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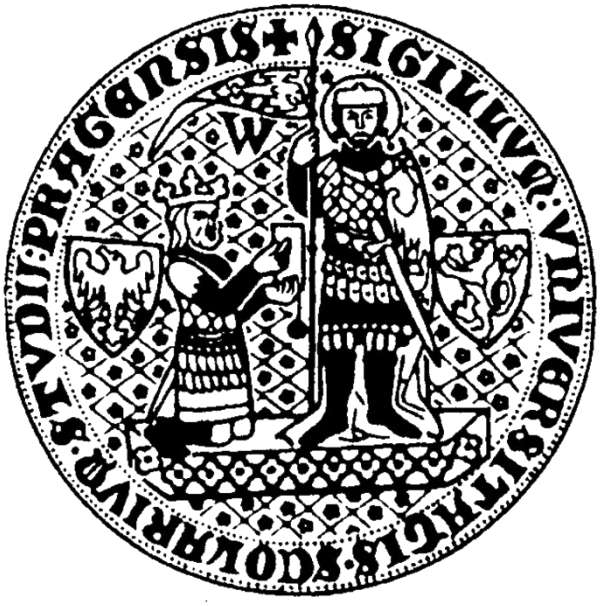

MASTER THESIS

Impact of Toll-like receptor 4 polymorphism on pro-inflammatory responsiveness in great tit (*Parus major*)

Vliv polymorfismu Toll-like receptoru 4 na prozánětlivou odpověď u sýkory koňadry (*Parus major*)

Bc. Jitka Vinklerová

Supervisor: Doc. Mgr. at Mgr. Josef Bryja, Ph.D.

Co-supervisor: RNDr. Michal Vinkler, Ph.D.

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Abstract

Toll-like receptor 4 (TLR4) belongs among chief bacteria-sensing Pattern recognition receptors. Endotoxin (lipopolysaccharide, LPS) recognition by TLR4 triggers signalling leading to release of cytokines that direct leukocyte infiltration into the inflammatory site and cause swelling. Effector mechanisms that ensure pathogen elimination include phagocytosis and oxidative burst. It has been repeatedly reported that the polymorphism in TLR4 may affect host resistance to various diseases. TLR4 may be, therefore, an important molecule in host-parasite co-evolution. Herein, I focused on TLR4 amino acid substitution Q549R which is associated with ornamentation in great tits. In tits I describe immune responsiveness to LPS stimulation on morphological and molecular level and examine effects of the Q549R substitution on inflammation and general body condition. In LPS-treated individuals I found decrease in heterophil-lymphocyte ratio (H/L) that might be caused by attraction of the blood-borne cells into the inflamed tissue. This is in striking contrast with increase in H/L in PBS-treated animals resulting from the stress response. There was no effect of Q549R on general condition and haematological parameters but I revealed a significant effect of the interaction between host Q549R genotype and the type of injected LPS (*E. coli* / *S. enterica*-derived) on the swelling responsiveness. This highlights the importance of the TLR4 Q549R genotype for tit immune defence.

Key words: condition, cytokine, expression, inflammation, leukocyte, oxidative burst

Abstrakt

Toll-like receptor 4 (TLR4) je jedním z nejdůležitějších Pattern recognition receptorů, jehož úkolem je včasné rozpoznání bakteriální infekce. Po navázání endotoxinu (lipopolysacharidu, LPS) na TLR4 dochází ke spuštění signální kaskády vedoucí k produkci imunomodulčaních molekul, cytokinů. Jednou z funkcí cytokinů je řídit infiltraci leukocytů z krevního řečiště do místa zánětu, která vede ke vzniku otoku. Patogen je následně eliminován těmito buňkami fagocytózou a oxidačním vzplanutím. Je známo, že polymorfismus v TLR4 může ovlivňovat resistenci hostitelského organismu k některým chorobám. TLR4 by tak mohl hrát důležitou roli v koevoluci hostitele a parazita. Ve své diplomové práci jsem se zaměřila na aminokyselinovou substituci Q549R v TLR4, která je u sýkory koňadry asociovaná s mírou exprese ornamentálního zbarvení. U jedinců, kterým bylo injikováno LPS, došlo k poklesu poměru heterofilů a lymfocytů (H/L) v krvi. Tento pokles mohla způsobit infiltrace krevních buněk do zanícené tkáně. Naopak u zvířat, kterým bylo injikováno pouze PBS, došlo k nárůstu H/L, patrně v důsledku stresu. Prokázala jsem, že substituce Q549R nemá vliv na celkovou kondici či hematologické parametry jedince. Zjistila jsem však vliv interakce mezi genotypem Q549R hostitele a typem LPS (*Escherichia coli* / *Salmonella entrica*) použitého ke stimulaci zánětu na míru otokové reakce. Toto zjištění dokládá možný význam genotypu TLR4 Q549R při obraně organismu proti působícím patogenům.

Klíčová slova: cytokiny, exprese, kondice, leukocyty, oxidační vzplanutí, zánět

Preamble

The data used in this work originate from field measurements and experiments performed during two study seasons in 2011 and 2012. I took a major part in collecting most of these data, though in many tasks the assistance of other members of our team was necessary. The present thesis, therefore, results from a collaborative work of several people. Involvement of other people in resolving individual tasks is clearly described in the acknowledgement to this thesis.

I declare that the thesis "Impact of Toll-like receptor 4 polymorphism on pro-inflammatory responsiveness in great tit (*Parus major*)" I wrote all by myself on the basis of the material which is cited in the text and with my supervisor's and colleagues' consultations. This thesis or its part was submitted to obtain neither another nor the same academic degree.

Prague, 10th August 2013

Jitka Vinklerová

Contents

List of abbreviation

PRR	Pattern Recognition Receptors
MAMP	Microbe-Associate Molecular Patterns
DAMP	Damage-Associated Molecular Pattern
TLR	Toll-like Receptors
CR	Complement Receptors
NOD	Nucleotide-binding Oligomerization-Domain
NLR	NOD-like Receptors
RIG-I	Retinoic acid-Inducible Gene 1
RLR	RID-I- Like Receptors
CLR	C-type Lectin Receptors
SNP	Single Nucleotide Polymorphism
MD-2	Myeloid Differentiation factor 2
LPS	Lipopolysaccharide
LPS_{Ecol}	Lipopolysaccharide derived from *Escherichia coli*
LPS_{Sent}	Lipopolysaccharide derived from *Samlmonella enterica*
PHA	Phytohaemagglutinin
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
MPO	Myeloperoxidase
APC	Antigen Presenting Cell
LRR	Leucine-Rich Repeat
CD14	Cluster Differentiation 14
IL	Interleukin
TNFα	Tumor Necroses Factor-α
INFγ	Interferon-γ
Th	T-helper cell
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
HRMA	High Resolution Melting Analyses
E. coli	*Escherichia coli*
S. ent	*Salmonella enterica*

I. Introduction

Inflammation is one of the chief immunological mechanisms defending host bodies from pathogen incursion (Ashley et al. 2012). This type of complex immune response takes part in most severe cases of homeostasis disbalance, such as infection, injury or exposure to various contaminants. Inflammation is induced by Pattern recognition receptors (PRRs), a set of receptors of the innate immunity recognition apparatus. Among the most well-known and characterised PRRs belong Toll-like receptors (TLRs). TLRs and other PRRs directly recognize structures derived from pathogens and damaged and necrotic cells (Akira et al. 2006). After their initial activation by their specific ligands a complex signalling cascade is triggered which results in alternations in gene expression affecting both surface membrane molecules and secreted soluble factors (Danilova 2006). Especially the soluble signalling molecules termed cytokines possess the ability to stimulate a sophisticated network of immunological, physiological, and behavioural changes aimed against the potential pathogen infection.

Although the molecular basis of inflammation is well studied in mammals, we know surprisingly little about the inflammatory network in non-mammalian vertebrates, such as birds. Despite the know similarity between birds and mammals, birds possess many novel and unique molecular traits and, on the other hand, lack many traits described in mammals (Kaiser 2010). Avian immunology might, therefore, highly benefit from a specific research focused on avian inflammation. Furthermore, apart from avian immunologists, the role of inflammation in mediating the outcomes of host-parasite interactions has received only minimal attention also by evolutionary ecologists (Ashley et al. 2012). Given that there are remarkable differences in different types of immune response in their costs and benefits (Vinkler et al. in press), ecologists should precisely differentiate at least between innate and adaptive immunity and cellular- and humoral-based effector mechanisms. Inflammation represents a mostly cellular-based innate immune response and, hence, its advantages consist of fast inducibility and broad specificity. Among disadvantages of inflammation we can name its compromised efficiency against specialised pathogens and wider self-destruction of the host in the course of defence. Revealing the molecular basis of inflammation as well as the ecological factors influencing this type of immune response is undoubtedly an essential step towards proper understanding the principles of host-parasite interactions.

Equally important, to comprehend the evolutionary aspect of the host-parasite interactions, is the understanding of the genetic basis of differences in inflammatory immune responsiveness. This is rather a tough issue as disclosing identity of genetic traits with subtle effects on quantitative traits is a demanding research activity (see e.g. Calenge et al. 2009). There are several methodological approaches to resolving this dilemma, the simplest of which is a research focused on genetic polymorphism in candidate genes. Under natural condition a strong inflammation may be triggered

by immune cell exposure to endotoxin, a bacterial lipopolysaccharide (LPS). The extensive gene expression changes in cell response to LPS exposure are dependent on TLR-signalling-pathway activation (Bliss et al. 2005) the molecular level LPS is detected namely by receptor TLR4 in association with myeloid differentiation factor 2 (MD-2) accessory molecule (Park et al. 2009). It has been repeatedly shown in various vertebrate species that genetic polymorphism in TLR4 may affect the intensity of pro-inflammatory immune responses to LPS and various pathogens (see, e. g. Leveque et al. 2003; Hawn et al. 2005; Swiderek et al. 2006; Ferwerda et al. 2007; Belforte et al. 2013). Most importantly, Hana Bainová has shown in her thesis that one particular amino acid substitution in this gene in great tit (*Parus major*), the Q549R substitution, is associated with variability in adult plumage colouration (Bainová 2011). Hence, presumably, there may be some direct association between TLR4 Q549R genotype, immune responsiveness, health, condition and ornamentation in this species. The goal of this thesis of mine was, therefore, to explore the possible relationship between the Q549R genotype, pro-inflammatory responsiveness, health and condition in a convenient model of great tit nestlings and verify whether the genotype of this single nucleotide polymorphism (SNP) may or may not influence the whole outcome of the great tit immune response to LPS. In the following subchapters of the introduction I summarise current knowledge of individual aspects of this problematic and name evidence that was important for designing the present study.

I.1 Conceptual background of the research in evolutionary immunology

Similarly to ecological immunology also evolutionary immunology is a newly emerging field of interdisciplinary research in biology (Norris and Evans 2000; Schulenburg et al. 2009). One of the main issues solved by evolutionary immunology is the investigation towards understanding the mechanisms underlying evolution in the ability to recognize foreignness (Galaktionov 2004). As this branch of research is generally realised in free-living animals (Pedersen and Babayan 2011), evolutionary immunology can be viewed as a cutting edge topic of evolutionary ecology (Ardia and Schat 2008) Despite both ecological and evolutionary immunology represent joint disciplines of zoology, ecology and immunology, ever since their emergence zoologists and ecologists are much more involved in the exploration than immunologists (Seed 1993). That is why there were several recent calls in ecology for deeper involvement of researchers with immunological background in the investigation (Martin et al. 2011; Pedersen and Babayan 2011). As proposed by Vinkler and Albrecht (2011), this lack of collaboration between zoologists and ecologists on one side and immunologists on the other side may be due to much misunderstanding between these two fields. While zoologists and ecologists tend to oversimplify the immunological basis of the problematic, immunologists, on the other hand, tend to ignore the evolutionary and ecological aspects of immunity. Example of misunderstanding between these two fields can be found in the usage of the term immunocompetence (Vinkler and Albrecht 2011). Although it has been repeatedly shown that immune system function cannot be measured as a single character (Adamo 2004) and that higher

response is not always the more efficient one (Graham et al. 2005), still many ecologists claim in their articles the opposite.

In this thesis, I will not view the immune system as a "black box" but will try to describe the studied mechanisms on both molecular and cellular level. My understanding of adaptations in immune function is based on the evolutionary models of host-parasite interactions known as the Red queen / Arms race principle (Woolhouse et al. 2002). Briefly described, this principle postulates that both host and parasite population needs to evolve to be able to react on evolutionary countermoves of the other – host needs to improve its immune defence to resist the parasite while parasite needs to overcome the host defence to be able to survive. This association makes out of this interaction one of the fastest evolving biological systems in the living world. As a result, much polymorphism and variability can be found both in host immune defence and parasite virulence factors. Several models have been published to describe the maintenance of polymorphism in the host and parasite populations (Bílková 2012). In these models direct interaction between host and parasite genotype is assumed (genotype by genotype interaction, GxG; Wolinska and King 2009; Berenos et al. 2012). The two basic models, from which most other ones are derived, are the Gene-for-gene model (Frank 1993a) and the Matching alleles model (Frank 1993b). In general terms, the former postulates that polymorphism is maintained in the populations as a result of high costs for unlimited resistance/virulence, while the later model expects that precise combination of host genotype and parasite genotype is responsible for resistance/successful parasite incursion. Although originally these models were proposed as independent models, it has been shown that they represent two extremes of the same continuum of possible causes for polymorphism maintenance in the course of host-parasite coevolution (Agrawal and Lively 2002). The position of a particular host-parasite relationship in question within the continuum is based on the costs, benefits and precise molecular mechanisms of the certain host-parasite interaction. It has been, however, also proposed that the Gene-for-gene model better fits the conceptual features of the effector mechanisms of immunity while the Matching alleles model can better explain the host-parasite coevolution in the receptor-ligand detection systems (Agrawal and Lively 2003). When discussing the evolution of the Q549R polymorphism in great tits in the present study I will, therefore, proceed mainly from the Matching alleles concept.

As mentioned earlier, mechanisms of the host defence against pathogens are highly diversified and there is no single measurement of immune function to be used in ecological research (Adamo 2004; Pedersen and Babayan 2011). Several independent methods investigating different aspects of immunity have been, therefore, adopted (summarised e.g. Vinkler 2005). In fact, several novel approaches have been developed recently to widen the spectrum of methods applicable in the ecological research in free-living animals (Millet et al. 2007; Demas et al. 2011). Despite this methodological improvement it is very inefficient to study too many immunological mechanisms at

time as the common result is then only the disagreement between individual results without any further explanation (Gonzalez et al. 1999; Blount et al. 2003; Adamo 2004; McGraw and Ardia 2005; Saks et al. 2006; Bonato et al. 2009; Drury 2010). Given, that the TLR4, in which the Q549R substitution has been revealed in the great tits, is functioning as a Pattern recognition receptor of innate immunity, I decided, when measuring the immune responsiveness, to focus on several aspects of the innate immune response. Contrary to adaptive immunity which allows the immune system to limit self-damage on one hand, but lacks generality of the response and is considerably time demanding on the other, innate immunity comprises of defence mechanisms that are rapid and general (Sorci and Faivre 2009; Ashley et al. 2012). These benefits from investment into innate immunity are traded-off against lower efficiency of innate immunity to specialised parasites and wider self-destruction of the host in the course of defence (Vinkler et al. in press). The TLR4 pathway triggers many different innate immune functions but one of its main effects is on induction of inflammation (Bode et al. 2012). Inflammation and pro-inflammatory potential can be tested by several different approaches. One of them is also the commonly used phytohaemagglutinin (PHA) skin-swelling test (Vinkler et al. 2010a, Vinkler et al. in press). Though commonly used in ecology, PHA is not a natural ligand of PRRs and the measurement of the pro-inflammatory potential based on stimulation with this activator, hence, lacks any specificity. Methodologically an analogous test to measure cellular activation during inflammation can be, however, performed using the LPS as the activator (Berthouly et al. 2008). LPS is a natural ligand of TLR4 and the test is, therefore, specific for activation of the TLR4-triggered pro-inflammatory immune response. Furthermore, novel in vitro techniques based on LPS stimulation have been also used recently in ecology to measure oxidative burst of blood-borne phagocytes (Sild and Horak 2010).

I.2 Inflammation

Acute inflammatory response is caused by infection or tissue injury which leads to migration of leukocytes and plasma from the blood to the site of injury or infection. Inflammation is accompanied by four cardinal signs: red colouration (rubor) and warmth (calor) of the inflamed tissue caused by increase of blood flow, swelling (tumor) because of exudates of plasma protein-rich fluid and recruitment of circulating leukocytes and pain (dolor) caused by mediators released by leukocytes on C-type sensory nerve fibres (Pober and Sessa 2007). Initial recognition of infection is mediated by receptors on the surface of tissue-localized macrophages and mass cells and leads to production of variety of inflammatory mediators including chemokines, cytokines, vasoactive amines and products of proteolytic cascades. Main effect of these mediators is recruitment and activation of leukocytes, primarily neutrophils (mammals) or heterophils (birds) which eradicate source of inflammation by killing the microbes and removing cellular debris by phagocytosis (Ashley et al. 2012). If successful, acute inflammation is then resolved and normal tissue architecture is restored or connective tissue scar is formed. If the source of inflammation persists, inflammatory process evolves further. Composition of the infiltrate changes from

neutrophils / heterophils to mixture of mononuclear phagocytes and T cells. Later on, in case of prolonged inflammation, the inflammatory response is changing from the innate phase (mediated by recognition ligands on PRRs) towards adaptive phase in which antigen recognition by receptors on B and T cells is becoming crucial. Effectors mechanisms of B and T cells, such as recruitment of eosinophils, are more specific and usually are successful in elimination of inflammatory source (Pober and Sessa 2007; Medzhitov 2008).

Among the most important effector mechanisms of innate immunity belongs phagocytosis (Danilova 2006). During this process, microbes, dead cells and environmental debris are engulfed by professional phagocytic cells such as macrophages, dendritic cells and neutrophils / heterophils. There is a wide range of receptors on the surface of professional phagocytic cells that enable microbe/debris detection. Some of these receptors are involved in transmitting signals that directly trigger phagocytosis, while others participate only in binding or increasing the efficiency of internalization. Receptors can bind either to bare target particles (non-opsonic phagocytosis) or to particles opsonized, e.g., by immunoglobulins (Ig) or complement components (opsonic phagocytosis). The best known phagocytic receptors are Fcγ receptors (FcγRs) from Fc receptor family which bind IgG opsonised targets (Daeron 1997). There are two classes of FcγRs: the first one contains ITAM motifs in their intracellular domain that recruit kinases and activate phosporylation cascade; the second one contains ITIM motifs that recruit phosphatases and inhibit further signalling. Complement-opsonised particles are detected by the phagocytic complement receptors that include Complement receptor 1 (CR1), 3 (CR3) a 4 (CR4). Fc receptors and complement receptors cooperate on internalization of particles. For example, macrophages do not internalize particles opsonised by suboptimal concentrations of IgG antibodies but internalize these particles when also opsonised by complement. From the non-opsonic receptors we can name e.g. Scavenger receptors (SR-A) that are expressed on most macrophages bind whole bacteria as well as the microbial cell wall components, lipoteichoic acid and LPS, or mannose receptor and Dectin-1 belonging among surface lectins. Mannose receptor binds α-mannan and Dectin-1 binds β-glucan which are both compounds of zymosan particle of *Saccharomyces cerevisiae* yeast cell walls (Underhill and Ozinsky 2002; Goodridge et al. 2012). Activation of phagocytic receptors leads to particle internalization through actin polymerization, membrane recruitment and outward extension to surround the particles and finally to particle engulfment. The signalling cascade leading to phagocytosis involves dozens of signalling molecules which are responsible for rearrangement of the actin cytoskeleton, extension of plasma membrane and membrane ruffling ensuring the engulfment. This signalling is associated with activation of transcription factors such as for NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and may effect cytokine production (Chimini and Chavrier 2000; Underhill and Ozinsky 2002; Tachado et al. 2008).

The main effector mechanism of microbial killing after phagocytosis is oxidative (respiratory) burst (Sorci and Faivre 2009). In this process Flavoprotein nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), which is a membrane-bound enzyme, catalyses the production of large amounts of superoxide anion radicals (O_2^-) from NADPH and oxygen.

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + 2H^+$$

Cytosolic NADPH generates the superoxide anion by transferring electron across the cell membrane or the membrane of phagosomes. Production of superoxide anion out of the cell may cause oxygen-mediated inflammation, tissue injury and other immunopathologies. Superoxide anion is relatively unreactive, but is spontaneously dismutated into hydrogen peroxide (H_2O_2).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction proceeds more efficiently at low pH or through enzyme-catalysis by superoxide dismutase (SOD). H_2O_2 has the capacity of diffuse within and between cells. Hereafter H_2O_2 interact with chloride anion Cl^- to hypochlorous acid ($HOCl$) in the presence of myeloperoxidase (MPO).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl^- + H_2O$$

Hypochlorous acid is the most bactericidal oxidant in oxidative burst which is ca. 100-1000 times more toxic than either O_2^- or H_2O_2. MPO is a lysosomal protein that is most abundantly expressed in azurophilic granules of neutrophils. It is also present in avian eosinophils, monocytes and macrophages but there is no MPO in their heterophils. So although heterophils are essential phagocytic cells of avian innate immunity system they are involved only in distinct non-oxidative mechanisms of phagocytosis. Furthermore, there are other highly reactive forms of oxygen that originate from H_2O_2: especially extreme reactive hydroxyl radical (OH^-) and singlet oxygen (O_2).

Phagocytosis and oxidative burst are usually accompanied by activation of pro-inflammatory mechanisms (Naik and Dixit 2011). Some of the phagocytic receptors induce pro-inflammatory response directly (e.g. FcR) whereas others (such as CRs) do not and initiation of the pro-inflammatory response requires some additional signals, mostly from PRRs. It has been, for instance shown, that phagocytosis stimulates expression of PRRs, namely TLRs (Liu et al. 2006).

I.3 Toll-like receptors

Innate immunity system represents the first line of host defence against invading pathogens. Innate immunity response is initiated by phagocytic cells and antigen presenting cells (APCs), such as macrophages, granulocytes and dendritic cells. These cells can recognize evolutionarily conserved structures of pathogens, termed microbe-associate molecular patterns (MAMPs), by pattern recognition receptors (PRRs). PPRs play a key role in host defence against danger and consist of

five receptor families: Toll-like receptors (TLRs), NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs), RIG-I (retinoic acid-inducible gene 1)-like receptors (RLRs), C-type lectin receptors (CLRs) and pentraxins (Palsson-McDermott and O'Neill 2007). While TLRs and CLRs are expressed into cell membranes, RLRs and NLRs are expressed into cytoplasm where they are key sensors of intracellular microbes and danger signals (Mogensen 2009). Pentraxins are soluble. MAMPs are characterized as small molecular motifs conserved within a class of microbes. PRRs identify MAMPs as non-self-structures and trigger proinflammatory and antimicrobial response by activating intracellular signalling pathways. Not only MAMPs can be recognized as signals of danger. Endogenous molecules created upon tissue injury may stimulate a similar, yet slightly distinct, immune response. These are called damage-associated molecular patterns (DAMPs). DAMPs are usually endogenous cytosolic or nuclear proteins (e.g. heat shock proteins, HSPs) released into the extracellular milieu as a result of cell necrosis or tissue injury (Piccinini and Midwood 2010). At the end of the signalling cascades leading from MAMPS and DAMPS lie alternations in gene expression and synthesis of wide range of inflammatory mediators including cytokines, chemokines, cell adhesion molecules and immunoreceptors. The innate immunity pathogen detection based on PRRs is a highly developed system with great specificity to discriminate self from non-self which is essential for timely pathogen recognition. This system is also closely linked with activation of adaptive immunity in later stages of the response (Mogensen 2009).

One of the most important and most well-known families of PRRs are TLRs. The first TLR was identified in 1997 by Medzhitov (Medzhitov et al. 1997) as a human homologue of *Drosophila melanogaster* gene Toll. *Drosophila* Toll encodes a receptor involved in developmental patterning and more importantly in resistance to fungal infections (Lemaitre et al. 1996). Apparently Toll originally had a purely developmental role and only secondarily evolved its function in resistance against infectious pathogens (Kanzok et al. 2004). Animal TLR genes are absent from non-animal phyla but they are present in most eumetazoans (Leulier and Lemaitre 2008). There are six major families of vertebrates TLRs (Roach et al. 2005) which currently contain 23-25 members (Temperley et al. 2008). Not all TLRs are present in all vertebrate species. Each species is usually equipped with about a dozen of TLRs. Some of the TLRs are common to all vertebrates while others are restricted only to some single vertebrates lineages (Vinkler and Albrecht 2009). The main TLR signalling pathway is conserved in such diverse classes of species as arthropods and higher primates suggesting that this cascade is at least 900 million years old (Lemaitre et al. 1996; Medzhitov et al. 1997). Despite the function of this pathway is highly conserved, there exist taxon-specific differences among several components of TLRs signalling pathway. These differences may be very important for species-specific defence against the pathogens (Cormican et al. 2009).

In birds there are ten TLRs (Brownlie and Allan 2011). TLR2a, TLR2b, TLR3, TLR4, TLR5 and TLR7 are clear orthologues to the mammalian TLRs. Avian TLR1LA and TLR1LB emerged from TLR2 through double gene duplication early in avian evolutionary history. These duplications were parallel to duplication of TLR1 into TLR1, TLR6 and TLR10 in mammals. Avian TLR21 seems to be orthologous to TLR21 found in fish and amphibians, and TLR15 (phylogenically related to the TLR2 family) is probably unique to avian species (Brownlie and Allan 2011). Avian TLRs recognise similar ligands as their mammalian counterparts, e.g. TLR4 binds LPS (Keestra and van Putten 2008), TLR5 binds flagellin (Keestra et al. 2008), and TLR7 binds viral singl-stranded RNA (Philbin et al. 2005). All TLR genes have been identified so far only in the domestic chicken (*Gallus gallus domesticus*; Fukui et al. 2001; Iqbal et al. 2005; Leveque et al. 2003; Lynn et al. 2003; Philbin et al. 2005; Smith et al. 2004; Yilmaz et al. 2005). Sequences of most of the TLR genes are also known in the red jungle fowl (*Gallus gallus gallus*) and three other *Gallus* species (green jungle fowl, *Gallus varius*, Ceylon jungle fowl *Gallus lafayetti*, gray jungle fowl, *Gallus sonneratii*; Downing et al. 2010). Most of these TLRs were also predicted in zebra finch (*Taeniopygia guttata*; Warren et al. 2010) and domestic turkey (*Meleagris gallopavo;* Dalloul et al. 2010), especially thanks to the whole genome sequencing. But there is for example no precise description of TLR function in passerine except TLR4 (Vinkler et al. 2009).

TLRs are type I integral membrane glycoproteins characterized by the extracellular domains and cytoplasmic signalling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill 2000). The TIR domain is evolutionarily highly conserved in a wide range of taxa from plants to animals and signalization through this domain results in inflammatory response (Temperley et al. 2008). Molecular variability in protein TLRs structures, especially in their ligand binding sites of the extracellular domain, might significantly influence host resistance to various diseases or disposition to autoimmune damage (Vinkler and Albrecht 2009). The TLR extracellular domain contains leucine-rich repeat (LRR) motifs that form the horse-shoe shaped structure responsible for recognition of various ligands in different TLRs, ranging from proteins and lipoproteins to nucleic acids and saccharides. Only several X-ray crystallographic structures of the extracellular domain-ligand complex are solved in humans, mice or fish. These include TLR3 and a hydrophilic double-stranded RNA (Choe et al. 2005; Bell et al. 2005; Liu et al. 2008), heterodimers TLR1-TLR2 and hydrophobic triacylated lipopeptide (Jin et al. 2007) and TLR2-TLR6 and diacylated lipopeptide or TLR2 and lipotechoic acid (Kang et al. 2009). The protein 3D structure has been resolved also for TLR4 with its co-receptor MD-2 in association with LPS (Park et al. 2009) or endotoxin antagonist eritoran (Kim et al. 2007). There is no crystallographic structure of any of the TLR exodomains in birds.

I.4 Toll-like receptor 4 and its effect on phenotype

TLR4 is expressed into the outer cell membrane in a broad range of tissues and by various cell types (Iqbal et al. 2005). High expression of TLR4 has been detected mainly in macrophages and also in heterophils (Kogut et al. 2005a; Iqbal et al. 2005; Vinkler et al. 2009; Farnell et al. 2003). In most vertebrates TLR4 recognizes a wide spectrum of agonistic substances: conserved bacterial surface molecules such as LPS, fungal mannan, viral envelope proteins, protozoan glycoinositolphopholopids and also some DAMPs (for example heat-shock proteins; Akira et al. 2006). Like other TLRs also TLR4 consists of a horseshoe-shaped LRR-rich extracellular domain, transmembrane domain and a TIR domain (Kim et al. 2007; Park et al. 2009). The TLR4 exodomain consists of three sub-domains: the N-terminal, central and C-terminal subdomain. TLR4 forms a binding complex with a MD-2. The MD-2 dimerization sites are located in the concave surface of the N-terminal and central subdomains. At least in mammals, where this process is better known, the mechanism of LPS binding is rather complex. At first soluble Lipopolysacharide-binding protein (LBP) captures LPS molecules released from bacterial membranes and transfers them to the host cell membranes. Here is the LPS-LBP complex recognized by CD14 (Cluster of Differentiation 14). The structure of MD-2 forms a large hydrophobic pocket to which the LPS molecule is transferred from CD14. Simultaneously with LPS binding to MD-2 the complex is bound to TLR4, which then mediates dimerization of two TLR4-MD-2-LPS complexes (Park et al. 2009). Binding of the ligand to the extracellular domain, hence, causes rearrangement of the receptor complex that brings into close proximity two TIR domains, which leads to binding of specific adaptor proteins, mainly the cytosolic myeloid differentiation primary response protein 88 (MyD88), to the intracellular domain and initiation of the signalling cascade (Kim et al. 2007). This signalling pathway leads to activation of transcription factor NF-κB (Akira and Takeda 2004). This mechanism of LPS-induced signalling is very well known in mammals (described in human and mice) but although important similarities to mammals have been confirmed in birds (Kogut et al. 2007), the references concerning partial aspects of this issue are rather inconsistent. For example, the function and even existence of LBP in avian species is unsure. Kogut and colleges (2005a) show in their *in vitro* experiment that activation of chicken heterophils is dependent on the presence of LBP but in their study they used recombinant human LBP instead of the specifically chicken one. However, according to Keestra and colleges (Keestra and van Putten 2008) there cannot be any orthologs of LBP identified in the chicken genome. It is, nonetheless, well evidenced that also in birds TLR4 plays a key role in LPS detection (Farnell et al. 2003a; Keestra and van Putten 2008; Vinkler et al. 2009), though in a species-specific manner with birds being presumably functionally closer in LPS responsiveness to mice than humans (Lien et al. 2000; Keestra et al. 2008).

Although the structure and function of TLR4 is conservative as in many other TLRs, genetic polymorphism in TLR4 is frequent both on the interspecific and intraspecific levels. Vinkler at al.

(2009) has shown that there is about 74% homology in the amino acid sequence between zebra finch and chicken. The variability present in the sequences may be partially adaptive. Vinkler at al. (2009) identified 75 positively selective sites in the gene in vertebrates. Nearly all of these sites occur in TLR4 extracellular domain which is responsible for LPS binding. On the other hand, 116 from 264 significantly negatively selected sites were found in the cytoplasmatic TIR domain. These results are consistent with other similar findings in mammals (Smirnova et al. 2000) and supported the hypotheses that extracellular domain is exposed to higher selective pressure of pathogens. Relationship between TLR4 polymorphism and susceptibility to several inflammatory diseases as, e. g., atherosclerosis (Kiechl et al. 2002), endotoxine-associated asthma (Arbour et al. 2000) and malaria (Ferwerda et al. 2007) in humans, resistance to *Mycobacterium tuberculosis* in mice (Abel et al. 2002) and resistance to salmonellosis in chickens (Leveque et al. 2003) have been shown.

The main transcription factor for TLR4-mediated gene expression is NF-κB (Mogensen 2009). Activation of NF-κB leads to expression of different transcripts encoding proinflammatory and Th1 cytokines (Kogut et al. 2005b; Bliss et al. 2005). Interestingly, activation of NF-κB does not require new protein synthesis, which permits a rapid response (Ashley et al. 2012). There are also some others transcription factors which are activated during the pro-inflammatory response, but these are involved in other signalling cascades then the TLR ones. It has been shown, that while LPS activates only the transcription factor NF-κB through binding on TLR4, the whole bacteria engage multiple surface receptors, not only TLRs and activate multiple signalling cascades involving multiple transcription factors (Bliss et al. 2005). The result of the signalling cascade is start-up of expression of inflammatory cytokines, such as e.g. interleukin-1-beta (IL1β), IL6, IL12, IL18, tumor necrosis factor-α (TNFα), interferon-γ (INFγ), chemokines (CCL2, CXCL8) and other various costimulatory proinflammatory molecules (prostaglandins). Effector mechanisms of phagocytosis are the main contributors to tissue damage and that is why these mechanisms must be regulated and suppressed in the course of inflammation (Ashley et al. 2012).

TLR4-associated LPS-induced inflammation affects and stimulates also the activity of adaptive immune defence. Naive T-helper (Th0) cell, Th cell which have never been exposed to their antigen, can differentiate into several types of effector and regulatory cells – proinflammatory Th1, Th17 and Th22, anti-inflammatory Th2, regulatory T cells (Treg) and Th3 and Th9 the function of which is not fully clear (Kaiser 2010). Each of these cell types is characterised by a specific cytokine profile. Th1 cells are generally known as inflammatory cells stimulated by IL-12. Their main effector cytokine is INFγ. Th0 development into Th2 cells is triggered by IL-4 and the main Th2 products are IL-4, IL-5 and IL-13. Th3 cells grow from Treg cells in the presence of IL2 while in vitro is their differentiation enhanced by TGFβ, IL-4 and IL-10. Th3 cells protecting mucosal surfaces develop in the environment rich in TGFβ and IL10 and inhibit development of Th1 and Th2 immunity (Sakaguchi et al. 2006). Th0 cells differentiate into Th9 cells in environment

combining TGFβ and IL-4. They secrete mainly IL-10 and IL-9, which is a cytokine involved in immunity against intestinal worms and allergy reactions. It is assumed that these cells suppress differentiation of Th1 cells (Veldhoen et al. 2008; Soroosh and Doherty 2009; Veldhoen 2009). Surprisingly, despite their important function in inflammation and autoimmunity, the subpopulation of Th17 cells was discovered only very lately, in 2005 (Harrington et al. 2005; Park et al. 2005). Cytokine factors which play a role in differentiation of Th17 cells are especially IL-6, TGFβ; important are probably also IL-1β, IL-21, and IL-23. The effector cytokines associated with this cell type are IL-17, IL-21 and IL-22. These cells are important in host defence against extracellular pathogens. Aberrant regulation of Th17 cells may cause multiple inflammatory diseases and autoimmunity disorders (see above; Harrington et al. 2005; Park et al. 2005; Stockinger and Veldhoen 2007). The last subpopulation of T hepler cells is the subpopulation of Th22 cells. These cells have been also described recently and their function is difficult to generalize. They are neither inducing an anti-inflammatory nor really proinflammatory response. Th22 cells secrete IL-22 and TGFα and they are involved in skin inflammation where IL-22 enhances wound healing (Eyerich et al. 2009). Given the lack of any specific markers for many of these cell types, still a lot of things remain unclear about their biology and functioning. LPS-induced TLR4-mediated inflammatory response might contribute especially to development of Th1, Th17 and possibly Th22 responses with potential regulatory effects of the other types of T helper cells.

I.5 Infection, health and condition of an individual

During various pathogen challenges an appropriate function of TLR4 after stimulation with pathogen-derived LPS may be the key signalling component inducing an adequate (type, magnitude and timing) immune response to the pathogen incursion ensuring good health of an individual. TLR4 genotype may be an important factor effecting the magnitude of the immune response. It has been shown both in mammals and birds that conspecific individuals expressing different variants of TLR4 exhibit a distinct immunophenotype after LPS or other pathogen treatment (see, e.g., Leveque et al. 2003; Ferwerda et al. 2007). It may be assumed that genotypes ensuring a stronger pro-inflammatory activity may ensure faster parasite clearance. In studies dealing with inbred lines it may be, however, difficult to differentiate the effect of a particular genetic variability from the general effect of the whole genetic background of the line. Except of genetic factors, also ecological factors have an important impact on the intensity of the pro-inflammatory response. This has been repeatedly demonstrated in studies investigating avian pro-inflammatory responsiveness after cutaneous experimental challenge with phytohaemagglutinin (PHA). For instance, the intensity of the PHA swelling response may be modulated by stress prior the treatment (Lazarevic et al. 2000; Ewenson et al. 2003; Morales et al. 2006) as well as by temperature or food abundance (Lifjeld et al. 2002). If possible, it is, therefore, important to keep all the examined individuals under the same conditions in the course of the same study. The response may also vary between sexes (Cheng and Lamont 1988; McGraw and Ardia 2005) and

fluctuate in time (e.g. as a response to seasonal changes in animal activity; Moller et al. 2003; Bourgeon et al. 2006). Reported is temporal variation in PHA responsiveness in the course of a day (Navarro et al. 2003; Martinez-Padilla 2006). It has been also proven that the intensity of inflammation is age-dependent, being higher in adults then in young ones but impaired in senescent individuals (Lavoie et al. 2007). These effect of ecological factors on inflammation do not seem to be specific to PHA-induced responses. Similarly, also for LPS-triggered response it has been found that stress effects the cellular performance (Bailey et al. 2009) and that there is important time variation in the immune responsiveness (Hegemann et al. 2013).

Although active inflammation is undoubtedly one of the most efficient mechanisms in defence against early pathogen invasion, if it persists for a long time, it can lead to tissue damage and immunopathology (Graham et al. 2005). There are even diseases during which immunopathology caused by the inflammatory response is more serious than damages caused by pathogens (e.g. cerebral malaria caused by *Pasmodium* ssp., tuberculosis caused by *Mycobacterium tuberculosis*, meningitis and pneumonia caused by *Streptococcus pneumoniae*, *Neisseria meningitides*; Ashley et al. 2012). Another serious problem associated with overreaction in immunity is that the local inflammation caused by wound or infection may rapidly spread to the periphery and there evoke a systematic response. This systematic response is triggered by pro-inflammatory cytokines especially TNFα, IL1β and IL6. These cytokines are released to the blood flow and activate secretion of acute phase proteins from liver and also activate fever and sickness behaviour (Ashley et al. 2012; Medzhitov et al. 2012). Sickness behaviour can be characterized as a dramatic change in behaviour, resulting in fatigue, anorexia, social withdrawal, fever and sleep alteration. This behaviour is generally considered to be adaptive. For example fever enhances an immune function and so makes from the host a less suitable niche for the pathogens (Pecchi et al. 2009). However, when the inflammation exists without any serious infection then it is maladaptive and usually leads to inflammatory diseases and immunopathology. That is why balance between immunity and immunopathology is essential (Graham et al. 2005; Graham et al. 2011; Leonard and Maes 2012), overreaction on any kind of stimulation is inappropriate (Viney et al. 2005) and in case of inflammation leads e.g. to too severe oxidative damage of the host tissues (Sorci and Faivre 2009; Ashley et al. 2012). The protective effect of inflammation may be also tissue-specific (Cunningham et al. 2009). That means that the whole process of inflammation must be properly timed and regulated. Also the restoration of tissues must occur immediately after the acute phase of inflammation to reduce damage but not before the parasite clearance (Sorci and Faivre 2009). In humans the inflammatory diseases (especially systematic inflammatory diseases) and also stress are associated with increasing risk of diabetes, obesity, pre-term birth, stroke, coronary artery diseases and atherosclerosis (Gibson III and Genco 2007; Dimsdale 2008). To avoid this, several regulatory mechanisms involving tolerance evolved. For instance, during the acute inflammatory response the activated state of macrophages may be, under specific conditions, only transitional and change into

a state of tolerance characterized by diminished signalling, repressive chromatin modifications and alternative gene expression program (Ivashkiv 2011). As a classic example can be named the endotoxin tolerance, a state when organisms exposed to suboptimal levels of endotoxin (e.g. LPS) enter a transient unresponsiveness and are unable to react on further contacts with LPS (Prendergast 2008; Biswas and Lopez-Collazo 2009). Endotoxin tolerance has also an important impact on sickness behaviour (Prendergast 2008). It seems that this type of pathogen tolerance evolved to save energy, decrease tissue damage and thus increase survival in energetically challenging conditions or in cases in which promoted inflammation would not improve pathogen clearance.

I.6 Prospects for investigation of TLR4 evolution in immune defence

No matter if a higher or lower pro-inflammatory response is appropriate to ensure individual health, genotypes that enable the appropriate immune response that improves recovery and health in the particular ecological context should be favoured by natural selection (Clayton 1997; Sorci and Faivre 2009; Ashley et al. 2012). The genetic and functional variability known in TLR4 and other TLRs on the interspecific level (Keestra et al. 2007; Keestra et al. 2008; Walsh et al. 2008; Keestra and van Putten 2008; Alcaide and Edwards 2011) suggests that there may be some evolutionary relationship between health / immunofenotype and TLR genotype. Although reasonably lower, similar variability can be revealed also among conspecifics (though the functional aspect of the relationship is rarely studied on the intraspecific level in natural host populations; Leveque et al. 2003; Cormican et al. 2009; Alcaide and Edwards 2011; Adelman et al. 2013). In the present thesis I focused on the effect of TLR4 genotype on pro-inflammatory responsiveness in the study population of great tit nestlings. Following the pieces of knowledge already gathered by other members of our team (especially those reported in the thesis by Hana Bainová) I focused on a single candidate amino acid substitution in TLR4: Q549R. This single nucleotide polymorphism (SNP) was predicted to remarkably alter the structural features of the resultant protein and it is frequent in our population, which suggests the effect of current positive or balancing selection. Most importantly Bainová (2011) reports the effect of this substitution on great tit sexually-selected colouration. Considering the Indicator hypothesis explaining the signalling function of ornamentation (Hamilton and Zuk 1982), this association suggests the existence of a relationship between Q549R genotype and health in this species. The basis and mechanism of such a relationship between TLR4 genotype and health remains, however, unknown. In the present study I aimed at revealing a bit of this intriguing puzzle. I focused on pro-inflammatory responsiveness to a single model ligand – the LPS, in two types: originating from bacteria *Escherichia coli* and *Salmonella enterica*. If there is any on-going selection, I expect that the allele frequencies would be out of the Hardy-Weinberg equilibrium. If there is any association between Q549R and health-affecting pro-inflammatory immunophenotype I expect to find the bearers of one allele responding more than the bearers of the other allele to the experimental challenges (tested *in vitro* and *in vivo*).

To be able to interpret the test results correctly I needed to investigate or confirm some of the aspects of the immune response to LPS in great tits, especially the type of the immune response and the systemic effect of the local subcutaneous treatment. I also predicted that if there was any selection on TLR4 genotype, the Q549R substitution may have some effect on condition, health and growth of the nestling

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II. Aims and hypotheses

Task 1: Describe frequency of the A1646G (Q549R) substitution in a population of great tit nestlings

H1: Due to selection the distribution of genotypes will be out of the Hardy-Weinberg equilibrium

Task 2: Investigate the course of the immune response to LPS on morphological (swelling response) and molecular (cytokine expression) level and assess its influence on individual condition

H2: LPS induces pro-inflammatory immune response that affects condition

H3: Different types of LPS differ in their capacity to trigger the inflammation

Task 3: Examine the effect of the Q549R substitution on body condition and pro-inflammatory responsiveness

H4: Presence of the Q549R substitution has effect on general condition of the nestlings

H5: Presence of the Q549R substitution influences the immune responsiveness to LPS

III. Material and methods

III.1 Study area

This study was conducted on a great tit population breeding in Čimický and Ďáblický háj woods, Prague, Czech Republic, EU (N 50° 7'39.44"- 50° 8'25.39", E 14°26'0.57" - 14°28'31.50", ~ 315-360 m above the sea level, total area ca. 0.9 km^2). Data and material sampling were performed in the period of two study seasons in years 2011 and 2012, in each year between 1st April and 25th June. The habitat represents a temperate deciduous forest (formed dominantly by oaks *Quercus petraea* and *Q. robur*, supplemented locally with European hornbeam *Carpinus betulus*, Linden tree *Tilia cordata*, common beech *Fagus sylvatica*, maples of genus *Acer* and silver birch *Betula pendula*) with minor admixture of coniferous trees (European larch *Larix decidua*, silver fir *Abies alba*, pines of genus *Pinus*, and Norway spruce *Picea abies*). Most of the trees in the woods are between 60 and 120 years old and were artificially planted. In 2007 Čimický and Ďáblický háj woods received a certificate from the international organization Forest Stewardship Council (FSC), which means that these woods fulfil ecological criteria of the environment-friendly management. Some parts of the wood are even excluded from all artificial management and the usage of chemicals and clear cutting are restricted in the entire area (EVIS http://envis.praha-esto.cz/(mlijti45awilrw55ay3ydoy3)/zdroj.aspx?typ = 2&Id = 81922&sh = -1703724117).

Fig. 1: Geographic position of the locality Čimický and Ďáblický háj woods in Prague

In 2010 244 nest boxes have been installed in this area in a regular grid with 50 m distances between individual nest boxes (based on the previously designed GPS coordinates). No nest boxes were installed into a 50 m-wide marginal zone around the forest to minimise the possible edge effect (Wilkin et al. 2007). Each nest box was of the same design, constructed of the same material. All nest boxes were placed between 2 and 3 m above ground with entrance oriented to south. In 2011 60 of our nest boxes were inhabited by the great tit, 79 by the blue tit (*Cyanistes caeruleus*), 1 by the crested tit (*Lophophanes cristatus*) and 8 by the nuthatch (*Sitta europaea*). In 2012 the avian population breeding in the nest boxes further increased, comprising of 98 great tit pairs, 82 blue tit pairs, 1 crested tit pair and 14 nuthatch pairs.

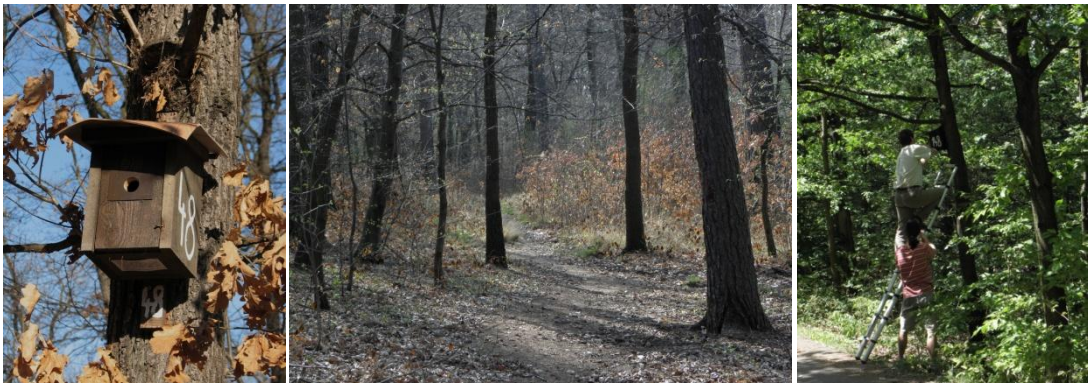

Fig. 2: Study habitat (photo H. Bainová, J. Vinklerová)

III.2 Study species: Great tit, *Parus major*

The Great tit, *Parus major* Linnaeus, 1758, is a small sized (ca. 18g) passerine bird belonging to the family Paridae, (Gill et al. 2005). The plumage is basically blue-green above and yellow below, with a white-cheeked black head and a black central stripe on the breast and belly (Cramp et al. 1993). Though both sexes are closely similar, the size of the stripe is sexually dimorphic and serves for sex discrimination. In general appearance the females are duller than males. Nestlings grow basically the same-coloured juvenile plumage that is, nonetheless, separable from the adult plumage by the saturation of the black and yellow contour feathers. Birds in their first year of life can be distinguished based on coloration of great primary wing covers (Jenni et al. 2011). Birds in their first calendar year may be, therefore, distinguished from older birds based on the colour of the un-moulted great wing covers (Jenni et al. 2011).

The species is distributed all over the Palearctic region including north-west Africa, Middle East, and also in India and south-east Asia. There are 11 races distinguished in west Palearctic and in total 34 subspecies are reported in the literature (Cramp et al. 1993; Šťastný et al. 2011). Out of these, the nominate *P. m. major* is distributed throughout most Eurasia, including the Czech Republic (Šťastný et al. 2011). In west Palearctic breeds this species from higher to lower middle latitudes, continental and oceanic, with July isotherms from 12 to 32°C (Cramp et al. 1993). In the

Czech Republic the species is abundantly breeding across the board with total estimated breeding population counting 3-6 million breeding pairs (Šťastný et al. 2006). Although able to ascend mountains great tits are more numerous in lowlands, disliking the pure coniferous forest and preferring mixed types or the pure deciduous forest or fragmented vegetation and scattered trees. Great tits are well able to live in human neighbourhood, such as gardens and city parks where trees or shrubs are present. The species is resident in the central part of its range (where the Czech Republic is situated) and only short-distance movements are frequent in this region (Cepák et al. 2008).However, in autumn when the population is high, large numbers of individuals may move more than 100 km far. Within these fluctuations birds from the Czech Republic move mainly southwest while in the same season birds from northeast Europe are reported in the Czech Republic. The sedentary part of the population (>80%) tends to form flocks during winter that roam through the countryside (Cepák et al. 2008). European populations of the great tit are well connected, which is mirrored by their relative genetic homogeneity (Kvist et al. 1999).

The European great tit population is contemporarily estimated to 37,000,000-52,000,000 individuals (European Bird Census Council and Hagemeijer 1997) and in the Czech Republic the breeding population counts approximately 3,000,000-6,000,000 pairs (Šťastný et al. 2006). Annual mortality is 70-90% in nestlings and 1st-year old birds and about 50% in adults (Cramp et al. 1993). The food of the great tit is composed of variety of insects (mainly in spring and summer), seeds and fruit (mainly in winter; Cramp et al. 1993; Šťastný et al. 2011).

Great tits are typical territorial tree-hole breeders with up to three clutches per year (only a part of breeding pairs manages more than one clutch in the breeding season; Cramp et al. 1993; Šťastný et al. 2011). In the Czech Republic the species willingly inhabits artificial nest boxes (Šťastný et al. 2011), which is a convenient feature of the species as a model organism for various biological studies. Great tit represents a socially monogamous species with moderate rates of extra-pair paternity, ranging from 3% to 20% of nestlings being sired by extra-pair males in 3%-58% nests in the population (Lubjuhn et al. 2007, Patrick et al. 2012). In Palearctic including the Czech Republic the breeding season of this species starts in April. In our study population the egg-laying period started on 1st April, egg-lying took 6-13 days and was followed by 12-to-15-day long incubation. Though great tit clutches may contain between 3 and 18 eggs (Cramp et al. 1993), in our study population we recorded only clutches of 6-13 eggs. Eggs are incubated 12-17 days by the female alone and in the first clutch the incubation starts at the time of laying the last or last but one egg in the clutch (Cramp et al. 1993). This information can be used to predict the earliest possible hatching date of the nestlings in the brood, which is convenient for planning the control nest visits (see below). In our study population all nestlings were hatched within 1 to 3 days after the hatching of the first nestling in the brood. Both parents then feed the nestlings, mainly with small invertebrates. The fledging period lasts 16-22 days and the parents take care of their offspring for

further 6-8 days after leaving the nest. Young birds start breeding in second calendar year of their life (Cramp et al. 1993).

Fig. 3: One-day old (left) and 16-day old (right) nestlings of the great tit; individual colourful marking is visible on the left photograph (photo H. Bainová, J. Vinklerová)

III.3 General field procedures

In both years 2011 and 2012 the same setup of field procedures has been adopted. Before the breeding season (in winter) all nest boxes were cleaned and maintained. From the beginning of April all nest boxes were controlled in one-week intervals to detect the onset of the breeding. During these controls we recorded any signs of breeding, i.e. presence of nest rudiment, complete empty nest or nest with eggs. If there were no eggs in the nest, the nest box was visited in about a week time again to verify the breeding event. If any eggs were detected, then the nest box was visited again in the following 4-10 days (depending on the contemporary egg number) to learn the ultimate number of eggs in the nest. Thereafter, the approximate earliest date of hatching was estimated (calculated as the date 10 days after laying the final egg in the brood, 1 egg was assumed to be laid per day; Šťastný et al. 2011). Since this anticipated hatching date one control was performed per day (in the afternoon) until all young hatched. After finding a newly hatched nestling in the nest the date and time of the control were noted and the nestling was individually marked on the back with a permanent marker (Visible Implant Elastomer Tags, Northwest Marine Technology, Inc, Shaw Island, WA, USA) to enable later discrimination. Then the nestling was weighted with a digital scale (Pesola PPS200, 200 g, d = 0,02 g). On day 1 post-hatch of the last nestling hatched in the nest the cross-fostering was performed by exchanging the whole brood with a brood from another nest containing nestlings of the same age (±1 day). After all offspring hatched and were cross-fostered no more visits of the nest occurred until the first nestling was 15 days-post-

hatch old. Then every nestling was ringed with a darkened steel ring of the Czech Ringing Station (N Museum Praha). At the age of 15 days post-hatch (individually for each nestling according to its hatching date) three to six selected nestlings out of each nest (the number depending on the size of the brood and precise date of hatching of the individual nestlings) were weighted with the digital scale and their tarsus length was gauged using a digital calliper Kinex, type 6040.2 (accuracy 0.01 mm). In these nestlings blood samples (50-100 μl) were collected aseptically from the jugular vein for haematological examination, oxidative burst analysis and genotyping and LPS-induced inflammation test were performed (please see below). Later, on day 16 post hatch the swelling responses in these nestlings were measured and their weights and tarsus lengths were recorded. Thereafter, digital image of ornamentation was taken in each investigated nestling and a feather sample for ptilochronological analysis was collected (these data are not included in the present study). The growth rate was calculated as the difference between tarsus length on day 15 and 16 ($\Delta L = L_{16}-L_{15}$). During no manipulation event the nestlings spent more than 30 minutes out of the nest. We followed only the first breeding attempt of each breeding pair, no second or replacement clutches were included into our dataset.

III.4 Analysis of in vivo immunological responsiveness to bacterial lipopolysaccharide (LPS)

In three to six nestlings per brood (the number depending on the size of the brood and nestlings' hatching dates) the immune responsiveness to bacterial lipopolysaccharide (LPS) was measured at the age of fifteen days after hatching. LPS is a superantigen that induces infiltration and aggregation of inflammatory cells at the site of injection (Parmentier et al. 1998). The test was performed in the same way as the PHA skin-swelling test that is commonly used in ecological studies (Smits et al. 1999), for similar approach see Berthouly et al. (2008). The spot in the centre of the wing web (patagium) was marked and the thickness of the wing web was measured with a digital thickness gauge with accuracy to 0.01 mm (product No. 547-313, Mitutoyo, Kawasaki, Japan). Then we injected the wing web of the nestlings with 0.01 mg of LPS dissolved in 0.02 ml of phosphate buffered saline (DPBS, product No. D-5652, Sigma-Aldrich, St Louis, MO, USA). In each nest one or two nestlings were injected with LPS isolated from *Escherichia coli* serotype O55:B5 (LPS_{Ecol}, product No. L2880, Sigma-Aldrich) and one or two nestlings were injected with LPS isolated from *Salmonella enterica* serotype *enteritidis* (LPS_{Sent}, product No L6011, Sigma-Aldrich). 27 nestlings were injected with pure DPBS and served as controls. All inocula were injected using a microsyringe (type 1705 RN, 50 uL, Hamilton, Reno, NV, USA) with RN needle (26s/51/pst2/tap, S, 6/pk, Hamilton) subcutaneously into the marked place of patagium skin. After the inoculum application the time of the injection has been recorded and the nestling was returned back into its nest. At a selected time point (see below) the swelling response was measured by a repeated measurement of the wing web thickness with the digital

thickness gauge. It has been previously shown that in chickens the swelling response to LPS is highest 4 hours following the injection (Parmentier et al. 1998). On the contrary, in great tit nestlings in Switzerland (Berthouly et al. 2008) did not show any response after 4 or 12 hours but they reported the strongest response 24 hours following the treatment. To verify this effect in our study population we performed a time experiment. The swelling response was measured 3 hours (± 10 minutes) after the LPS injection in 11 nestlings, 6 hours (± 30 minutes) after the LPS injection in 75 nestlings, 24 hours (± 1 hour) after the LPS injection in 186 nestlings, 48 hours (± 2 hours) after the LPS injection in 13 nestlings and 72 hours (± 2 hours) after the LPS injection in 2 nestlings. Based on the results (please see the Results) only the 6-hour and 24-hour time periods have been adopted for further experiments. During each measurement the thickness of the skin wing web tissue was measured in the marked spot by three independent measurements. The repeatability of the three successive measurements was considerably high (r = 0.97; calculated on 525 measurements of left wing webs prior and post treatment injection, all performed in triplicates). For further analyses the mean of these three measurements was used. A greater swelling was assumed to reflect a stronger inflammatory response (Parmentier et al. 1998). Immediately after measuring the thickness of the wing web swelling a biopsy sample of the tissue from the middle of the inoculation site has been collected with a tissue corer 2.0 mm in diameter (FST 18035-02, Fine Science Tools GmbH, Heidelberg, Germany). In each bird also a control biopsy sample was collected from the opposite untreated (control) wing web. Both biopsy samples were immediately placed into RNALater (Qiagen, Hilden, Germany), stored overnight at +8°C and frozen to -80°C on the next day. These biopsy samples were later used for qPCR assessment of the cytokine expression (see below). After performing all necessary measurements the nestlings were placed back into their nest.

Fig. 4: From left: manual measurement of wing web, biopsy collection, and healed wounds 48 hours after biopsy sampling (photo: J. Vinklerová)

III.5 Measurement of cytokine mRNA expression during LPS-induced inflammation

To analyse cytokine expression in the LPS-treated tissues of the experimental individuals we used the biopsy samples stored in RNALater at -80°C. From these biopsy samples (tissue samples after the LPS-treatment as well as control samples) total RNA was isolated using High Pure RNA Tissue Kit (Roche Applied Science, Basel, Switzerland). Firstly, we prepared a mixed sample from 10 individual samples (both 6 and 24 hours after LPS stimulation) and performed a reverse-transcription of the total RNA into cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and random AP primers according to the manufacturer's instructions. In this cDNA sample we tried to identify partial coding DNA sequences (CDS) of the following cytokine genes: *IFNγ*, *IL-1β*, *IL-6*, *IL-10*, *IL-18*, *TNFSF15*, *CXCLi2*. In each case a polymerase chain reaction (PCR) was performed using 1.00 µL PCRbuffer (10x), 0.30 µL $MgCl_2$ (50mM), 0.20 µL dNTPs (10mM), 0.25 µL gene-specific forward primer (10µM), 0.25 µl gene-specific reverse primer (10µM), 0.30 µL Taq W1 polymerase 5 U/µL (Cat. No. 18038-026, Invitrogen, Carlsbad, CA, USA) and 1 µL of template cDNA (10 ng/µL). The primer sequences and primer-pair PCR features are summarised in Tab. 1.The PCR programme was as follows: 1) 94°C for 3 min., (2) 94°C for 45 sec., 3) gradient 51-62°C for 30 sec., 4) 72°C for 1:30 min.) x 35 cycles, 5) 72°C for 10 min., 6) 4°C hold. All PCR products were visualised by electrophoresis on 1% agarose gel with Sybergel (Invitrogen).

Tab. 1: Primer sequences and primer-pair PCR features used for amplification of the great tit cytokine genes.

Gene	Primer name	Tm	Primer sequence	Pred. product size
CXCLi2	PaMaCXCLi2-1-F	63.9°C	GGCTGTCCTGGCTCTTTTC	216 bp
	PaMaCXCLi2-1-R	64.4°C	TCCAAGCACACCTCTCTGC	
IFNγ	PaMaIFNg-1-F	62.6°C	GCCTTCCAAACTTACAGCTTG	482 bp
	PaMaIFNg-1-R	62.1°C	AGCCTCTGAAACTGGCTTCT	
IL-1β	PaMaIL1B-1-F	62.5°C	GCTGATAGTGACCTTGGCAG	545 bp
	PaMaIL1B-1-R	65.6°C	GCAATGTTCACCTGGTCTGG	
IL-6	PaMaIL6-1-F	66.2°C	AACAACCTCAACCTCCCCAAG	402 bp
	PaMaIL6-1-R	66.5°C	TCAAGCACTGAAACTCCTGGTG	
IL-10	PaMaIL10-1-F	64.8°C	CTACATGGAGGAGGTGCTGC	203 bp
	PaMaIL10-1-R	64.6°C	CCTTGTAGATTCCGTTCCTGTTC	
IL-18	PaMaIL18-1-F	61.8°C	ACAGACCAGGAGATGCAATC	246 bp
	PaMaIL18-1-R	62.5°C	GGAGCAGCAAGATGTAAATGTC	
TNFSF15	PaMaTNFSF15-1-F	62.9°C	AGGGGCTCTCACTTTCTGAA	372 bp
	PaMaTNFSF15-1-R	61.3°C	GTTCTCACTGAGGGTCTTGG	

As only *IL-1β*, *IL-6*, *IL-18*, *TNFSF15* and *CXCLi2* could be amplified from our mixed cDNA sample, we started further work only with these genes. All detected products of the correct size (originating from any annealing temperature) were excised from the gel and weighed. For DNA extraction we used QIAquick Gel Extraction Kit (Cat. No. 28704, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified products were then ligated into pTARGET expression vector S1 (Promega, Fitchburg, WI, USA) by incubating the ligation reaction prepared according to the manufacturer's manual at 4°C overnight. Vectors containing the cytokine inserts were then cloned into JM109 competent cells (Promega), seeded on IPTG-X-gal ampicillin LB plates and cultured overnight at 37°C. From 3-6 positive colonies per plate colony PCR were performed as follows. Part of the colony was transferred to a new LB plate while the rest was pipetted into 50 µL of PCR grade water. The sample was heated to 95°C for 5 min., spun at 13000 g for 10 min. and 1 µL of the supernatant was used as a template in PCR reaction containing 5 µL of PCR Buffer (10x), 1 µL of dNTPs, 1.5 µL of $MgCl_2$, 1.25 µL of primer T7 (10µM, sequence: TAATACGACTCACTATAGG), 1.25 µL of primer pTARGET-Rev (10µM, sequence: TTACGCCAAGTTATTTAGGTGACA), 0.3 µL of Taq polymerase (Invitrogen), 2.5 µL W1 solution (Invitrogen) and 36.2 µL of water. The PCR conditions were: 1) 94°C for 3 min., (2) 94°C for 45 sec., 3) 53°C for 30 sec., 4) 72°C for 1:30 min.) x 30 cycles, 5) 72°C for 10 min., 6) 4°C hold. In all 5 genes we obtained vectors of an appropriate size of the insert as confirmed by gel electrophoresis. Two colonies per gene were then selected for sequencing, transferred into LB medium (2.5 mL with 5 µL of ampicillin) in 5mL universals and cultured overnight at 37°C in shaker. Out of these the DNA was purified using the QIAprep Spin Miniprep Kit (Cat. No. 27106, Qiagen) following the manufacturer's instructions. For sequencing with T7 primer (5 µL of the primer with 5 µL of DNA) we used the commercial service of GATC Biotech. As confirmed by BLAST analysis, in all cases we obtained sequences of orthologues of the cytokines in focus. The great tit sequences of *IL-1β*, *IL-6* were later used to design primers and probes for quantitative PCR (qPCR; see Tab.2).

Tab. 2: Sequences of primers and probes used for qPCR in IL-1β and IL-6.

Gene	Primer name	Tm	Primer sequence
IL1β	PaMaIL1B F	59,7°C	CTTGGCAGCTTCTTGGATGATATTT
	PaMaIL1B-Fw	57,5°C	TGGCAGCTTCTTGGATGATATTT
	PaMaIL1B R	59,3°C	AACGGGTGGCCTGGTATAACT
	PaMaIL1B-Rev	59,8°C	AGTGTAGCGGAAAACGGGTG
IL6	PaMaIL6F	57,7°C	CTGGCACGTACCCTAAGAGAGAT
	PaMaIL6-Fw	56,9°C	GAGATGCTGATCAATCCCAAAGA
	PaMaIL6R	57,9°C	GCTTTGTGTGAGGGATTCC
	PaMaIL6-Rev	58,2°C	GAGGATGAGATGGATGGTGATTTTC

Gene	Probe name	Tm	Probe sequence
IL1β	PaMaIL1B -Probe	66,6°C	FAM-TGAGCCCATCTCCTTCCAGTGCATC-BBQ
IL6	PaMaIL6-Probe	66,9°C	FAM-TGATCATCCCCGATGCAGCTACCC-BBQ

There are two common approaches how to quantify gene expression. The first one is quantification relative to a housekeeping gene. The second one is absolute quantification which is slightly less sensitive to changes in cellular composition of the tissue due to infiltration of novel cell types and, thus, more appropriate for analyses of inflammatory processes (Whelan et al. 2003). Based on this information we decided to use the absolute quantification.

For optimization of qPCR was used the same mixed sample of cDNA as for identifying partial sequences of the target genes. qPCR was performed using LightCycler® 480 Probes Master (Roche Applied Science) and primers and hydrolysis TaqMan probes designed according to the partial sequence of IL-1β and IL-6 of great tit (described above; summarized in Tab. 2). As the reporter we used FAM (6-carboxyfluorescein) at the 3'-end and as the quencher we used BBQ (BlackBerry Quencher) at the 5'-end. As a positive control was used plasmid DNA of the particular gene.

Firstly, we started with optimization pairs of primers and their annealing temperature. PCR was performed using 1.4 μL PCRbuffer (10x), 0.14 μL dNTPs (10mM), 0.21 μL gene-specific forward primer (10μM), 0.21 μL gene-specific reverse primer (10μM), 0.1 μL Taq (Roche Applied Science), 4.44 μL ddH_20 and 0.5 μL of template cDNA (10 ng/μL). The PCR programme was as follows: 1) 94°C for 2 min., (2) 94°C for 30 sec., 3) gradient 56-62°C for 30 sec., 4) 72°C for 30 sec.) x 35 cycles, 5) 72°C for 5 min., 6) 4°C hold. All PCR products were visualised by electrophoresis on 1% agarose gel with Sybergel (Invitrogen). Optimal temperature for all primers pair was 58°C. Then, we continued with optimization of primer concentration (0.2, 0.4, 0.6 and 0.8μM final concentration in master mix) and concentration of plasmid as a positive control (serial dilution from concentrated plasmid, plasmid diluted to 2×10^{-2} and then ten-fold serial dilutions down to plasmid dilution 2×10^{-10}). The qPCR was performed in a 96-well optical plate using 5 μL Probe Master Mix, 0.1-0.4 μL (depends on desired final concentration in master mix) gene-specific forward primer (10μM), 0.1-0.4 μL (depends on desired final concentration in master mix) gene-specific reverse primer (10μM), 0.25 gene specific probe, and 1.45-2.05 μL ddH2O (depends on desired final concentration of primers in master mix). The programme for qPCR was as follows: 1) 95°C for 10 min., (2) 95°C for 10 sec., 3) 58°C for 30 sec., 4) 72°C for 15 sec.) x 55 cycles, 5) 40°C for 10 sec. In all measurements a negative control had the same dynamics of reaction as the positive samples of lower concentrations. We repeated measurements with all new components but without better results. We tried even qPCR performed using intercalating fluorescence dyes EvaGreen instead of probes but with the same results.

III.6 Health assessment by haematological methods

The haematological analysis performed in this study follows generally the methods used previously by Vinkler et al. (2010b). In each investigated nestling 50-100 μL of blood were collected directly from the jugular vein by a sterile and heparinised 500 μL syringe. Out of this volume 15 μL were

pipetted (again the tip was heparinised) into 2985 μL of Natt-Herick staining solution (for formulation of the solution see Campbell and Ellis 2007), gently shaken and stored at +4-+10°C until the total white blood cells count (i.e. number of leukocytes per volume unit of blood, hereafter TWBC) analysis. TWBC was assessed according to Campbell and Ellis (2007) within 24 hours after sample collection. The cell numbers were counted in the Bürker's counting chamber (100 large squares for leukocytes were scanned). From another ca. 10 μl of blood two smears were prepared in the field: one single drop of blood placed on one side of the smear glass was spread along it to the other edge by a spreading glass. These smears were left to air dry and were kept on a dry place until their staining. The smears were not fixed in methanol before staining (which is usual in mammalian haematology) because methanol fixation decreases the stainability of basophil granules, which impairs correct identification of basophils (Robertson and Maxwell 1990; Dubiec et al. 2005; Vinkler et al. 2010b). Later in the laboratory all smears were stained with Wright-Giemsa modified staining solution (product No. WG128, Sigma-Aldrich, St. Louis, MO, USA) using the dip method according to the manufacturer's instruction. Briefly, approximately 50 ml Wright-Giemsa Stain was placed into a staining dish and another dish was filled with distilled water. The blood smears were dipped in a slide rack into the Wright-Giemsa Stain for approximately 30 seconds, then removed from the stain and placed into the water for approximately 10 minutes. Thereafter the smears were rinsed briefly in running water and air dried thoroughly. This staining method enables the recognition of all basic blood cell types under the light microscope and so it is convenient for assessment of leukocyte differential counts that may provide useful information about the general health of examined animals (see e.g. Ots et al. 1998; Horak et al. 1998; El-Lethey et al. 2003). All smears were scanned with Olympus CX-31 microscope (Olympus Corporation, Tokyo, Japan) under magnification of 1000x to count the proportions of lymphocytes, heterophils, eosinophils, basophils, monocytes and immature leukocytes within a sample of 110-140 leukocytes in the smear. Great tit leukocytes are morphologically similar to leukocytes described from other avian species. Individual leukocyte types were therefore identified according to Lucas and Jamroz (1961) and Campbell and Ellis (2007). The immature stages of any leukocyte type could not be distinguished properly and were, therefore, assigned to a single category. The repeatability of measuring the leukocyte frequencies was calculated based on a sample of 25 individuals for which counts were taken two separate times. It was $r = 0.91$ for lymphocytes, $r = 0.75$ for heterophils, $r = 0.88$ for basophils, $r = 0.53$ for eosinophils, $r = 0.63$ for monocytes and $r = 0.79$ for immature cells. The low values for repeatability in eosinophils, monocytes, heterophils and immature cells were caused by their low frequencies among blood leukocytes. The remaining blood volume of the samples collected from the nestlings was stored in 96% ethanol and was later used for preparation of DNA samples for TLR genotyping.

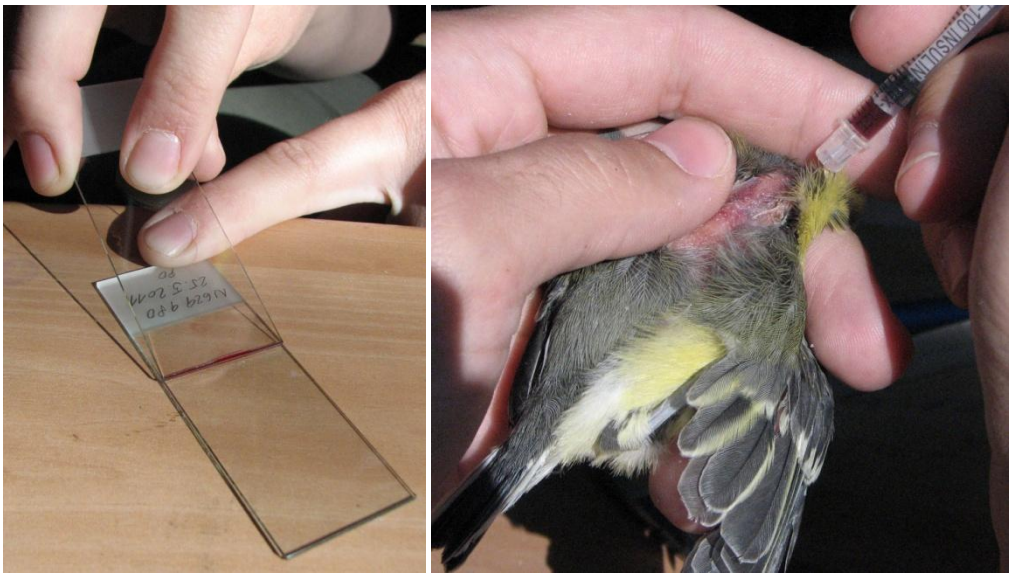

Fig 5: Field procedures (left: preparation of blood smear; right: blood collection from jugular vein; photo: J. Vinklerová)

III.7 Oxidative burst analysis

The oxidative burst was measured from the whole blood samples by using ABEL® Cell Activation test kit with Pholasin® and Adjuvant-K™ (Knight Scientific, Plymouth,UK). Pholasin is photoprotein from bioluminescent mollusc *Pholas dactylus* that emits light and enhances this chemiluminiscent signal in the presence of reactive molecules such as superoxide anion, singlet oxygen, hydroxyl and ferryl radicals, nitric oxide, hypochlorous and hypobromous acids, chloramines, bromamines, peroxynitrite and peroxidases. Traditionally, for the measurement of an oxidative burst optical probe such as luminol or cytochrome c have been used, but Pholasin® utilised in ABEL® Cell Activation test kit enhances the light signal 50-100 times more than the above mentioned probes. This property is very useful especially for avian blood samples. Avian phagocytic cells possess different bactericidal mechanisms than mammalian cells for which this kit was developed, so their reaction through the oxidative burst is weaker than in mammals. The suitability of Pholasin for avian studies was assessed and validated by Slid and Hõrak (2009). Our measurements were performed according to this study.

Measurements were performed on 261 whole blood samples according to the manufacturer's instruction with minor modification. The blood samples were stored in a fridge at 4°C before analysis and were processed within 3 hours after blood collection. 5 µL of whole blood were diluted in 500mL dilution buffer warmed to room temperature. Then 70 µL of reconstitution and assay buffer, 20 µL of Adjuvanst-K, 50 µL of Pholasin and 20 µL of the diluted whole blood were added to a microplate well and incubated at 37°C for 1 minute in luminometr (Immunotech LM-01T, Meopta, Přerov, Czech Republic). Thereafter, 40 mL of stimulant were added. As stimulants were used the same LPS as for the *in vivo* treatment (LPS_{Sent} and LPS_{Ecol}) but in twice a higher concentration (the response was too weak and difficult to detect). Thus, 0.04 mg of LPS was

dissolved in 0.04 mL of DPBS. From each nest blood from one individual was stimulated with LPS_{Ecol}, one individual with LPS_{Sent} and two individuals separately with both LPS_{Ecol} and LPS_{Sent}. Activation of an oxidative burst starts within the first 5s after addition of the stimulant. Manipulation with the samples after the LPS stimulation had to be very quick, so maximum four samples were analysed at once. Measurements of luminescence were recorded every two seconds for each sample for 3 minutes. We recorded two variables from each sample. The first one was the peak of oxidative reaction (maximal relative light unit; RLUmax) and the second one was the area under the curve (AUC). These variables were subsequently included in further statistical analyses.

III.8 TLR genotyping

The TLR4 CDS sequence has been described previously by Hana Bainová (Bainová 2010). In this study I focused only on the potentially functionally important A1646G polymorphism responsible for Q549R amino acid substitution (as described in Bainová 2010). The TLR4 A1646G genotyping was performed by using High resolution melting analysis (HRMA). HRMA detects SNPs in fragment of amplified DNA by comparing fluorescence as a function of temperature. Different alleles produce distinct melting curves that can be compared with reference sample. The original comparison of the melting curves with reference sequence (AA/AG/GG genotype) was performed by Anna Bryová (unpublished data). DNA was extracted from all blood samples with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Primer set for the analysis was designed using Primer3 web tool (http://frodo.wi.mit.edu/primer3/; Rozen & Skaletsky 2000) and primers were placed at a very short distance from the desired substitution A1646G. The sequence of the forward primer TLR4-HRM1614 is 5′-TAAGCTCCTGACCTTCGATCC-3′, its melting temperature is 56°C, length 21 bases and GC content 52,4%. The sequence of the reverse primer TLR4-HRM1690 is 5′-GCTGGTTCCTGCTGAAATCC -3′, its melting temperature is 56,4°C, length 20 bases and GC content 55%. The product length is 76 bases. HRMA was performed by using LightCycler® 480 KAPA HRM FAST PCR kit (Kapa Biosystems) according to the manufacturer's instruction with minor modifications. In each case HRMA was performed using 1.2 µL Master Mix (KAPA HRM FAST), 0.1 µL *Taq* DNA polymerase, 0.36 µL $MgCL_2$ (50mM), 0.6 µL intercalating fluorescent dyes EvaGreen®, 0.24 µL gene-specific forward primer (10mM), 0.24 µL gene-specific reverse primer (10mM), 0.24 µL dNTPs (10mM), 7.02 µL ddH_2O and 2 µL of template cDNA. Each sample was measured in duplicates in a white 96-well plate. The HRMA programme was as follows: 1) polymerase activation 95°C for 5 min., (2) denaturation 95°C for 10sec., 3) touchdown 68-62°C for 15sec., 4) extension 72°C for 10sec.) x 45 cycles, 5) melting curve 98°C for 1min, 40°C for 1min, 65°C for 1sec 6) cooling 40°C for 30s. Data analysis was performed by using the instrument software (LightCycler480 software1.5.0 SP3). Furthermore, the curves for each sample were also controlled manually. Measurements of samples with unresolved genotype were repeated.

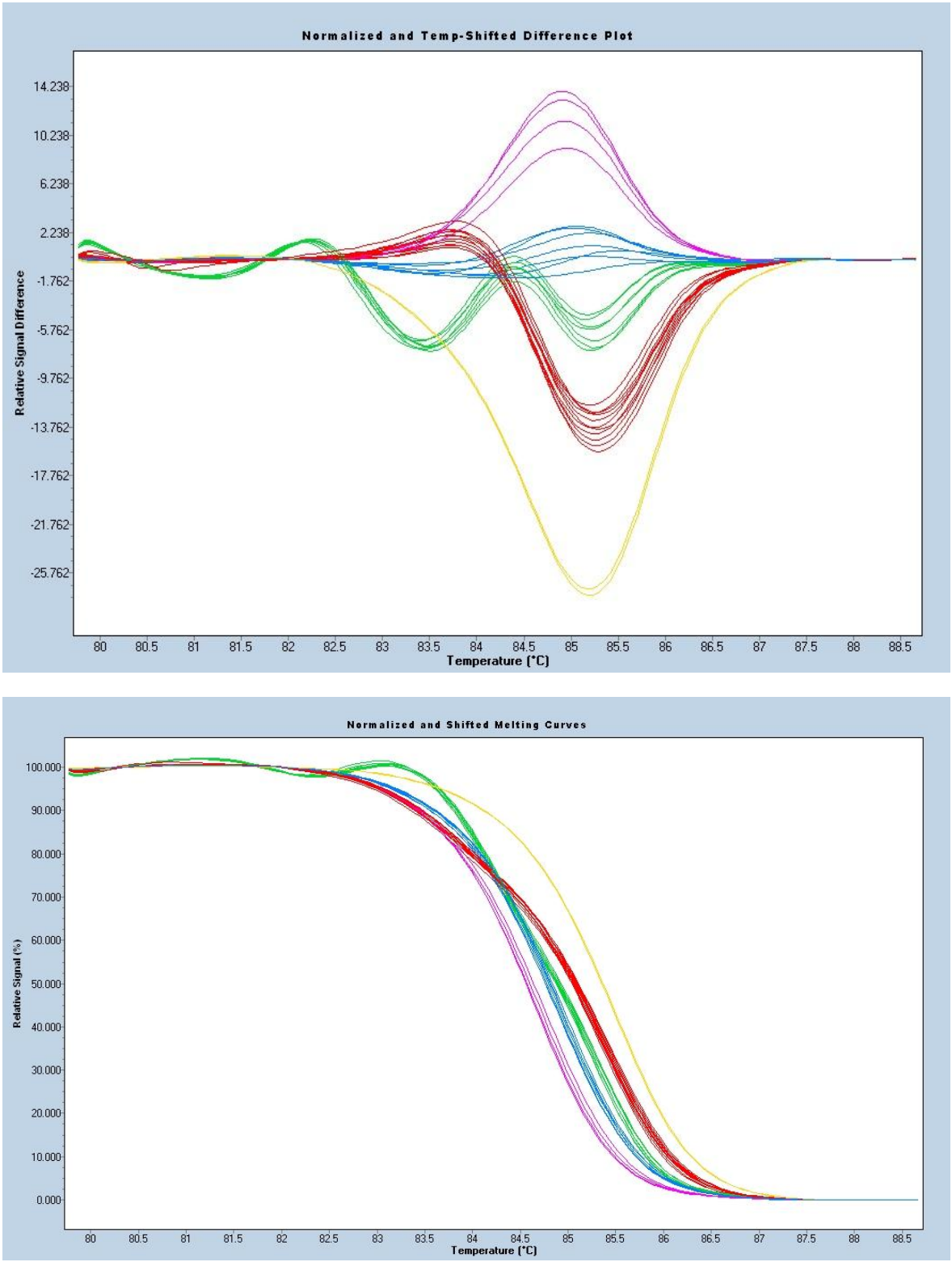

Fig. 6: Illustration of software sample processing (LightCycler480 software1.5.0 SP3), each colour represent one genotype TLR4: yellow-1646GG, red-1646AG, blue-1646AA, pink-1646AA with substitution on position 1664, green-unknown genotype.

III.9 Statistical analyses

Statistical analyses were performed using the statistical software R version 2.12.1 (R Foundation for Statistical Computing 2010). Prior to each analysis, data were checked for normality using the Shapiro-Wilk normality test. Correlations were tested by Pearson's product-moment correlation test. Pairwise comparisons of the basic traits were performed either by t-test (in case of Gaussian distribution) or by Wilcoxon signed rank test (in case of non-Gaussian distribution). Wherever appropriate (e.g. differences between LPS-treated and control wings or measurements prior and post challenge), the paired setting of the test was adopted (i. e., Paired t-test or Paired-sample Wilcoxon signed rank test, respectively). To analyse more complicated associations the general linear mixed-effects models were utilised. In these models the year (2011-2012) and nest identity were always treated as random effects. In each analysis the Minimum adequate model (MAM; i.e. a model with all terms significant) was obtained by backward eliminations of non-significant terms ($p > 0.10$) in candidate models using likelihood ratio test assuming Chi square distribution of change in deviance. The order of term elimination was determined based on the minimal decrease in AIC values between the original model and its reduced forms. Marginally non-significant terms ($0.10 < p < 0.05$) were retained in the MAMs due to the relatively small sample size. The significance of a particular term adjusted to the effects of other terms was based on the change in deviance between the full and reduced models, distributed as *Chi* with degrees of freedom equal to the difference in the degrees of freedom between the models with and without the term in question. Gaussian or Gamma distribution of model residuals was assumed. Difference between categories was tested using the Tukey's HSD test. Hardy-Weinberg equilibrium was tested using a Chi-squared test with observed and expected frequencies of individual genotypes. Repeatability was assessed according to Lessells and Boag (1987). The significance level was set to $p = 0.05$.

IV. Results

IV.1 Describe frequency of the A1646G (Q549R) substitution in the great tit population of nestlings (Task 1)

In the first instance I examined the distribution of allele frequencies of the SNP A1646G (nsSNP responsible for the Q549R substitution on the amino acid level) in our study population and tested if it is consistent with Hardy-Weinberg (H-W) equilibrium. In total we have 245 individuals with frequency of allele A f(A) = 0.79 and frequency of allele G f(G) = 0.21. We calculated the observed frequency and expected frequency of each genotype (summarized in Tab. 3). We found a marginally non-significant tendency for deviation to H-W equilibrium (Chi-squared test: X-squared = 5.286, $df = 2$, $p = 0.071$), but given the limited samples size we, therefore, cannot exclude the possibility of weak selection acting on the population.

In the following models we distinguished between presence and absence of the Q549R substitution in the nestlings' diploid genotype (i.e. AA→Q, AG+GG→R).

Tab. 3: Summarization of expected and observed genotype in TLR4 in study population of great tit.

	1646AA	1646AG	1646GG
observed number of individual/genotype frequency	147/0.60	93/0.38	5/0.02
expected number of individual/genotype frequency	153/0.62	81/0.33	11/0.04

IV.2 Description of the immune response to LPS (Task 2)

To describe the effect of LPS on tissue swelling I compared the tissue magnitude increase after 24h in LPS-treated wing webs (a combined category of LPS_{Ecol} and LPS_{Sent} treated birds has been analysed) and PBS-treated wing webs (paired samples from the same individuals, both years included, n = 142). I found a significant difference between PBS- and LPS-treated wing webs in the tissue magnitude increase (Paired-sample Wilcoxon signed rank test: $V = 9996$, $p << 0.001$). This was due to the swelling response in the LPS injected wings (Fig. 7). In 22 control individuals I compared identical changes in skin tissue thickness between untreated and PBS-treated wing webs (no LPS stimulation in the individual). The PBS treatment had no effect on the tissue swelling response (Student pair T test, $p > 0.1$).

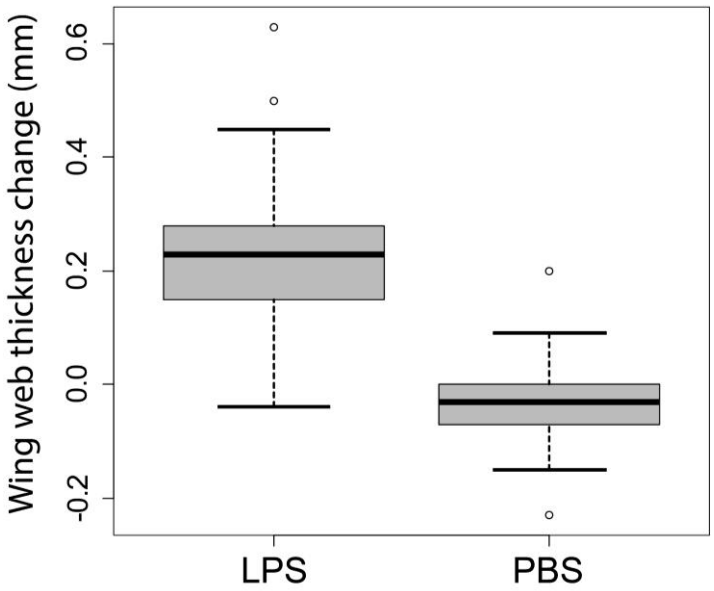

Fig. 7: Comparison of the wing web swelling response in LPS- and PBS-treated nestlings measured 24 hours post injection. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

There are two commonly used calculations of the swelling response used in ecological immunology (Smits et al. 1999). The first one compares the swelling response (thickness before and after stimulation) between stimulator-treated and PBS-treated wing webs, while the second one compares only the thickness of the stimulator-treated wing before and after the swelling response. I tested the correlation between values of the swelling index obtained by both of these approaches in nestlings measured 24h post treatment (combined category LPS_{Ecol} and LPS_{Sent}). Results of both methods are highly correlated (Pearson's product-moment correlation: n = 142, $r = 0.85$, $t = 19.45$, $df = 140$, $p << 0.001$). In the following analyses I, therefore, used only the difference in magnitude of swelling response before and after stimulation according to the recommendation by Smits (1999).

To explore the temporal dynamics of the swelling response after the LPS treatment I measured the skin thickness change in LPS_{Sent}- and LPS_{Ecol}-treated nestlings at 3, 6, 24, 48 and 72 hours after the LPS injection (N = 175; LPS_{Sent}: 3h = 4, 6h = 31, 24h = 43, 48h = 7; LPS_{Ecol}: 3h = 3, 6h = 32, 24h = 47, 48h = 6, 72h = 2). I revealed that the magnitude of swelling response constantly increased in time in both LPS_{Sent}- and LPS_{Ecol}-treated wing webs and apparently not even after 72 hours it reached its maximum (Fig. 8).

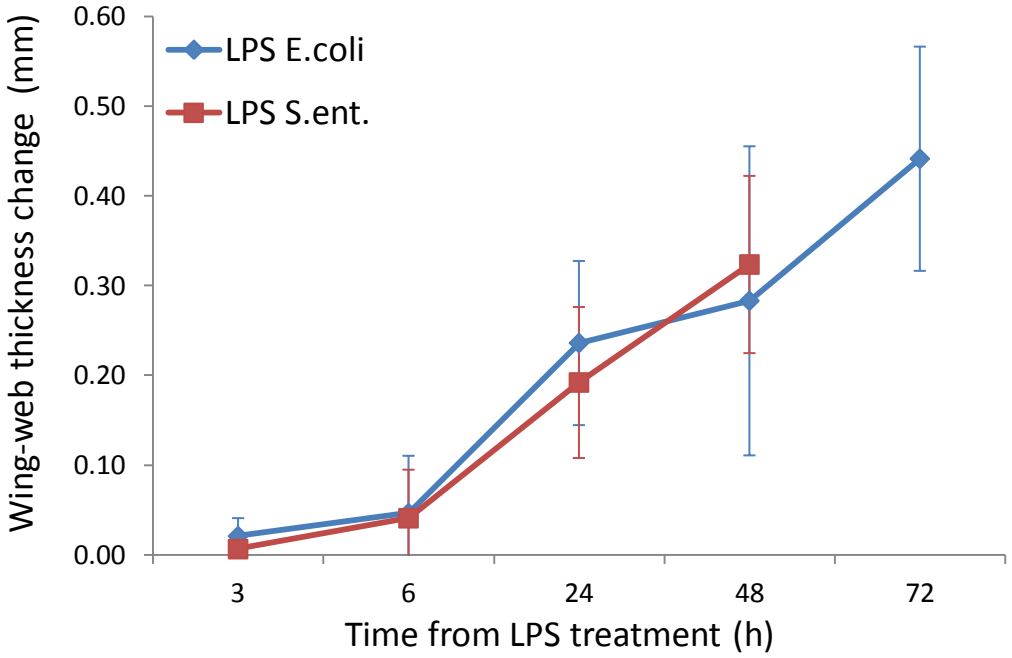

Fig. 8: Time dynamics of the LPS-triggered skin swelling response in great tit nestlings. Blue line with diamond points represents swelling in LPS_{Ecol} treated nestlings, red line with square points represents swelling in LPS_{Sent} treated nestlings. Mean in each time point is indicated by diamond/square point, whiskers represent standard deviation from mean (SD).

The sample size available for the measurement at time of 72h post treatment did not allow any statistical testing of the difference in tissue magnitude between LPS-treated and PBS-treated wing webs. However, similarly to the wing webs measures at 24h, there was a significant difference between LPS-treated and PBS-treated wing webs also at 48h (Paired-sample Wilcoxon signed rank test: n = 13, combined sample $nLPS_{Sent}$ = 7, $nLPS_{Ecol}$ = 6, $V = 91$, $p < 0.001$) and 6h (Paired-sample Wilcoxon signed rank test: n = 63,combined sample $nLPS_{Sent}$ = 31, $nLPS_{Ecol}$ = 32, $V = 1110$, $p << 0.001$). In birds measured at 6h post treatment the thickness difference between LPS and PBS treated wing webs was only minor (see Fig. 9).

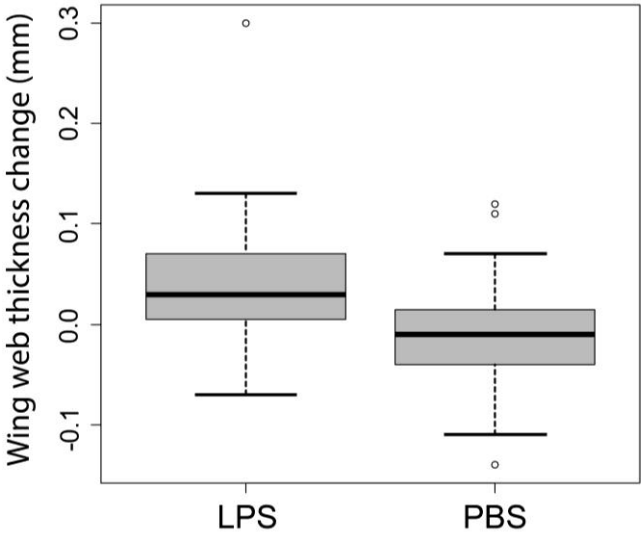

Fig. 9: Comparison of the wing web swelling response in LPS- and PBS-treated nestlings measured 6 hours post injection. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

In the mixed-sample cDNA originating from 10 individuals (biopsies collected 6 hours after LPS stimulation) we tried to identify partial CDSs of the following cytokine genes *IFNγ*, *IL-1β*, *IL-6*, *IL-10*, *IL-18*, *TNFSF15*, *CXCLi2*. Only *IL-1β*, *IL-6*, *IL-18*, *TNFSF15* and *CXCLi2* could be amplified. These genes were cloned into the vector and consequently sequenced. In all cases we obtained sequences of ortholoques of the target genes. For primary optimization of qPCR we decided to use only *IL-1β* and *IL-6*. The optimization failed mainly due to repeated positive response in the negative controls. Further attempts with other settings could not be performed for limited time possibilities.

IV.2.1 Influence of LPS treatments on general haematological state and condition

In our great tit nestling study population we assessed the range of values of several basic haematological parameters on day 15 post hatch. The TLC was estimated on a sample of 233 nestlings to mean ± SD = $6.17\times10^6 \pm 3.38\times10^6$ cells/mL (min–max = 0.50×10^6–32.00×10^6). In a sample of 61 nestlings we assessed the frequencies of individual leukocyte types detectable in avian blood. The results are shown in Tab. 4. The H/L ratio ranged from 0.09 to 1.34 (n = 61, mean ± SD = 0.52 ± 0.28).

I did not find any effect of Q549R substitution on nestling TLC (n = 210, all tested parameters in the model including Q549R $p > 0.10$) or H/L ratio (N = 59, resultant MAM $p > 0.10$).

	Mean (%)	SD	Min (%)	Max (%)
Lymphocytes	47.14	11.19	23.02	80.00
Immature leukocytes	1.93	3.42	0.00	15.83
Heterophils	22.26	8.06	6.90	44.44
Basophils	13.72	7.19	0.00	34.92
Eosinophils	9.715	4.57	0.79	22.12
Monocytes	5.24	4.27	0.00	25.41

Tab. 4: Frequencies of individual leukocyte types detectable in great tit blood, N = 61.

Thereafter, I investigated whether there was any effect of the LPS treatment on haematological parameters in peripheral blood. In total we assessed TLC in 28 LPS_{Ecol} treated and 19 PBS treated nestlings before (day 15) and after (day 16) the treatment. There was no significant difference in TLC between the two time points in any of the treatments (Wilcoxon signed rank test, in both cases p>0.10). In a small subsample of nestlings we also estimated the leukocyte differential count before and after PBS and LPS treatments. After the PBS treatment there was a marginally non-significant difference between day 15 and 16 post hatch in H/L ratio (Paired t-test: n = 6, t = -2.27, df = 5, p = 0.072). In LPS-treated nestlings there was a change in H/L ratio significant (Paired t-test: n = 12, t = 2.29, df = 11, p = 0.043). Intriguingly, the association had opposite directions in the two treatment groups. While in LPS-treated nestlings H/L decreased, in PBS-treated individuals there was an H/L ratio increase (Welch Two Sample t-test: n = 6PBS+12LPS, t = -3.20, df = 10.14, p = 0.009; Fig. 10). This was due to significant decrease in absolute heterophils count (calculated as the difference in absolute heterophils count post minus prior treatment) in LPS treated birds and simultaneous increase in the absolute heterophiles count in PBS treated individuals (significant difference between LPS and PBS treated birds, Welch Two Sample t-test: t = -4.28, df = 8.258, p = 0.003; Fig. 10). A similar difference has been found in the change of the absolute lymphocyte count (t = -2.15, df = 15.79, p = 0.047; Fig. 10) but not in the change of the absolute basophils count (p > 0.10).

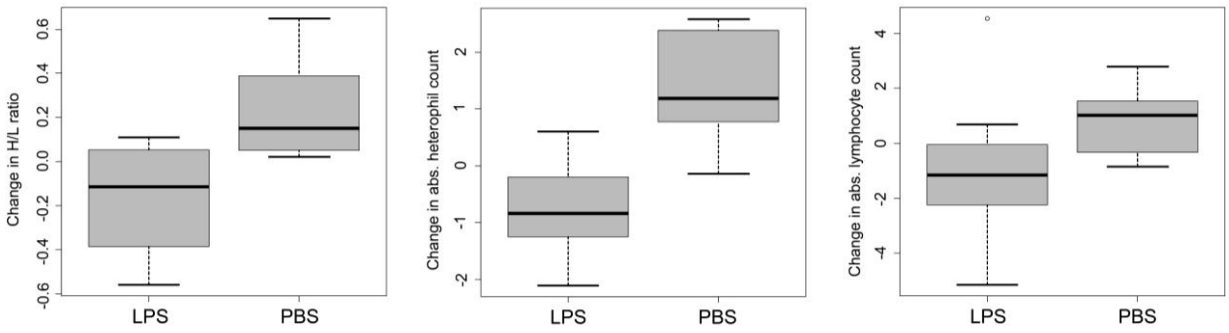

Fig. 10: Comparison of haematological changes (H//L ratio, absolute heterophils count, absolute lymphocyte count) in response to LPS- and PBS-treatment measured 24 hours post injection. Absolute cell counts are given in cells×10^6/ml. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

Finally, I tested the effect of the LPS treatment on growth and condition of the nestlings. In the whole dataset (LSP-treated nestlings n = 214 (LPS_{Ecol} = 110, LPS_{Sent} = 104) and PBS-treated nestlings n = 22) there was a significant tendency for decrease in body weight between day 15 and day 16 post hatch (Paired t-test: $t = 15.24$, $df = 235$, $p << 0.001$). Simultaneously, I observed a significant growth in the nestlings (Paired-sample Wilcoxon signed rank test: $V = 8656$, $p << 0.001$). As a result, the body condition of the nestlings significantly decreased between day 15 and 16 post hatch (Paired-sample Wilcoxon signed rank test: $V = 21384.5$, $p << 0.001$) but the same highly significant changes were found also if I estimated separately the LPS-treated individuals (decrease in weight: $t = 13.64$, $df = 213$, $p << 0.001$; growth: $V = 7452.5$, $p << 0.001$; decrease in condition: $V = 17030.5$, $p << 0.001$) and PBS-treated individuals (decrease in weight: $t = 9.46$, $df = 21$, $p << 0.001$; growth: $t = -2.79$, $df = 21$, $p = 0.011$; decrease in condition: $V = 253$, $p << 0.001$). There was no difference in growth between nestlings after the LPS and PBS treatments ($p < 0.10$).Although the first analysis showed that the nestlings lost significantly less weight after the LPS treatment than after the PBS treatment (Welch Two Sample t-test: $t = 2.78$, $df = 31.06$, $p = 0.009$) and also had lower decrease in body mass after the LPS treatment than after injection of PBS (Wilcoxon rank sum test: $W = 3151$, $p = 0.009$), this seems to be the effect of huge sample size difference and uneven representation of the variability in the PBS-treated subsample. In the following analysis I, therefore, compared the LPS-treated and PBS-treated nestlings from the same broods in random pairs. I did not find any significant difference between these two treatment classes either in growth or in weight or body mass (Paired t-test: n = 40, 20 pairs from 11 nests, in all three cases $p > 0.10$).

IV.2.2 Capacity of different types of LPS to trigger oxidative burst in phagocytes and initiate the pro-inflammatory immune response

Our *in vitro* measurements of the oxidative burst did not reveal any correlations between the responses to LPS_{Ecol} and LPS_{Sent} when measured in the same individuals (Pearson's product-moment correlation: n = 88; RLUmax, AUC,in both p>0.10). RLUmax (Wilcoxon signed rank test, n = 88, $V = 30$, $p << 0.001$; Fig. 11) and AUC (Wilcoxon signed rank test, n = 88, $V = 26$, $p << 0.001$; Fig. 11) were significantly higher in response to LPS_{Sent} than in response to LPS_{Ecol}. Oxidative burst induced by LPS_{Ecol} and LPS_{Sent} was therefore analysed separately in the subsequent analyses. In LPS_{Ecol} stimulated blood the oxidative burst correlated with TLC (Pearson's product-moment correlation, RLUmax LPS_{Ecol}: n = 30, $r = -0.52$, $t = -3.24$, $df = 28$, $p = 0.003$; AUC LPS_{Ecol}: n = 30, $r = -0.41$, $t = -2.40$, $df = 28$, $p = 0.023$), but not with H/L (Pearson's product-moment correlation, RLUmax LPS_{Ecol}: n = 30, $p > 0.10$). Importantly, the RLUmax was correlated with the absolute number of heterophils and monocytes in the blood (Pearson's product-moment correlation, RLUmax LPS_{Ecol}: n = 30, $r = -0.43$, $t = -2.51$, $df = 28$, $p = 0.018$; AUC LPS_{Ecol}: n = 30, $r = -0.46$, $t = -2.74$, $df = 28$, $p = 0.011$). In LPS_{Sent} stimulated blood there was no association between the oxidative burst and TLC (Pearson's product-moment correlation, RLUmax LPS_{Sent}:

n = 31, $p > 0.10$; AUC LPS_{Sent}: n = 31, $p > 0.10$) or the absolute number of heterophils and monocytes in the blood (RLUmax LPS_{Sent}: n = 31, $p > 0.10$; AUC LPS_{Sent}: n = 31, $p > 0.10$).

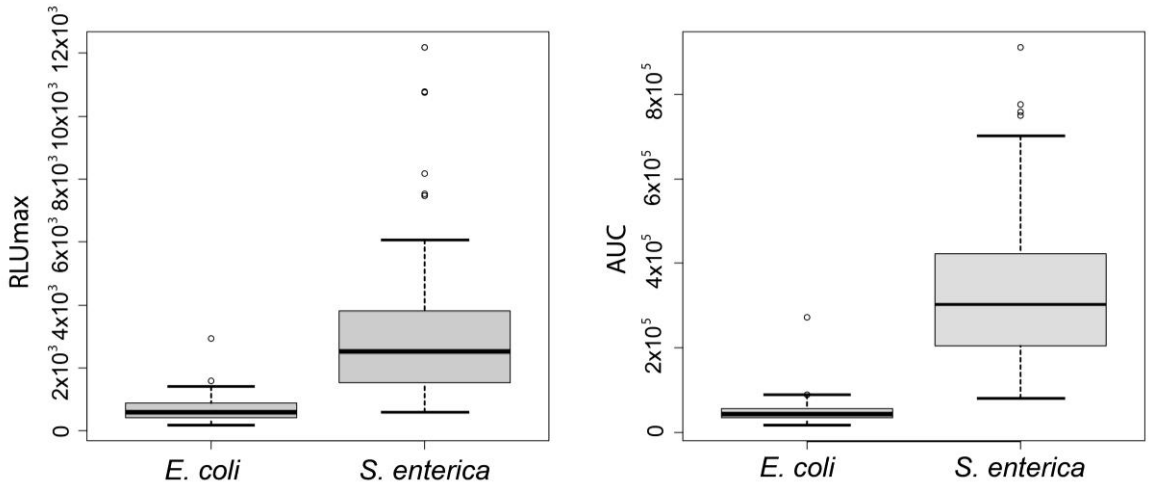

Fig. 11: Differences between LPS_{Ecol} and LPS_{Sent} in their capability to trigger oxidative burst in whole blood in vitro. RLUmax = maximal relative light unit, AUC = area under the curve. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

In vivo I failed to reveal any difference between LPS_{Ecol}-treated and LPS_{Sent}-treated individuals measured at 6h post treatment (Wilcoxon rank sum test: n = 63 (LPS_{Sent} = 31, LPS_{Ecol} = 32), $p > 0.10$). Nevertheless, I have found a significant difference in the capability of LPS_{Sent} and LPS_{Ecol} to induce the swelling response in nestlings 24h post treatment (Welch Two Sample t-test: n = 142, $t = -2.09$, $df = 134.06$, $p = 0.038$; Fig. 12). There was no significant difference between LPS_{Ecol} and LPS_{Sent} in their effects on growth, weight and body mass (n = 214, in all cases $p > 0.10$).

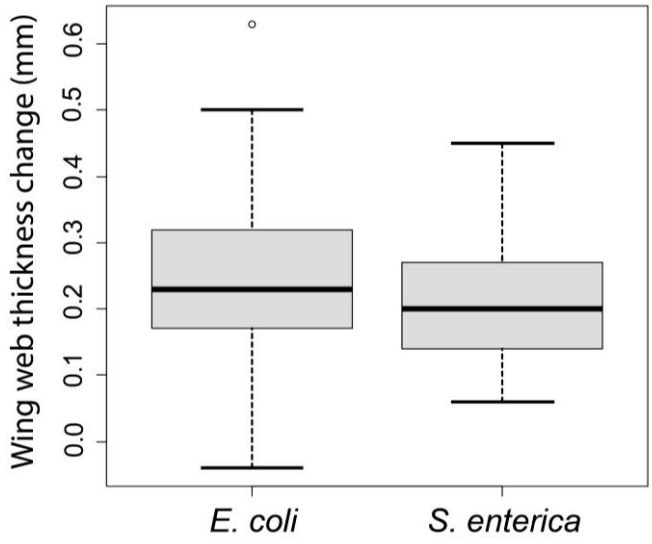

Fig. 12: Comparison of the wing web swelling response in LPS_{Ecol}- and LPS_{Sent}-treated nestlings measured 24 hours post injection. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

IV.3 Effect of the Q549R substitution on body condition and pro-inflammatory responsiveness (Task 3)

IV.3.1 Effect of the Q549R substitution on condition

The substitution Q549R has no effect on condition (weight, size, body mass; n = 216, in all p>0.10) on the day 15. My analysis, however, revealed that body mass is affected by the number of nestlings in the nest (Linear mixed-effects model: n = 216, slope±SE = 0.006±0.003, *Chi* = 4.339, *df* = 1/208, *p* = 0.037) and hatching day (Linear mixed-effects model: n = 216, slope±SE = -0.003±0.001, *Chi* = 4.644, *df* = 1/208, *p* = 0.031; MAM: *Chi* = 7.970, *df* = 2/208, *p* = 0.019). Furthermore, in the control group of PBS-treated (LPS-unstimulated) individuals I analysed the effect of Q549R on the nestling growth. I revealed a significant effect of the interaction between Q549R and brood size (Linear mixed-effects model: n = 23, interaction Q549R:brood size slope±SE = 0.055±0.026, *Chi* = 5.06, *df* = 1/18, *p* = 0.025; Q549R slope±SE = -0.479±0.254, *Chi* = 7.69, *df* = 2/19, *p* = 0.021; brood size slope±SE = -0.037±0.018, *Chi* = 5.84, *df* = 2/19, *p* = 0.054; MAM: *Chi* = 8.42, *df* = 3/20, *p* = 0.038).

IV.3.2 Effect of the Q549R substitution on haematological parameters

There is no effect of the Q549R polymorphism on TLC (n = 210; *p* > 0.01), H/L ratio (n = 59; *p* > 0.01) or basophil frequency in peripheral blood (n = 59; *p* > 0.01; all parameters measured on day 15 post hatch, i.e. before the LPS treatment).

IV.3.3 Effect of the Q549R substitution on *in vitro* immune responsiveness to LPS

In measurements of the oxidative burst induced by LPS_{Ecol} I failed to find any association between RLUmax and the Q549R genotype (Linear mixed-effects model: n = 113, MAM contained only hatching date as a significant member slope±SE = 5.219e-05±1.401e-05, *F* = 13.82, *df* = 1/111, *p* < 0.001). There was also no association between AUC and Q549R genotype (Linear mixed-effects model: n = 113, *p* > 0.010). The same absence of any relationships between the Q549R genotype and RLUmax (Linear mixed-effects model: n = 114, *p* > 0.010) and AUC (Linear mixed-effects model: n = 114, *p* > 0.010) has been revealed also among the LPS_{Sent} stimulated blood cell suspensions.

IV.3.4 Effect of the Q549R substitution on *in vivo* immune responsiveness to LPS

Testing the association between Q549R genotype and swelling responsiveness to LPS would be too complicated in one test as there would be too many interactions. Therefore, the dataset was tested separately for the measurements of the response 24h post-treatment and 6h post-treatment.

In the sample of individuals that were measured 6h post LPS injection I did not find any effect of the Q549R genotype or the LPS type on the magnitude of swelling response (the only significant member of the MAM was the hatching date n = 59, *Chi* = 5.63, *df* = 1/7, *p* = 0.018; marginally non-

significant effect of the body mass at day 15 *Chi* = 3.40, *df* = 1/7, *p* = 0.65; MAM: *Chi* = 8.70, *df* = 2/6, *p* = 0.013). In the sample of individuals that were measured 24h after the LPS injection I revealed association between the magnitude of the swelling response and the hatching date, type of LPS applied (LPS_{Ecol}, LPS_{Sent}) and the Q549R genotype; there was also a significant effect of interaction between the Q549R genotype and type of LPS injected (Linear mixed-effects model: n = 131, interaction Q549R:LPS type *Chi* = 11.36, *df* = 1/10, *p* < 0.001; Q549R *Chi* = 14.02, *df* = 2/10, *p* < 0.001; LPS type *Chi* = 15.88, *df* = 2/10, *p* < 0.001; hatching date *Chi* = 7.22, *df* = 1/10, *p* = 0.007; MAM: *Chi* = 25.27, *df* = 4/10, *p* << 0.001). The interaction between the Q549R genotype and type of LPS injected in their effect on the residuals of the swelling response in resultant MAM is shown in Fig. 13. The Tukey's test revealed that category 549Q-LPS_{Ecol} differs significantly from 549Q-LPS_{Sent} (*p* < 0.001) and 549R-LPS_{Ecol} (*p* = 0.003) and marginally non-significantly from 549R-LPS_{Sent} (*p* = 0.079). Although the number of individuals with 549RR genotype is extremely limited in our study sample I decided to test even this association with full genotype variants (i.e. 549QQ, 549QR, 549RR). Again, I revealed the association between the swelling magnitude and the hatching date, type of LPS applied (LPS_{Ecol}, LPS_{Sent}) and the Q549R genotype (i.e. heterozygosity of the Q549R = QQ/QR/RR) with a significant effect of the interaction between the Q549R genotype and the type of LPS injected (Linear mixed-effects model: n = 131, interaction Q549R:LPS type *Chi* = 11.02, *df* = 2/10, *p* = 0.004; Q549R *Chi* = 13.68, *df* = 4/8, *p* = 0.008; LPS type *Chi* = 15.61, *df* = 3/9, *p* = 0.001; hatching date *Chi* = 6.51, *df* = 1/11, *p* = 0.011; MAM: *Chi* = 24.93, *df* = 6/6, *p* < 0.001). Under this setting the Tukey's test revealed differences only between the category 549QQ-LPS_{Ecol} and 549QQ-LPS_{Sent} (*p* = 0.001) and 549QQ-LPS_{Ecol} and 549QR-LPS_{Ecol} (*p* = 0.011). The Figure 14 (Fig. 14), nevertheless, shows that there may be a non-significant tendency to a more complex pattern in the interaction between host genotype and type of LPS, in which 549Q is more responsive to LPS_{Ecol} and 549R is more responsive to LPS_{Sent}. This is also suggested by the absence of any significant difference between 549QQ-LPS_{Ecol} and 549RR-LPS_{Sent}.

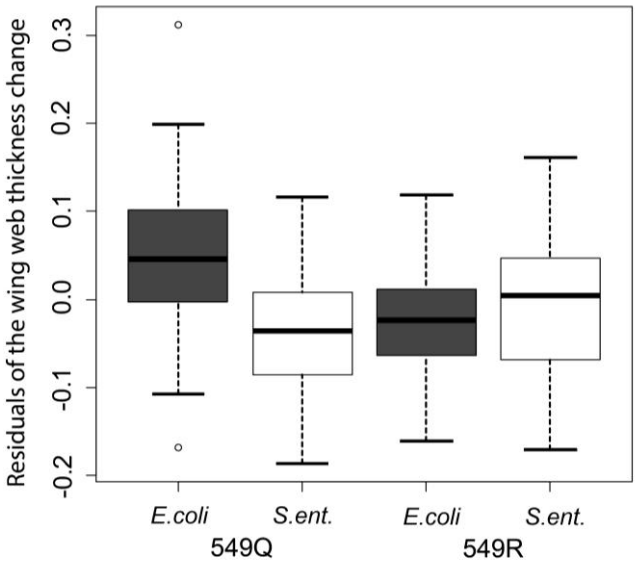

Fig. 13: Comparison of the wing web swelling response in LPS_{Ecol}- (dark-grey boxes) and LPS_{Sent}-treated (white boxes) nestlings of the TLR4 549Q and 549R genotype measured 24 hours post injection. The swelling response is shown as residuals of the wing-web thickness change after controlling for the effect of year, nest and hatching date. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

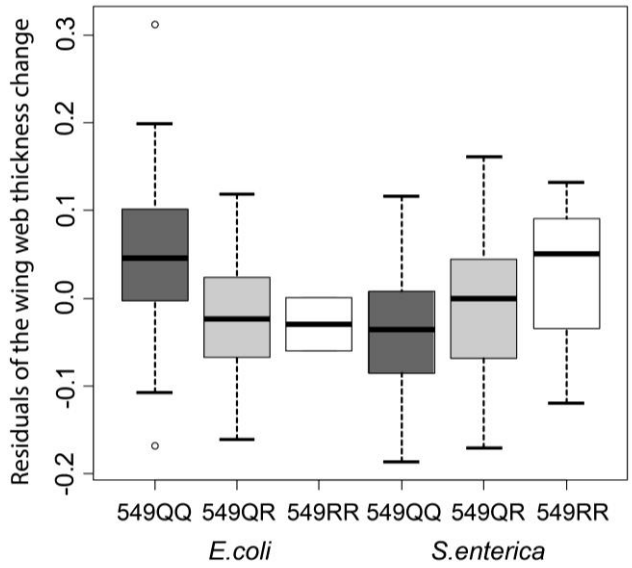

Fig. 14: Comparison of the wing web swelling response in LPS_{Ecol}- and LPS_{Sent}-treated nestlings of the TLR4 homozygous 549QQ (dark-grey boxes), heterozygous 549QQR (light-gray boxes) and homozygous 549RR (white boxes) genotype measured 24 hours post injection. The swelling response is shown as residuals of the wing-web thickness change after controlling for the effect of year, nest and hatching date. Median is indicated by thick solid line, upper and lower quartile by box, outlying samples are shown as open dots.

The effect of Q549R substitution on the change in haematological values between the time points prior and after the LPS treatment could not be tested due to the small sample size available (12 individuals in which haematological parameters were measured before and after LPS treatment, only 3 R genotypes). Although the Q549R substitution has a significant effect (in interaction with the number of nestlings in the nest) on nestling growth in PBS-treated nestlings (shown in chapter IV.3.1), in LPS-treated nestling (combined LPE_{Ecoli} and LPS_{Sent}) there was no significant relationship of this kind (Linear mixed-effects model: n = 193, $p > 0.10$). Neither there was any association with the change in nestling body weight nor the body condition between day 15 and day 16 post hatch and not even the LPS-type had any significant effect (Linear mixed-effects models: n = 193, in all cases $p > 0.10$).

V. Discussion

In this diploma thesis I describe the effect of the LPS subcutaneous injection on the pro-inflammatory immune response, general condition and haematological state, and the functional association of this response with interaction between LPS type and TLR4 Q549R genotype in a population of great tit nestlings. In our study population I have revealed that the frequency of the Q549 allele is 0.79 and the frequency of the R549 allele is 0.21. Hence, both alleles are relatively frequent. Intriguingly, the homozygous QQ genotype was extremely rare, less common than could be expected based on the allele frequency. This results in marginally non-significant tendency to deviation from the Hardy-Weinberg equilibrium which might be indicative of an ongoing selection. The subcutaneous LPS treatment induces an inflammatory immune response in which cytokine regulation by IL-1β, IL-6, IL-18, TNFSF15 and CXCLi2 may play significant role. The pro-inflammatory immune response causes swelling that is constantly increasing within the first 72 hours post LPS injection. I found a significant difference in the tissue magnitude between PBS- and LPS-treated wing webs after 6, 24 and 48h (comparison at 72h was not possible due to the limited sample size available). As the nestlings spontaneously tended to leave the nest on the third day post treatment, all analyses had to be performed only on birds measured 6 and 24 hours after the LPS injection. There was no swelling response in the PBS treated controls. The local inflammation affected also the quantities of the leukocytes in peripheral blood. Although I did not find any significant changes in TLC between day 15 and 16 post hatch in any of the treatments (PBS / LPS), there were significant changes in the H/L ratio in this period. Interestingly, while in LPS-treated nestlings the H/L decreased, in PBS-treated individuals there was a non-significant tendency for increase in the H/L ratio. It seems that while stress increased absolute heterophil and, to lesser extent, lymphocyte count in PBS-treated individuals, local inflammation attracted blood-borne heteropils and (again, to lesser extent) lymphocytes to the treatment site, causing decrease in the H/L ratio 24h after the LPS treatment. In all nestlings I observed a decrease in the body weight and growth, and resulting body mass decrease between day 15 and day 16 post hatch. There was, however, no clear effect of the LPS-induced skin inflammation on the weight change, growth or a change in the body mass. The two LPS types utilised in this study (LPS_{Ecol} and LPS_{Sent}) remarkably differed in their immunostimulatory potential. LPS_{Sent} triggered a stronger oxidative burst than LPS_{Ecol} when measured in vitro (RLUmax and AUC). On the contrary, when measured in vivo as a swelling response 24h post treatment, the LPS_{Sent} managed to induce only a weaker response than the LPS_{Ecol}. There was no significant difference between LPS_{Ecol} and LPS_{Sent} in their effects on growth, weight change, and body mass change. The substitution Q549R had only a limited effect on nestling vigour and no effect on the oxidative burst measured in vitro. When pro-inflammatory responsiveness was measured in vivo, the Q549R genotype had, however, a significant effect on the swelling response at 24h post treatment. Although the 549QQ homozygots responded more

intensively to LPS_{Ecol} than the 549QR heterozygots (and 549RR homozygots, in which the difference was, however, non-significant), the relationship seemed to be reversed in response to LPS_{Sent} to which the 549QQ homozygots responded only weakly, while the 549QR heterozygots and 549RR homozygots tended to respond more strongly. Thus, there is a significant interaction between the Q549R genotype and the type of LPS injected in their effect on the tissue swelling response that is consistent with the matching alleles model of evolution of host-parasite interactions. It should be, however, admitted that although I tried to minimise the effects of all confounding factors on the tested variables of the pro-inflammatory responsiveness to LPS I, some factors may have been underestimated and overlooked. The in vivo and in vitro responsiveness to LPS was measured only in 15-day-old nestlings to minimise the effect of age, all sampling has been performed in only two years, to reduce the possibility of changes in the selection pressures posing on the population. Still, the budget constrains prevented me from analysing some of the factors potentially valuable in explaining the immune responsiveness to LPS. Among these mainly the effects of sex and paternity remained untested. Hopefully, both variables will be analysed additionally and included in the future work.

V.1 Q549R polymorphism in a population of great tit nestlings (Task 1)

I have revealed that the frequency of Q549 allele is 0.79 and the frequency of R549 allele is 0.21. The homozygous QQ genotype was extremely rare in our sample, resulting in marginally non-significant tendency for deviation from the Hardy-Weinberg equilibrium. It has been proposed that recent selection on specific alleles may cause deviations from the Hardy-Weinberg equilibrium in immune related genes. It has been, for instance, reported that alleles of MHC class I gene HLA-C depart from the Hardy-Weinberg equilibrium in the Mixtec native-American population (Cao et al. 2001). Access of heterozygotes, similar to the one observed in the present study, has been described for TNF-α -308G⁄A SNP in Northern Ivory Coast (Santovito et al. 2012). The authors suggest that their results indicate a possible selective advantage of the heterozygote genotypes, associated with intermediate levels of TNF-α expression, against infectious diseases endemic in Western Africa. In TLRs similar evidence from natural populations has been lacking so far. In TLR4, however, fixation of certain alleles in domestic cattle breeds causes significant deviations from the Hardy-Weinberg equilibrium (Mariotti et al. 2009). On the other hand, several studies reported that even functionally important SNPs in TLR4 (such as D299G/T399I in humans) are in Hardy-Weinberg equilibrium (Iliadi et al. 2009; Zaki et al. 2012; Bali et al. 2013). It is obvious that the SNP features in the particular ecological context may affect the outcome of selection. The disequilibrium may be, thus, observed only in some specific groups of individuals.

V.2 Course of the immune response to LPS and assessment of its influence on individual condition (Task 2)

In the present study I confirmed that subcutaneous injection of LPS induces an inflammatory immune response. Although at the end I was unable to perform a quantification of the gene transcription, by cloning the IL-1β, IL-6, IL-18, TNFSF15 and CXCLi2 genes from the swelling-isolated RNA we have shown that the swelling response is associated with pro-inflammatory cytokine expression. The course of the response is thus likely very similar to the one induced by the subcutaneous application of PHA. Also after dermal application of PHA expression of various pro-inflammatory cytokines is triggered in birds (Vinkler et al. in press), which is associated with infiltration of various types of leukocytes, including those involved in innate and adaptive immunity, into the tissue (McCorkle et al. 1980; Martin et al. 2006). The leukocyte infiltration leads to a tissue swelling, which is metrically measurable (Smits et al. 1999). This local alternation in homeostasis may have further, systemic effects. It has been shown that PHA injection may lead to changes in haematological traits in peripheral blood (Buchanan et al. 2003) and after LPS injection birds can develop fever reaction (Maloney and Gray 1998; Berthouly et al. 2008; Hegemann et al. 2012). Finally, it has been shown in chickens that if intramuscular application of LPS is used, then gene expression in remote lymphatic tissues may be affected (St Paul et al. 2011).

Most studies, nevertheless, focus on description of the local changes in the inflamed tissue. In our study the LPS treatment induced a swelling which was constantly increasing at least within the first 72 hours post injection. As the great tit nestlings spontaneously start to leave the nest about the age of 17 days post hatch, most analyses were performed only on birds measured 6 and 24 hours after the LPS injection. Furthermore, since at the time of 6 hours after LPS application the swelling response was still rather small, most significant results gained in my thesis come from birds measured 24 hours after the LPS injection. This is fully consistent with other similar studies in ecology where in the vast majority of cases the swelling response to an experimental stimulator (most commonly PHA) is measured 24 hours after the injection (Smits et al. 1999). In chickens, however, the swelling response was higher after 4 hours than 24 hours after the LPS injection (Parmentier et al. 1998). In contrast, no swelling response could be measured 4 and 12 hours after LPS injection in another study in the great tit, while a significant response was present after 24 hours (Berthouly et al. 2008). It, therefore, seems that our data fit the results of other similar studies.

In my study the LPS treatment did not significantly affect the TLC but altered the H/L ratio through changes in the absolute heterophils and absolute lymphocytes count in the great tit. Both the H/L ratio and heterophils and lymphocytes counts tended to decrease within the 24 hours after the LPS injection suggesting possible efflux of these cells from blood into the inflamed skin tissue. This result is in contrast with other similar studies published. This scenario is rather different from

what was described by Bowen et al (2009) in chickens. Bowen et al (2009) found increase in H/L ratio only in the initial phases of the response and at the time 24 hours post injection all both parameters were close to the normal values. Interestingly, in male chickens they found a significant increase in TLC and both absolute heterophils and lymphocytes count in the same time. In their study, however, the type of the immune response was completely different from the immune response observed in our study. Bowen et al (2009) injected the LPS directly into the basilica wing vein, which led to a systematic immune reaction in the bloodstream. Thus, the location of the response was in vein and bloodstream, and not in tissues. This might explain the proportional increase of heterophils and lymphocytes there. In contrast to them, we injected LPS to the subcutaneous space in the centre of wing web, which led to a local immune response, attracting the blood-borne cells to skin. Nearly similar results (increase of proportion of heterophies, no change in proportion of lymphocytes and H/L ratio after 13h after LPS treatment) as Bowen at al (2009) obtained also Hegemann at al. (2013) in their study in skylarks (*Alauda arvensis*). Hegemann at al (2013) injected the birds intra-abdominally, which is also inconsistent with our study. A study similar to ours in terms of the site of immune challenge is the one by Buchanan et al (2003), who, however, used PHA instead of LPS to induce the swelling response. Unfortunately, neither Bowen et al (2009) nor Hegemann et al (2013) or Buchanan et al (2003) used PBS treated birds as controls. This approach has its pitfalls. Puncturing the skin and injecting the PBS or saline fusion may cause stress and result in minor local inflammation per se. Thus, when the aim is to discriminate the effect of LPS which stimulates specifically TLR4 from unknown bacterial infection, we have to use PBS or saline fusion treated individuals as a control group. It is probable that PBS treatment alone may alter the H/L ratio in the birds as a result of stress caused by the handling procedure.

I observed a decrease in the body weight, growth and a resulting condition decrease between day 15 and day 16 post hatch. When the whole dataset was analysed, the nestlings lost significantly less weight after the LPS treatment than after the PBS treatment. This was, nevertheless, most probably an effect of unevenly represented variability in weight in the relatively small PBS subsample. When I re-analysed the same relationship in a random-sibling-pair design only in a subsample of nests with both PBS-treated and LPS-treated nestlings, I did not find any significant effect of the LPS-induced skin inflammation on either weight or body mass. In contrast to this study, Bonneaud et al (2003) and Hegemann et al (2012) reported a decrease in weight after the LPS treatment. The higher loss of weight after the LPS-treatment than after no treatment or after the PBS-treatment during the night (after 13h/16h) may reflect the effect of metabolic changes after the LPS treatment (Hegemann et al. 2012). Our results are, therefore, difficult to compare with these two studies due to the different timing of the weight measurement. Since we measured the weight change in nestlings as late as after 24hours, our results are likely a mixture of the effects of metabolic changes and parental feeding rate.

In the present study the swelling response was associated with the hatching date of nestling (if TLR4 genotype was considered), which is consistent with other similar studies focused on ecological factors explaining variation in the pro-inflammatory responsiveness (Lifjeld et al. 2002). As proposed by Lifjeld et al (2002) this relationship may be based on varying weather and changing availability of food resources in the course of the breeding season. This environmental factor, hence, may or may not play a role. Berthouly et al (2008), for instance, did not find any effect of the hatching date on LPS-induced swelling.

The two LPS types utilised in this study (LPS_{Ecol} and LPS_{Sent}) had remarkably different immunostimulatory properties. LPS_{Sent} triggered a stronger oxidative burst than LPS_{Ecol} when measured in vitro (RLUmax and AUC). In contrast to my findings, Sild and Horak (2010), reported that the oxidative burst response induced by LPS_{Ecol} measured in vitro was six time higher than the one induced by LPS_{Sent}. Sild and Horak (2010) used, nonetheless, a different serotype of *Salmonella* (*S.enterica* serotype Typhimurium instead of *S.enterica* serotype Enteritidis used in my study), which could explain the observed difference. The results by Sild and Horak (2010) are, however, consistent with our own findings from the swelling response measured in vivo (in our study the swelling response measured 24h post injection was stronger to the LPS_{ecol} treatment than to the LPS_{Sent} treatment). In our study only in LPS_{Ecol}-stimulated blood the oxidative burst intensity correlated with TLC (but not with H/L). Importantly, the RLUmax was negatively correlated with the absolute number of heterophils and monocytes in the blood. The same negative correlation has been described also by Sild and Horak (2010). Their explanation was that the intensity of the oxidative burst depends more on functioning of phagocytic cells than their number in peripheral blood and that in some case peripheral heterophilia may cause suppression of the functional activity of heterophils. This does not seem to be a convincing explanation as, according to our and even their data, the birds examined in any of the studies did not suffer from clinical heterophilia. The oxidative burst in heterophils was previously described (Farnell et al. 2003a; Farnell et al. 2003b) but there is no report of a relationship between intensity of the oxidative burst and number of heterophils in blood published in the literature. In my opinion, a more plausible explanation of our results is that the Pholasin assay detects also some other and very different reactive molecules than expected, and these may be produced in a different context by different cell types than anticipated in the experimental design (possibly there may be even a negative correlation between the other unknown factors and the heterophils count). Another possible explanation may involve the stress response induced by some blood-sucking exoparasites. Stress causes an increase in numbers of heterophils in blood and parasite-mediated injury may lead to aggregation of thrombocytes and their decrease in the blood stream. Because thrombocytes have also a phagocytic activity and exhibit an oxidative burst (Wigley et al. 1999; St Paul et al. 2012) we might explain our observations by a negative correlation between heterophils count and thrombocyte count (explaining the intensity of the oxidative burst). Unfortunately, neither in our study nor in the study

reported by Sild and Horak (2010) the thrombocyte count had been measured. In LPS_{Sent} stimulated blood there was no association between the oxidative burst and TLC or the absolute number of heterophils and monocytes in the blood, which makes any conclusions even more problematic.

V.3 Effect of the Q549R genotype on body condition and pro-inflammatory responsiveness (Task 3)

It has been previously shown that several polymorphic sites in the extracellular domain of TLR4 are the key for to the TLR4 functioning and variability at these sites may be responsible both for variation in immunological profiles and resistance to diseases. In humans is the presence of specific alleles of TLR4 associated, for instance, with susceptibility to cancer (Kutikhin 2011), pulmonary tuberculosis (Najmi et al. 2010), malaria (Ferwerda et al. 2007), atherosclerosis (Kiechl et al. 2002), and endotoxine-associated asthma (Arbour et al. 2000). Similar results are known in model laboratory species especially in mice and chickens, and also in livestock (susceptibility to pulmonary lesion in swine (Yang et al. 2012), infectious bovine keratokonjunctivitis (Kataria et al. 2011) Johne's disease in cattle (Mucha et al. 2009), bacterial infection of the mammary glands in sheep bacterial (Swiderek et al. 2006). In contrast, there is a lack of studies investigating similar associations between polymorphism in TLR4 and prevalence of diseases (or effect on condition) in free-living birds, namely passerines. To my knowledge, the diploma thesis by Bainova (2011) is the first study on this topic in passerine birds. She revealed a marginally significant relationship between Q549R polymorphism in TLR4 and ornamental colouration (specifically to the melanin-based and carotenoid-based ornamentation) in great tits. This suggests a linkage between Q549R genotype and sexual selection in this species. Ornamental plumage coloration represents a condition-related trait associated with individual health. From this reason, in this study, I also investigated the effect of Q549R polymorphism on condition and health in our study population of great tit nestlings. Contrary to the original expectations, I revealed only a limited effect of Q549R genotype on nestling vigour. Similarly to Bainová (2011) but on a bigger sample and with additional analyses I conclude that Q549R polymorphism has no effect on general condition (weight, growth, size, body mass), haematological parameters or intensity of the oxidative burst in great tits when investigated on the whole population level in individuals untreated with any experimental pathogen. Interestingly and in contrast to this general finding, the in vivo measurement of inflammatory responsiveness was significantly affected by the Q549R polymorphism (the swelling response measured 24h after the LPS stimulation). Furthermore, there was a significant interaction between Q549R polymorphism and the type of LPS injected. This result seems to reflect the structural changes induced by the substitution at the LPS binding side of TLR4. My observation is consistent with the matching alleles model of host-parasite interactions which describes the polymorphism in host and parasite genes as adaptations of the hosts to match perfectly and, hence, detect the parasite structures (Agrawal and Lively 2003). The 549Q variant

seems to respond more to LPS_{Ecol} and less to LPS_{Sent} while in the 549R variant the situation tends to be the opposite. In ecological studies it has been repeatedly postulated that the greater swelling response in a skin test reflects better immunity or immunocompetence (see, e.g., Berthouly et al. 2008). Contrary to this statement it has been emphasized in several articles that the magnitude of an immunological response (skin swelling response or any other) does not necessarily mean better parasite resistance (i.e. immunity) or better lifetime fitness (Graham et al. 2005; Viney et al. 2005; Owen and Clayton 2007; Vinkler and Albrecht 2011). Terms "better" and "worse" are only relative when the activity of the immune system is described and should be used with much caution. The efficiency of any immune response is specific to a certain host-parasite interaction. Exactly this pattern seems to be nicely illustrated by the present results. However, which is "better" is difficult to judge. It has been shown that overreaction to a particular stimulation is equally or even more harmful to the host than the activity of the pathogen itself. The higher immune responsiveness may therefore not be associated with higher fitness. For example, it has been shown in scarlet rosefinch that individuals exhibiting higher skin-swelling responses to PHA were less ornamented than the low-responding individuals (Vinkler et al. 2012). This result suggests that overreaction on an inadequate stimulation is more a sign of imbalance in the immune system that the evidence for more efficient immune function. Some caution in generalisation of my results is needed since the substitution Q549R had no effect on the oxidative burst measured in vitro. This may suggests that the difference between genotypes may not be a matter of differential effector activity of stimulated cells but a matter of variability in cellular signalling associated with tissue infiltration. Further investigation is needed to reveal whether the difference in TLR4 genotype variants is associated with differential cytokine signalling and resistance to any pathogens naturally occurring in the great tit population (see, e.g., call for carefulness by Owen and Clayton 2007).

VI. Conclusion

Inflammation is an essential element of the vertebrate immune function. This vitally important complex of immunological mechanisms plays a key role in many aspects of innate as well as adaptive immune defence against invading pathogens. PRRs, including TLRs play a cornerstone role in activation of inflammation because they trigger the very first immune response which may prevent spreading of the pathogen to the host organism. TLRs are expressed on many different cell types and recognise conservative structures derived from the pathogens. TLR4 is responsible for recognition of bacterial structures such as LPS derived from the cell walls of Gramm-negative bacteria. In the present study I investigated the TLR4-mediated response to LPS in a model free-living passerine bird, the great tit. LPS binds to the TLR4 which triggers the signalling cascade leading to expression of immunomodulatory molecules, the cytokines. Herein, I verified that in 15-day-old great tit nestlings several important pro-inflammatory cytokines, such as *IL-1β*, *IL-6*, *IL-18*, *TNFSF15,* and *CXCLi2*, are expressed in the skin tissue after the experimental LPS inoculation. The cytokine function is to attract leukocytes into the place of infection and regulate their activity in effort to modulate the local inflammatory response. I have shown that LPS treatment induces a significant wing-web swelling response that is presumably associated with leukocyte infiltration into the site of the LPS treatment. Elimination of pathogens occurs mainly throughout phagocytosis and oxidative burst in the infiltrating cells. The results of my in vitro experiments have shown that LPS induces a strong oxidative burst in blood-borne cells. These effector mechanisms are highly effective but can also cause serious tissue damages leading to the organ failure and autoimmune diseases. A proper regulation of inflammation is thus as important as inflammation itself and its optimal function may be crucial to the host survival. Unfortunately, my attempt to quantify the cytokine expression at the site of LPS treatment in different time points failed and, hence, I could not correlate the immunological performance of the nestlings with their condition and growth. I did not find any significant effect of the local LPS-induced inflammation on changes in nestlings' condition. Individuals bearing genotypes of the immune related genes that are better adapted to the actual ecological context are expected to be healthier and exhibit higher fitness. In my thesis I focused on a TLR4 Q549R genetic variation which had been previously proposed as an important genetic trait affecting fitness in great tits. I have found that both alleles are frequent in the study population and, given the tendency for deviation from the Hardy-Weinberg equilibrium, they may be under some current selective pressure. Polymorphism in immune receptors is maintained by the balancing selection evoked by pathogens. One of the hypotheses of host-parasite co-evolution towards polymorphism maintenance is known as the Matching alleles model. This model was proposed as particularly suitable for receptor evolution and postulates that the host-parasite interactions lead to specific adaptation of the host molecules (receptors) to perfectly match (detect) the specific parasite structures (sensu lock and key). In the great tit nestling population I revealed

an effect of the Q549R substitution in TLR4 on the inflammatory responsiveness to LPS. Intriguingly, I found a significant effect of the interaction between host TLR4 Q459R genotype and LPS type on the magnitude of the LPS-induced skin-swelling response. My results, thus, fully support the Matching alleles evolutionary hypothesis. On the other hand, the Q549R genotype had no effect on the oxidative burst response, which might indicate that the difference between genotypes did not cause differences in cellular effector activity but rather affected activation of signalling cascades. This variability may cause a different cytokine release and, hence, differentiate the development of the immune response. Further work should reveal whether the difference in TLR4 genotype is associated with differential cytokine signalling or not. The present study, however, confirms the important role of the Q549R substitution in maintenance of health in great tits.

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