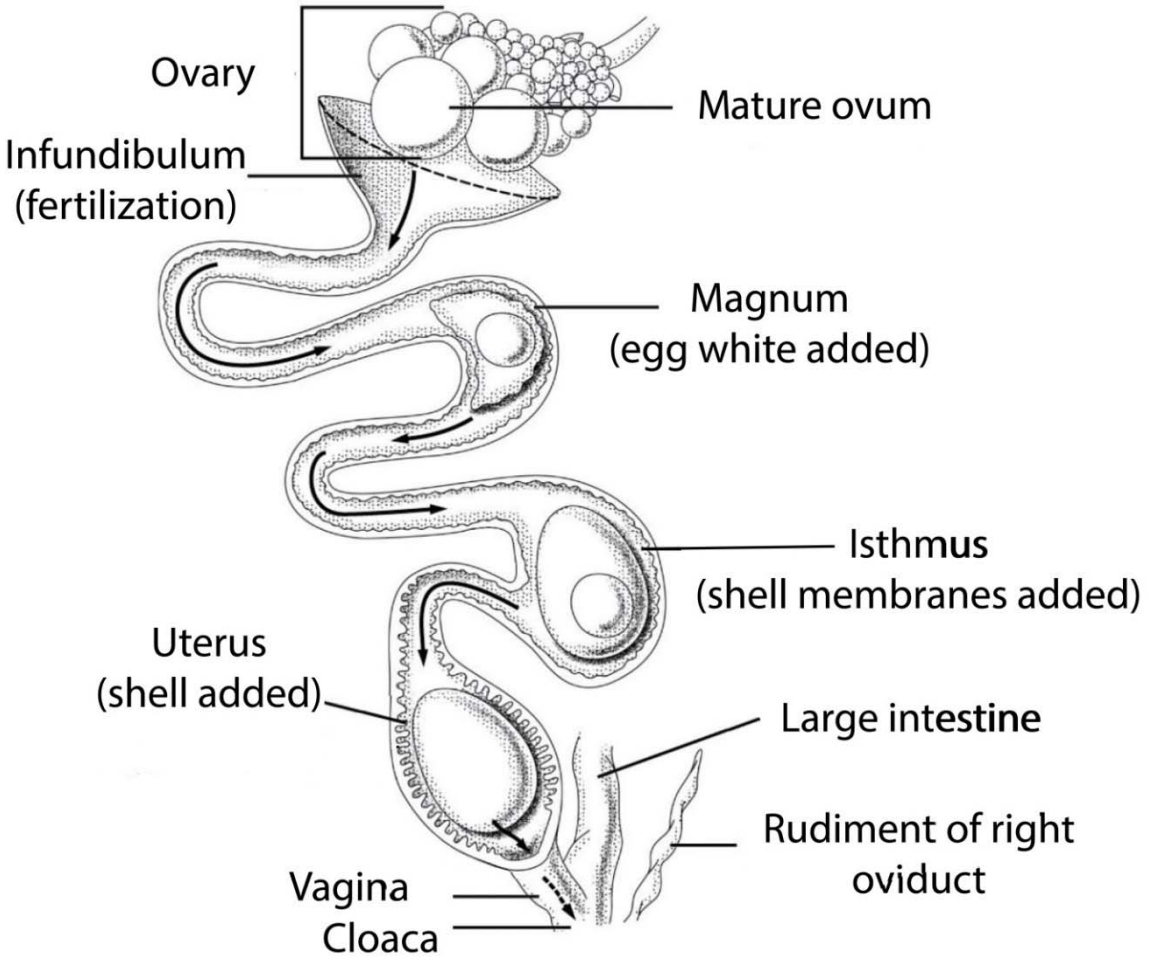

Figure 3 Formation of egg in hen reproductive tract. Mature ovum is released from ovary and then egg is successively formed in distinct parts of oviduct. Taken from Gill (2007) and edited

Egg white creates up to two thirds of the egg volume and accordingly the magnum is the largest part of the oviduct (Nys & Guyot 2011). About 88 % of the egg white material is formed by water, while the proteins represent 90 % of dry egg white matter (Lesnierowski & Stangierski 2018). Including our study (**paper III**), there have been so far only five studies conducted describing egg white proteome by modern methods of a mass spectrometry (Mann 2007; D'Ambrosio *et al.* 2008; Mann & Mann 2011; Sun *et al.* 2017). These studies identified from 78 to 202 distinct egg white proteins, but only 41 were consistently detected in all these studies. This range in number of proteins is probably caused by the use of different protocols for sample manipulation and processing. In our study, after excluding potential contaminants, we were able to identify 116 egg white proteins. Overall, the amounts of respective proteins described in our study correspond with previously published results, with only few minor differences (Mann & Mann 2011).

Eggs can be contaminated with pathogens, potentially causing pathology in the developing embryos and bringing biosecurity risks to humans through transmission of dangerous foodborne diseases after consumption. Large attention has been paid mainly to contamination of eggs with bacteria of the genus *Salmonella*, since approximately 94 million people are annually infected with salmonellosis worldwide and in about 155 000 cases this infection is lethal (Majowicz *et al.* 2010). We also detected nine proteins of pathogenic origin in our egg white samples. All of them were avian virus proteins, found in low abundances and therefore we excluded them from further analysis.

Two possible ways of a pathogen transmission from chicken to an egg were described (De Buck *et al.* 2004; De Reu *et al.* 2006). The first way is the direct transmission of pathogens from an oviduct of hen into the newly formed egg white during its creation (Takata *et al.* 2003; Gantois *et al.* 2009; Yoshimura 2015). Most common is probably bacterial colonisation of the hen magnum in particular, resulting in contamination of egg white, which accents the importance of immune defence in those tissues (Hoop & Pospischil 1993; Keller *et al.* 1995; Schoeni *et al.* 1995; Gantois *et al.* 2009). The second way of transmission take place after laying of egg, it could be described as horizontal or external transmission, as pathogens from environment (mostly spread by faecal shedding from the maternal organism) intrude the egg trough eggshell (De Reu *et al.* 2006; Rathgeber *et al.* 2013; Ahmed *et al.* 2017).

The egg defence against pathogens is affected by multiple factors, including mechanical characteristics and a biochemical composition of an eggshell, eggshell membranes, vitelline membranes and an egg white and yolk (Dunn 2004; Mann 2008; Bain *et al.* 2013). Similarly as in the case of disease resistance of chickens, resistance of eggs can be enhanced by artificial selection on known protective traits (Dunn 2012; Sun *et al.* 2012; Bain *et al.* 2013; Rathgeber *et al.* 2013; Thiruvenkadan & Prabakaran 2017).

It has been previously shown that the largest arsenal of proteins with antimicrobial function involved in natural defence mechanisms of the egg can be found in the egg white (Baron *et al.* 1997; Rehault *et al.* 2007; Mann & Mann 2011; Baron *et al.* 2016; Guyot *et al.* 2016b). During the process of incubation, antimicrobial defence proteins in the egg play main role in providing protection to the newly developing embryo which itself is not yet capable of any type of adaptive immune response. Therefore, variance between chicken breeds in egg white proteomic composition may have also great impact on maintenance of natural immunological protection of eggs (Dunn 2004).

By our study (**paper III**) we confirmed that chicken egg white contains large amounts of proteins with previously described antimicrobial defence function. We found 19 proteins participating in egg defence which together compose approximately 51 % of the total amount of egg white proteins (Table 2). Thus, we affirmed large importance of immune protective function off the egg white.

The results of Wang *et al.* (2012) gained by two-dimensional electrophoresis suggest that egg white protein composition could importantly differ between egg varieties from different commercial chicken lines, including differences in antimicrobial proteins. Also our between-breed comparison showed significant variance in the egg white proteome composition (**paper III**). As the egg white is crucial component in development of the embryo and as we show, it contains high amounts of proteins with defence function, such a variation might affect egg quality and biological security. Nevertheless, described patterns of the between-breed variability in haematology of adult hens (**paper II**) and in the egg white proteome are different and cannot be linked together. In case of egg white composition significant differences were observed between small breeds (Booted Bantam, Rosecomb Bantam) and large breeds (Araucana, Czech Golden Brindled, Minorca; **paper III**). In particular, there were 15 egg white proteins found in different relative amounts between small and large breeds, five of them, ovalbumin-related protein X, ovoinhibitor, ovodefensin B1, lymphocyte antigen 86 and ovocalyxin-32, possessing functions contributing to the egg defence against pathogens (**paper III**). All these proteins were found in a lower proportion in albumen of small breeds, implying reduced capacity of pathogen defence of eggs of those breeds. The explanation of this phenomenon possibly lies in origin and breeding purpose of the chosen small breeds. Both Rosecomb Bantam and Booted Bantam are primarily kept as fancy pet breeds that are selected strongly for an external appearance and much less for their egg production. Therefore, their egg durability and pathogen defence could be compromised. Similarly, artificial selection for the egg resistance could enhance egg white antimicrobial capacity of large layer breeds.

Table 2 Proteins with described antimicrobial defence function detected in egg white. Abundance - mean relative abundance of protein in egg white samples. Function - described antimicrobial effect.

Protein	Abundance	Function	References
Ovotransferrin	31.43 %	iron sequestering; iron independent bactericidal and antiviral activity	Wu & Acero-Lopez (2012); Baron *et al.* (2014)
Lysozyme	7.42 %	hydrolysis of bacterial cell wall peptidoglycan	Ragland & Criss (2017)
Ovomucoid	4.57 %	inhibition of the bacterial proteases	Nagata & Yoshida (1984)
Ovalbumin-related protein X	3.32 %	heparin binding properties; unknown mechanism of antibacterial function	Rehault-Godbert *et al.* (2013)
Ovoinhibitor	1.86 %	inhibition of the bacterial proteases	Bourin *et al.* (2011)
Ovoglobulin G2	1.59 %	bactericidal permeability-increasing protein; unsure mechanism of antibacterial function	Maehashi *et al.* (2014); Whenham *et al.* (2014)
Ovostatin	1.20 %	bacterial protease inhibitor	Nagase *et al.* (1983); Lim *et al.* (2011)
Gallin	0.26 %	inhibition of *Escherichia coli* grow by unknown mechanism	Whenham *et al.* (2015)
Avidin	0.14 %	sequestering biotin	White *et al.* (1992)
Cystatin	0.11 %	inhibitor of cysteine proteases	Wesierska *et al.* (2005)
Lipocalin 8	0.10 %	siderophore binding lipocalin	Correnti *et al.* (2011)
Histone H2B	0.02 %	unknown mechanism of bactericidal function	Silphaduang *et al.* (2006); Hoeksema *et al.* (2016)
Histone H2A.Z	0.02 %	unknown mechanism of bactericidal function	Silphaduang *et al.* (2006); Hoeksema *et al.* (2016)
Vitellogenin-2	< 0.01 %	bacterial cell disruption by metal-chelating ability in cell membrane	Choi *et al.* (2004)
Histone H4 type VIII	< 0.01 %	unknown mechanism of bactericidal function	Lee *et al.* (2009); Nys *et al.* (2011); Hoeksema *et al.* (2016)
Gallinacin-11	< 0.01 %	cell wall disruption, exact mechanism unknown	Herve-Grepinet *et al.* (2010)
Histone H2A	< 0.01 %	unknown mechanism of bactericidal function	Silphaduang *et al.* (2006); Hoeksema *et al.* (2016)
Ovodefensin B1	< 0.01 %	inhibition of *E. coli* grow by unknown mechanism	Whenham *et al.* (2015)
Lymphocyte antigen 86	< 0.01 %	modulation of liposaccharide response	Sekelova et al. (2017)
Ovocalyxin-32	< 0.001 %	carboxypeptidase inhibitor	Gautron et al. (2001; Xing et al. (2007)

Gene Expression in Oviduct of Maternal Organism

Especially recently, there has been growing body of evidence showing high expression of antimicrobial proteins in a chicken oviduct (Gautron *et al.* 2001; Mageed *et al.* 2008; Gong *et al.* 2010; Mageed *et al.* 2011; Whenham *et al.* 2014; Yoshimura *et al.* 2014; Whenham *et al.* 2015; Socha & Hrabia 2018) and changes of this expression after treatment of hens with bacterial stimuli (Yoshimura *et al.* 2006; Mageed *et al.* 2008; Sonoda *et al.* 2013). These findings confirm the irreplaceable role of antimicrobial proteins in defence of chicken eggs. Furthermore, it was shown that environmental microbial contamination of hen breeding facility could affect the bactericidal properties of an egg white (Bedrani *et al.* 2013). It implies that defence capacity of the egg could be influenced by immunological processes in the maternal organism and this relationship deserves further investigation.

Therefore, in **paper III** we, for the first time, described a whole transcriptome of the chicken magnum and also its relationship with the whole proteome of the egg white. By description of the whole magnum transcriptome, we have shown that, similarly as in the case of proteins in chicken egg white, mRNA transcripts encoding proteins with antimicrobial defence function belongs to the most highly expressed ones. The most expressed defence proteins are on the transcriptomic level ovotransferrin, lysozyme C, ovoglobulin G2, ovalbumin-related protein X and ovoinhibitor. The mean expression of mRNA of individual genes across all hens correlates significantly with the mean abundance of respective proteins in eggs ($r = 0.66$). Nevertheless, in transcriptome, we were unable to observe the same between-breed variability as described for the egg white proteome. On individual level there is thus no co-structure between magnum transcriptome and the egg white proteome. Similarly, Kim & Choi (2014) did not observe the same changes in egg white proteins and mRNA expression after corticosterone treatment in chickens. We propose that this discrepancy might have arisen from post-transcriptional regulation of gene expression (Morris & Geballe 2000; Hershey *et al.* 2012; Curinha *et al.* 2014).

Summary and Conclusion

In this doctoral thesis, I describe variability in immune-related traits of hens and their eggs across several chicken breeds. Resistance of domestic animals against pathogen infections is crucial from the economical perspective as well as from the perspective of biosecurity control of a human disease transmission. Variability in the immune defence mechanisms between chicken breeds is also extremely interesting from the perspective of ecological immunology, as it could show different host-parasite coevolution in lineages evolving under different artificial and natural selective pressures.

In my research, I was able to show large effect of blood cellular composition on skin tissue inflammatory responsiveness (both swelling and cellular composition), which highlights the importance of haematological variability for avian defence against pathogens. By a novel method of whole-blood flow cytometry with fluorescently labelled antibodies, I was then able to evidence a significant haematological variability between the studied breeds of my choice. This was true for both parameters associated with erythrocytes and leukocytes. Since the chicken breeds originated at different places and experienced different evolutionary history in a distinct environmental context under varying directions of natural and artificial selection, their variability in haematological traits could reflect different adaptations of the immune system to relevant selective pressures. In my dataset, two breeds showed the most extreme values of the haematological parameters measured; the European breed Czech Golden Brindled with high $CD4^+$ T-cell frequencies and low H/L ratio and the South American breed Araucana with high monocyte frequencies and high H/L ratio. I speculate that the contrasting haematological values in these breeds may indicate adaptations on different pathogenic pressures selecting for increased adaptive T-cell mediated and humoral immune responses in Czech Golden Brindled, while for strong innate immune responsiveness in Araucanas. As the haematological parameters are tightly linked to other physiological traits, especially the difference in H/L ratios could suggest also differences in stress resistance in those breeds.

Apart from meat, the economically most important product of chicken is the egg. Eggs serve both as a natural source of nutrients and protection for chicken embryos and as valuable food source for humans. Since at the beginning chicken embryos do not have their immune system fully developed, the defence against pathogens relies on the protective substances deposited into eggs by the maternal organisms, the hens. The egg white forms

the largest part of the avian egg and also has the largest protein content. Majority of the egg white proteins (considered based on their amount) plays a role in antimicrobial defence of the egg. Therefore, proteomic composition of the egg white can largely influence the egg quality as well as its biosafety. My results indicate that chicken breeds vary significantly in egg white proteomic composition. From perspective of this thesis, the most interesting variability is in the relative amounts of proteins with the antimicrobial defence function. These were found in lower amounts in small breeds (Booted Bantam, Resecomb Bantam) than in large breeds (Araucana, Czech Golden Brindled, Minorca). This, again, could imply different adaptations of the breeds to different selective pressures. In this case, I speculate that selection on low size and appearance could compromise their antimicrobial capacity.

The egg white proteins are synthetized in a hen oviduct, inside cells of villiform mucous of magnum, and afterwards deposited to a newly developing egg. In my thesis I performed for the first time the next generation sequencing of the whole magnum transcriptome and showed its relationship to the egg white proteome. The most expressed proteins in magnum correspond with those highly abundant in egg proteome and there is generally strong correlation between gene expression on mRNA and protein levels. Despite this, I revealed discrepancy between transcriptomic expression in maternal organism and proteomic composition of egg white, meaning that the between-breed variability described in eggs is not observable on the level of hen oviduct transcriptome. This lack of co-structure may indicate a large role of posttranscriptional modifications on the emergence of variation in the egg white composition.

Taken altogether, the results of my thesis provide early insight into the high and potentially relevant immuno-phenotypic variability between the traditional chicken breeds. So far there are very few studies concerning chicken breed variability in immune-related traits and its effect on the resistance and susceptibility to parasitic infections. Since variability is the key factor determining sustainability of natural defence against pathogens constantly co-evolving with the host, understanding this variability is not only crucial from the perspective of evolutionary immunology, but also essential for future knowledge-based breeding of the domestic chicken.

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