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Summary of the Ph.D. thesis



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

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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.

Introduction

Domestic chicken (*Gallus gallus* f. *domestica*) is considered both one of the most valuable bird models in biology (Stern 2005, Burt 2007) and largely important farm animal (Food and Agriculture Organization; FAO 2016). In modern research and also in husbandry, usually inbred or monotypic commercial lines are used (Muir et al. 2008, Delany and O'Hare 2008). Unfortunately, unilateral breeding towards specialised and highly productive chicken breeds could contribute to decrease of phenotypic and genetic variability, higher susceptibility and loss of resistance to parasites (Schachner et al. 2018, Institute of medicine and national research council 2004, van der Most et al. 2011). Susceptibility of chicken to pathogens is fraught with the risk of economical loss, but more importantly with thread of foodborne diseases and spread of infection to human population (Majowicz et al. 2010).

It has been suggested, that for sustaining pathogen-free chicken population and biologically secure chicken products systematic breeding towards improved disease resistance is needed (Gavora and Spencer 1983, Kramer, Malek and Lamont 2003, Dunn 2004, Fulton 2004, Bagust 2013, Berghof et al. 2015, Schilling et al. 2018). Description and conservation of immunologically relevant variability plays pivotal role in this approach (Beaumont et al. 2003, Institute of medicine and national research council 2004).

Desirable phenotypic, genetic and possibly also immunologically important diversity could still be found in non-commercial and traditional chicken breeds (Notter 1999, Hillel et al. 2003, Toro, Fernandez and Caballero 2009, Groeneveld et al. 2010). Chicken breeds were formed by artificial selection mainly on required specific external traits (Jull 1932), evolving for hundred years in various condition of different environments. Their evolutionary past could thus shape their physiology, including immune system and ability of pathogen defence. There is rising number of evidence of chicken-breed variability in immune traits, including capability to fight bacterial and viral diseases (Redmond et al. 2009, Zhang et al. 2018), large variability in MHC receptors (Izadi, Ritland and Cheng 2011, Kannaki et al. 2017, Fulton et al. 2017) or Toll-like receptors (Ruan, An and Wu 2015, Ruan and Zheng 2011).

For purpose of this thesis we chose five morphologically different layer chicken breeds: Araucana, Booted Bantam, Czech olden Brindeled Minorca and Rosecomb Bantam and described variability in their immune traits, specifically in haematological parameters and amount of proteins with antimicrobial defence function in their eggs.

Blood belongs to most immunologically important tissues, as it transports the white blood cells (leukocytes) from place of their origin to site of their incidence. Leukocytes are

responsible for pathogen recognition and elimination by multiple pathogen specific and non-specific tools. There are five main leukocyte types in avian blood with different effector mechanisms: lymphocytes, heterophils, monocytes, basophils and eosinophils (Campbell and Ellis 2007, Lucas and Jamroz 1961). There is extensive evidence of change in blood leukocyte count in response to parasitic infection (Ruhnke et al. 2017, Soria, Bonnet and Bueno 2015, Matur et al. 2011) but also stress (Davis, Maney and Maerz 2008, Frigerio et al. 2017), and other external factors (Waheed et al. 2017, Cengiz and Kucukersan 2010, Matur et al. 2016). For this reason, assessment of blood cellular composition by haematological screening belongs to widely used methods of measuring health and physiological state of animals in both veterinary and eco-immunological studies (Pickler et al. 2013, Talebi et al. 2005, Davis et al. 2008, Neveling et al. 2017, Kar et al. 2018, Lentfer et al. 2015)

As different leukocytes have different functions in immune system, their abundance in peripheral blood could affect the ability of immune responsiveness of individual. Haematological variability may thus result into variable capability of response to pathogens. By description of relationship between experimentally induced inflammation and variability in haematological parameters, in **paper I** we confirmed large effect of blood cellular composition on pro-inflammatory immune responsiveness of birds.

Despite the great importance of haematological parameters, variability in those parameters in domestic chicken breeds was described only in few Asian and African chicken breeds by basic light microscopy analysis (Peters et al. 2011, Islam et al. 2004, Adenaike et al. 2016). To reveal variance between domestic chicken breeds, in **paper II** we performed novel method of whole blood flow cytometric analysis (Seliger et al. 2012).

Chicken health state and ability of immune responsiveness could importantly affect their egg laying performance (Iseri and Klasing 2014). Egg is an easy target for pathogenic microbial contamination, as in the beginning of ontogeny the embryo does not have their immune system fully developed yet (De Buck et al. 2004). Therefore protection of egg is completely dependent on substances deposited from maternal organism. It has been previously described that an egg possesses whole arsenal of antimicrobially active peptides and proteins guarding the egg against pathogenic threats from environment (Mann and Mann 2011, Rehault et al. 2007, Da Silva et al. 2015, Rehault-Godbert et al. 2013, Herve-Grepinet et al. 2010). These proteins are highly abundant in an egg white, which is created in magnum, the largest part of chicken oviduct (Nys and Guyot 2011). Variability in egg white protein composition was previously described only between two commercial breeds and their egg varieties (Wang et al. 2012). Such differences in egg white protein abundances, especially

concerning proteins with antimicrobial function might presumably affect quality and defensive capacity of the egg. Hence in **paper III** we explored proteome composition of the egg white and its between-breed differences.

It is known, that antimicrobial defence proteins are highly expressed also in the magnum of hens (Mageed, Isobe and Yoshimura 2008, Sonoda et al. 2013, Yoshimura et al. 2006), nevertheless straight association between magnum mRNA expression and proteome composition of egg white remains unknown. Kim and Choi (2014) showed that experimental corticosterone treatment changes both mRNA expression in magnum and egg white protein amount, but not necessarily in same direction. The relationship of whole magnum transcriptome and whole egg white proteome should therefore be properly examined. We are the first to present description of whole mRNA transcriptome of hen magnum in context of comparison with whole proteome of egg white, in **paper III**.

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

Material and Methods

For purpose of paper I, we used zebra finch (*Taenopygia guttata*) as model organism. To explore the relationship between haematological parameters and ability of immune responsiveness we applied standard method of Phytohaemagglutinin (PHA) skin-swelling test as described before in Vinkler et al. (2012). Solution of PHA lymphoproliferative subunit PHA-L was injected into wing web (patagium) of experimental animals. We determined the magnitude of swelling response as difference in patagium thickness before and 24 hour after PHA-L treatment. After the treatment biopsy of swollen tissue was taken for histological analysis. Also before and after the treatment, we collected peripheral blood samples from birds for haematological analysis of differential cell count. Cellular composition of haematological and histological samples was analysed under light microscope as described in Campbell and Ellis (2007).

In paper II and III we described variability in immune traits of domestic chickens (*Gallus gallus domesticus*) from five traditional breeds Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam. All animals were bred from egg to maturity in standardised condition of breeding facility of Czech University of Life Sciences, Prague. After reaching maturity, we took blood samples from all animals for cytometric analysis. Then eggs from hens were collected for proteomic analysis of egg whites. Hens were euthanized by rapid cervical dislocation and samples of magnum part of oviduct were taken for transcriptomic analysis. Cytometric analysis of blood samples providing absolute and differential cell counts (paper II) was performed on CytoFLEX flow cytometer (Beckman Coulter Inc., Brea, California, USA) with four fluorescently labelled specific antibodies against blood cell epitopes. We described proteome of chicken egg whites (paper III) by tandem mass spectrometry analysis on Thermo Orbitrap Fusion mass spectrometer. Transcriptomes from magnum tissue were obtained by NextSeq 500 Illumina sequencing (paper III).

Detail information on used methods is available in the thesis and in original publications.

Results and Discussion

In this doctoral thesis we were able to describe significant variability of five domestic chicken breeds, Araucana, Booted Bantam, Czech Golden Brindled, Minorca and Rosecomb Bantam in immune traits of hens and their eggs. At first, in PHA stimulated immune response, we affirmed large effect of variability of blood cellular composition on course of pro-inflammatory immune response (**paper I**). According to our histological analysis, PHA stimulates heterophil dominated tissue inflammation (**paper I**), which is in consistence with some previous studies (Turmelle et al. 2010, Vinkler, Bainova and Albrecht 2010, Brown, Shilton and Shine 2011, Vinkler et al. 2012, Finger et al. 2013, Salaberria et al. 2013, Maxwell and Robertson 1998). The magnitude of stimulated pro-inflammatory response concerning both swelling and cellular composition was highly influenced by differential count of lymphocytes and basophils in peripheral blood of experimental birds. Similarly, it was shown by Zekarias et al. (2000) that blood cellular composition in chickens influences their ability of response to bacterial infection with *Enterococcus faecalis*. Those results accent the large role of peripheral blood lymphocytes in immune response and great importance of haematological investigation in birds.

Therefore, in **paper II** we determined haematological parameters in our five selected chicken breeds reporting significant variability in numbers of both erythrocytes and leukocytes. So far there have been few studies concerning haematological variability between indigenous chickens (Peters et al. 2011, Islam et al. 2004, Adenaike et al. 2016), all performed by light microscopy analysis of blood samples. In our research, we validated and afterwards used innovative method of whole blood flow cytometry with fluorescently labelled specific antibodies (Seliger et al. 2012). We observed two extremes in leukocyte profiles of chicken breeds; on one side of the spectra there were Czech Golden Brindled with high proportion of CD4⁺ T-cells and low heterophils to lymphocytes ratio (H/L) and on the other side Araucanas with high monocytes frequencies and high H/L ratio. As CD4⁺ T-cells participate on B-cellular immunity and adaptive response, their increase was previously observed after vaccination (Bridle et al. 2006, Calefi et al. 2016, Zhu et al. 2016) and also in birds with high antibody production (Parmentier et al. 1995, Dalgaard et al. 2010). The high H/L ratio, as seen in Araucanas, is commonly used as sign of long term stress (Davis et al. 2008, Al-Murrani, Al-Rawi and Raof 2002), but as heterophils also provide protection mainly against bacterial infections, their higher levels could also indicate enhanced ability of anti-bacterial innate immune response. Czech Golden Brindled are traditional European breed, believed to

be adapted to harsh winter climate, on the other hand Araucanas are South American breed brought to Europe relatively recently (Pavel and Tuláček 2006). Concerning their different evolutionary history, we speculate that contrasting haematological values are sign of adaptation on different selective pressures. In Czech Golden Brindled haematological parameters presumably indicate strong T-cell mediated and humoral adaptive immune response or high stress-resistance, in Araucanas cellular composition might indicate enhanced innate immune responsiveness or susceptibility to stress environment.

Agricultural importance of chicken lies also in egg production. As eggs are one of the major sources of foodborne diseases (Majowicz et al. 2010), egg biosecurity and anti-parasite resistance belongs to main concerns in poultry husbandry. An egg is protected against pathogenic infections by multiple proteins with various defence mechanisms. Those proteins are deposited in large amounts mostly in egg white (Mann and Mann 2011, Bourin et al. 2011, Dombre et al. 2017, Rehault-Godbert et al. 2013, Herve-Grepinet et al. 2010, Herve et al. 2014). Our proteomic analysis revealed that antimicrobial defence proteins form approximately 51% of all protein amount of egg white (**paper III**). Five of these defence proteins, ovalbumin-related protein X, ovoinhibitor, ovodefensin B1, lymphocyte antigen 86 and ovocalyxin-32 were found in significantly different relative amount in eggs from small (Booted bantam and Rosecomb bantam) and large breeds (Araucana, Czech Golden Brindled and Minorca). The amount of mentioned proteins was in all cases lower in eggs derived from small breeds. We speculate, that this variance could result from distinct selective pressures acting in past. Booted Bantam and Rosecomb Bantam breeds are mainly kept as fancy breeds, and were probably strongly selected on appearance, more than on egg durability and resistance. On the other hand, large breeds kept for laying purposes could be under stronger selective pressure towards egg resistance improving their egg white antimicrobial capacity.

Egg white proteins are synthesized in magnum, largest part of hens oviduct, and afterwards deposited to newly forming egg (Nys and Guyot 2011). In **paper III**, we, for the first time in history, sequenced whole magnum transcriptome and described its relationship with complete egg white proteome. Similarly as in proteome of egg white, large expression of antimicrobial proteins could be observed also on mRNA levels in magnum tissue. There has been generally strong correlation between mean mRNA expression in magnum and abundance of respective proteins in egg whites. Nevertheless, between-breed variability described in proteome of eggs was not found in level of mRNA expression, meaning that there is lack of co-structure between proteome and transcriptome on individual level (**paper III**). Similarly, Kim and Choi (2014) observed different direction of change of mRNA

expression in oviduct and protein abundance in egg whites after corticosterone stimulation of hens. Those discrepancies probably indicates large role of posttranscriptional modification in between-breed variation of egg white composition.

Conclusions

This thesis provides insight into immuno-phenotypic variability between the traditional chicken breeds. Between-breed differences were described in both haematological parameters of hens and amounts of egg-derived proteins with antimicrobial defence function. Since variability is the key factor providing ability of sustaining natural resistance of host in constant parasite driven evolution, describing this variability is important from the perspective of evolutionary immunology, but could be also crucial for future breeding of the domestic chicken.

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Curriculum vitae

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Education

2014 - present

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Ph.D. Thesis: Variability of domestic chicken breeds in selected immune traits of chicken and egg

2012 - 2014

MSc study: Charles University, Faculty of Science, Department of Zoology

MSc thesis: Variability in health state of mice in *Mus musculus musculus* and *Mus musculus domesticus* hybrid zone

2009 - 2012

BSc study: Charles University, Faculty of Science, Biology

BSc thesis: Evolution of molecular interaction between host and parasite

Teaching experience

2015 – 2017

Department of Zoology, Faculty of Sciences, Charles University:

Morphology of animals practices

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STARS program for talented PhD students at the Faculty of Science, Charles University in Prague

International experience

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INRA Research Center at Jouy-en-Josas, France

Certificates

Certificate of professional qualification to propose experiments and projects of experiments pursuant to Art. 15d (3) of Act No. 246/1992 Coll., on the protection of animals against cruelty, as amended

List of publications

Publications included in this thesis

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 Lukas & 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, Sviderska 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 *PROTEOMICS*

Publication not included in this thesis

Bilkova Barbora, Albrecht Tomas, Chudickova Milada, Holan Vladimir, Pialek Josef & Vinkler Michal (2016) Application of Concanavalin A during immune responsiveness skin-swelling tests facilitates measurement interpretation in mammalian ecology. *Ecology and Evolution*, 6, 4551-4564.

Publication without impact factor

Michal Vinkler, **Barbora Bilkova**, Zuzana Swiderska, Jozef Janda, Lukas Zita (2018) Mají různá plemena slepic různou krev? Accepted to *Chovatel*.

Selected conference contributions

Bilkova Barbora, Sviderska Zuzana, Janda Jozef, Stopka Pavel, Zita Lukas, Laloe Denis, Charles Mathew, Benes Vladimir & Vinkler Michal: Jako vejce vejci? Proteom bílku a transkriptom oviduktu u tradičních plemen kura domácího. Zoologické dny 2018, Prague, Czech Republic

Bilkova Barbora, Bainova Zuzana, Janda Jozef, Stopka Pavel, Zita Lukas & Vinkler Michal: Variability in immune defence of chicken breeds. Conflict and cooperation - bridging evolution, ecology and immunology, PhD student meeting 2017, Bautzen, Germany

Bilkova Barbora, Bainova Zuzana, Janda Jozef, Stopka Pavel, Zita Lukas & Vinkler Michal: Slepice, vejce a imunita: Variabilita hematologických parametrů a proteinů vaječného bílku u plemen kura domácího. Zoologické dny 2017, Brno, Czech Republic

Bilkova Barbora, Bainova Zuzana, Janda Jozef, Zita Lukas & Vinkler Michal: Different breeds - different blood. XIV Avian Immunology Research Group Meeting AIRG meeting 2016, Herrsching Am Ammersee, Germany. Poster

Bilkova Barbora, Bainova Zuzana, Zita Lukas, Janda Jozef & Vinkler Michal: Antimikrobiální peptidy ve vejcích kura domácího. Zoologické dny 2016, České Budějovice, Czech Republic. Poster

