Charles University

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Microbiology

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Effects of microbiota on defense system of earthworms

Vliv mikrobioty na obranné mechanismy žížal

Ph.D. Thesis

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Prague, 2017

Čestně prohlašuji, že jsem nepředložil tuto práci ani její podstatnou část k získání jiného nebo stejného akademického titulu. Práci jsem zpracoval samostatně a uvedl všechny použité informační zdroje a literaturu.

V Praze dne 15. května 2017 Jiří Dvořák

Rád bych poděkoval svému školiteli RNDr. Martinovi Bilejovi, DrSc. za pomoc, vedení, cenné rady a věcné připomínky, které jsem využil při kompletaci této práce. Mé poděkování patří též mým kolegům z Laboratoře buněčné a molekulární imunologie Petře Procházkové, Radce Roubalové a Františkovi Škantovi za spolupráci při získávání údajů pro výzkumnou část práce. Dále bych rád poděkoval všem skvělým lidem, které jsem za posledních 14 let poznal ve studentském klubu Přírodovědecké fakulty Mrtvá ryba. V neposlední řadě bych rád poděkoval svým rodičům za podporu a pomoc, kterou mi po celou dobu mého studia poskytovali.

Table of contents

Abstract

Earthworms are important soil invertebrate organisms that participate in nutrient cycling in terrestrial ecosystems and in the formation of the soil profile from the physical, chemical and also microbial point of view. Soils are considered the most microbially diverse environments on earth. All invertebrates living in soil therefore need to possess a complex immune system. Earthworms are used as a model organism in immunology for decades. Their simple body plan consists of two main body cavities: true coelom and digestive tube. Both coelomic cavity and digestive tract represent open systems with permanent contact with soil microorganisms. *Eisenia andrei* species is used as a standard immunological model in our laboratory for many years. *E andrei* earthworms live in compost, microbially abundant environment, which is reflected in their well-developed immune system. Some new mechanisms of *E. andrei* defense system are described in this work. Two novel pattern recognition receptors (PRRs), Toll-like receptor (TLR) and lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI) were characterized in earthworms. These molecules are expressed in coelomocytes and their production is upregulated after microbial challenge. Moreover, both receptors were detected in digestive tract of earthworms. Intestine of earthworms contains significantly higher amount of microorganisms in comparison with coelom. Interactions between microorganisms and mucosal immune system of the gut are more complex and they also include digestive enzymes which can promote molecular recognition of bacterial components by immune system. We hypothesize that high microbial load of bacteria in the gut can trigger release of coelomocytes from the coelomic lining and the differentiation of free coelomocytes in coelom. Comparison of two epigeic earthworms, *E. andrei* and *E. fetida* revealed stronger immune response in coelom of *E. andrei* in some aspects, especially in the production of antimicrobial proteins. Moreover, adverse effects of dioxin pollution on earthworm gut were documented and the response to these toxins is reflected in higher expression of antioxidant molecules.

Abstrakt

Žížaly patří mezi významné půdní bezobratlé živočichy. Přispívají ke koloběhu živin v půdních ekosystémech a podílí se na tvorbě půd ovlivňováním jak fyzikálně chemických tak mikrobiálních procesů. Půdy jsou nejvíce mikrobiálně různorodým prostředím na Zemi. Půdní bezobratlí proto musí mít dobře vyvinutý imunitní systém. Žížaly se používají jako modelový organismus pro studium imunitních procesů několik desítek let. Mají jednoduchou stavbu těla. Její základní schéma je založeno na trávicí trubici, která leží uvnitř dutiny coelomu. Obě dutiny jsou otevřené vůči vnějšímu prostředí, a z toho důvodu obsahují mikroorganismy. V naší laboratoři používáme mnoho let jako imunologický model druh žížaly *Eisenia andrei*. Přirozeným prostředím těchto žížal je kompost obsahující velké množství mikroorganismů, a proto mají dobře vyvinutý imunitní systém. Objasnění nových obranných mechanismů tohoto druhu je předmětem této práce. Popsali jsme dva nové receptory rozpoznávající vzory u žížal, a sice *Toll-like* receptor (TLR) a protein rozpoznávající lipopolysacharid (LBP/BPI). Obě tyto molekuly jsou produkovány coelomocyty a jejich exprese se zvyšuje po mikrobiální stimulaci. Tyto receptory se také exprimují v trávicím traktu. Střevo žížal obsahuje mnohem více bakterií v porovnání s coelomem. Slizniční imunitní systém střev vytváří složité interakce se střevními mikroorganismy. Na těchto interakcích se podílejí také trávicí procesy, které mohou štěpením bakterií podpořit proces rozpoznávání bakteriálních komponent proteiny rozpoznávající vzory. Předpokládáme, že vysoká mikrobiální zátěž v trávicím traktu může podnítit uvolňování coelomocytů z výstelky coelomu a jejich následnou diferenciaci. Aktivita imunitního systému byla sledována u dvou epigeických druhů, *E. andrei* a *E. fetida.* Imunitní systém *E. andrei* je v některých ohledech efektivnější, konkrétně v produkci antimikrobiálních proteinů. Navíc bylo prokázáno, že dioxiny významně poškozují tkáně trávicího traktu. Žížaly reagují na tyto polutanty produkcí antioxidačních molekul.

List of Abbreviations

BPI	bactericidal/permeability-increasing protein
CCF	coelomic cytolytic factor
CFU	colony forming unit
COI	cytochrome c oxidase I
CRT	calreticulin
DNA	deoxyribonucleic acid
EaIRP	*E. andrei* earthworm IRP
EaLBP/BPI	*E. andrei* earthworm LBP/BPI
EaTLR	*E. andrei* earthworm TLR
EFAF	*Eisenia fetida andrei* factors
Hsp70	heat shock protein 70
IRE	iron-responsive element
IRP	iron regulatory protein
ISO	International Organization for Standardization
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeats
OECD	Organisation for Economic Co-operation and Development
PAH	polyaromatic hydrocarbons
PAMP	pathogen-associated molecular pattern
PCDD/Fs	polychlorinated dibenzo-*p*-dioxins and dibenzofurans
PCR	polymerase chain reaction
PRR	pattern recognition receptor
rEaIRP	recombinant *E. andrei* earthworm IRP
REMSA	RNA electromobility shift assay
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
UTR	untranslated region

1. Introduction and aims

Earthworms are important edaphic macroorganisms. They can be found in almost all types of soil habitats. Soils represent habitats accommodating probably the most complicated microbial communities of the world. For that reason, earthworms had to evolve efficient defense system which allowed them to survive in this microbially hostile environment. Earthworms as well as the other invertebrates rely only on innate immune system mechanisms. Both cellular and humoral parts of immune system of earthworms were described in last decades. Direct contact between microorganisms and immune components is mediated via mucosal surfaces of two body cavities of earthworms: coelom and digestive tract. Defense strategies of these two cavities are different. Multiplication of microorganisms in coelom is not desirable. Coelomocytes represent an effector part of coelomic immune system. They kill the bacteria by the mechanisms of phagocytosis and encapsulation and produce a broad spectrum of antimicrobial molecules into the coelom. In contrast, digestive tract of earthworms is in permanent contact with huge amount of ingested microorganisms from soil. This must be reflected in more complicated relationships between immune system of the gut and intestinal microbial communities, because permanent inflammatory response against gut bacteria should be restricted. Moreover some bacteria can have beneficial effects on the integrity and health of gut epithelium.

This thesis focuses on elucidation of relationships between microorganisms and immune system of earthworms both in coelom and in gut. Following aims of this thesis were settled:

- Identification of selective immune mechanisms of the earthworm *E. andrei* that allow its survival in compost, environment containing enormous number of bacteria.
- Characterization of new pattern recognition receptors of this species that can contribute to its ability to effectively resist bacterial infections.
- Description of relations between gut immune system and microbiota.
- Description of mechanisms that participate in the maintenance of iron homeostasis in earthworms taking into account its relations to immune system.
- Assessment of dioxin toxicity to immune system of the gut and coelom.

2. Scientific background

2.1. Earthworm anatomy

Earthworms are triploblastic coelomate invertebrates with bilateral body symmetry belonging to the phylum *Annelida*. The Annelids are considered to be a member of superphylum Lophotrochozoa that includes Mollusca, Brachiopoda, Bryozoa, Nemertea and Sipuncula (Halanych 2004). Earthworms belong to a group of Oligochaete. They are segmented worms adapted to the life in the terrestrial environment.

The tube-within-tube body plan of earthworms consists of digestive tract that runs through the entire length of the body. It is located in a cavity filled with fluid that functions as a hydrostatic skeleton. This body cavity is a true coelom which is lined by the coelomic epithelium derived from mesoderm (Anderson 1973) (Fig. 1A). The coelomic space is divided into compartments by the septa. This type of body segmentation represents an example of true metamerism consisting of segments with the same set of internal organs in each of them (Fig. 1B). Evolution of metamerism in annelids is proposed as an adaptation for burrowing (Clark 1964).

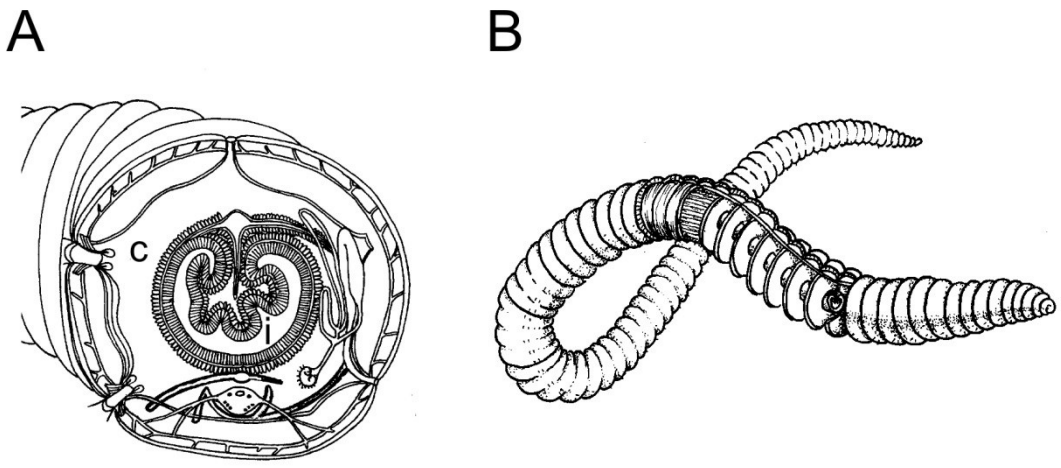

Fig. 1. **A** General scheme of earthworm's body organization; c coelom, i intestine (adapted from © BIODIDAC; Auth. Josée Soucie)

B Scheme of metameric body organization (adapted from © BIODIDAC; Auth. Ivy Livingstone)

The surface of earthworms is covered by a thin layer of cuticle, which protects the skin commonly pigmented in the scale from red to brown. Dorsal pores and nephridiopores represent external openings on the surface that contribute together with mucus producing cells to the moistening of the body of earthworms. Sexually mature individuals possess the ring-shape glandular structure, called the clitellum, which is located toward the anterior part of the animal. Clitellum represents the part of the reproductive system and produces egg capsules. Muscular system consists of two layers of muscles: a thin outer layer of circular muscles, and a thicker inner layer of longitudinal muscles. These layers of muscles are organized as a wall of coelomic cavity filled with coelomic fluid. Coelomic fluid contains cells called coelomocytes that possess various functions contributing to the maintaining of stable inner environment. These include: defense functions (Du Pasquier and Duprat 1968), trophic functions (Valembois and Cazaux 1970), excretion functions (van Gansen 1956) or synthesis of hemoglobin (Lindner 1965). At the center of this space is located digestive tract which is flanked by dorsal and ventral blood vessels and ventral nerve cord. Coelom is divided by perforated transversal septa, allowing the coelomic fluid to flow freely between segments. (Fig. 2).

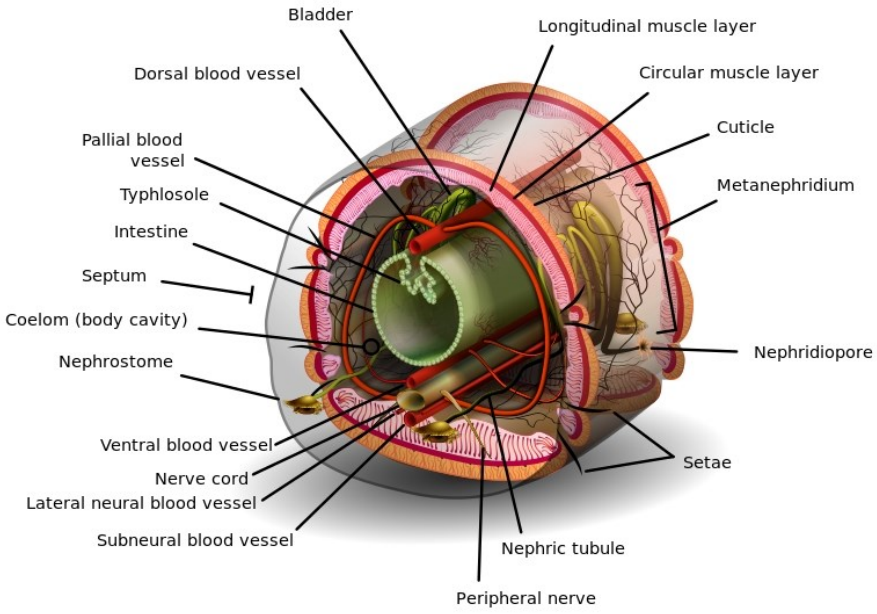

Fig. 2. Diagram of a post-clitellum segment (adapted from Wikimedia Commons; Auth. K. D. Schroeder; lic. CC-BY-SA 3.0)

Earthworms have a central and peripheral nervous system. The central nervous system consists of supra-pharyngeal and sub-pharyngeal ganglia which are connected with circum-pharyngeal connectives in the anterior part of body. This system is extended to the posterior part of body as ventral cord below the alimentary canal. The function of peripheral nervous system is to supply the innervation of the prostomium and buccal chamber as well as to supply the innervation of various structures of the body segments.

Earthworms have dual circulatory system: closed circulatory blood system and coelomic fluid. Both of these systems can transport nutrients, wastes and respiratory gases. The blood is pumped to the vessel system by aortic arches that are located in the anterior part of earthworms. The blood consists of amoeboid cells and hemoglobin particles dispersed in the plasma. There is no special respiration system. Gases are exchanged in capillaries, where the oxygen is picked up by the hemoglobin.

Excretory organs are almost in every segment of the body. Filtering organs are called metanephridia that remove metabolic waste from the coelomic fluid and expels it through nephridiopores.

Digestive system of earthworms is a straight tube which is differentiated into a buccal cavity, pharynx, esophagus, crop, gizzard and intestine (Fig. 3). The food is taken with the mouth. The pharynx is located directly after the mouth and acts as suction pump to transfer the food to the posterior part of digestive tract. It also excretes mucus to aid digestion in later stages. Behind the pharynx is a short, narrow, tube which is called the esophagus. In the esophagus the openings of calciferous glands are present. They release calcium into digestive canal to maintain pH of food and also proper blood calcium levels. From esophagus, the food passes to the crop and the gizzard. The crop is responsible for food storage. The gizzard is strong muscular organ, which grind the food with help of mineral particles. From the gizzard, food continues into intestine to further digestive processes. The digestion is done by the set of enzymes, such as amylases, cellulases, proteases and lipases (Tracey 1951, Kamat 1955, Nielsen 1962, Berner and Hammond 1970). Middle part of intestine is folded to the form of dorsal ridge, which is known as the typhlosole. This structure increases the digestive surface. Shape and size of this

organ differs among species from different feeding habitats (Semenova 1966). Indigested rest of food is expelled via anus in the posterior part of body.

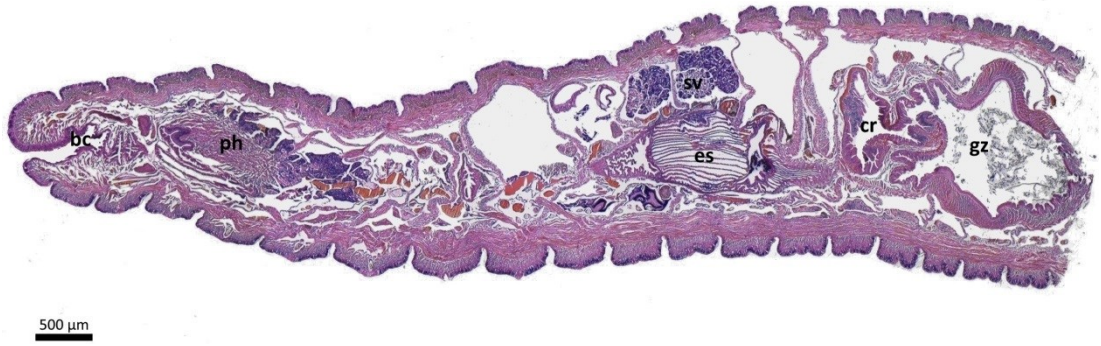

Fig. 3. Anterior body part of earthworm *Eisenia andrei* (sagittal section, 6 µm) stained with Hematoxylin and Eosin; **bc** buccal cavity, **cr** crop, **es** esophagus, **gz** gizzard, **ph** pharynx, **sv** seminal vesicles (Auth. Jiří Dvořák)

Earthworms are hermaphrodites and thus they have both male and female reproductive organs. Most earthworm species reproduce by cross-fertilization. Sperms are produced and stored in seminal vesicles. Eggs are produced in ovaries. Copulation and fertilization are separated in time. In the mating process, earthworms start to secrete mucous substances in the clitellum. This results in forming of a mucous ring around the anterior body parts of mating pair. During the copulation, sexual partners change their sperm cells and then store the sperm of their sexual partner in spermatheace. Eggs and sperm cells are ejected to this mucous ring, which is then transformed to a cocoon. Eggs are fertilized by sperm cells from spermatheace in the cocoon. The process of embryo development in the cocoon is dependent on the temperature and it takes several tens of days (Jensen and Holmstrup 1997).

2.2. Earthworm ecology

Earthworms represent important part of soil macrofauna in most terrestrial ecosystems (Edwards 1998). They occur all over the world with some exceptions like deserts and areas under constant ice and snow (Edwards and Bohlen 1996). Earthworms play an important role in many processes of soil transformation. Darwin was one of the first scientists who highlighted the importance of earthworms in pedogenesis (Darwin 1881). Their feeding and burrowing activities promote decomposition of organic debris, humus formation, cycling of nutrients and soil structural development (Mackay and Kladivko 1985, Lavelle et al. 1997). Earthworms are also an important part of the food chains. They serve as a food for other invertebrates as well as for birds and mammals.

Earthworms can be grouped according to their behavioral adaptation into three ecological groups: epigeic, endogeic and anecic (Bouche 1971, Bouche 1977) (Fig.4). Epigeic earthworms are active close to the surface of the soil. Species from this ecotype do not make burrows and accordingly consume decaying plant residues. These earthworms are responsible for converting surface debris to topsoil, an important process for the continual renewal of soil ecosystems. Epigeic earthworm species include *Dendrobaena octaedra, Lumbricus rubellus, Eisenia fetida, Eisenia andrei*. Endogeic earthworms inhabit organo-mineral, deeper soil profile. They make preferentially horizontal burrows through the soil and they feed with more humified organic matter mixed with soil. The horizontal burrows define their living space for relatively limited layer of soil. The representatives of endogeic earthworms include *Allolobophora chlorotica*, *Apporectodea caliginosa, Apporectodea rosea.* Anecic earthworm species create more permanent vertical burrows across the soil horizons. The burrows of anecic species may penetrate to depths of meter or more (Lee and Foster 1991). They feed on plant debris on the soil surface that they drag into their burrows. Anecic earthworms include for example *Lumbricus terrestris, Apporectodea longa*. From the evolutionary point of view, epigeic and anecic earthworms evolved from endogeic ancestors (Dominguez et al. 2015).

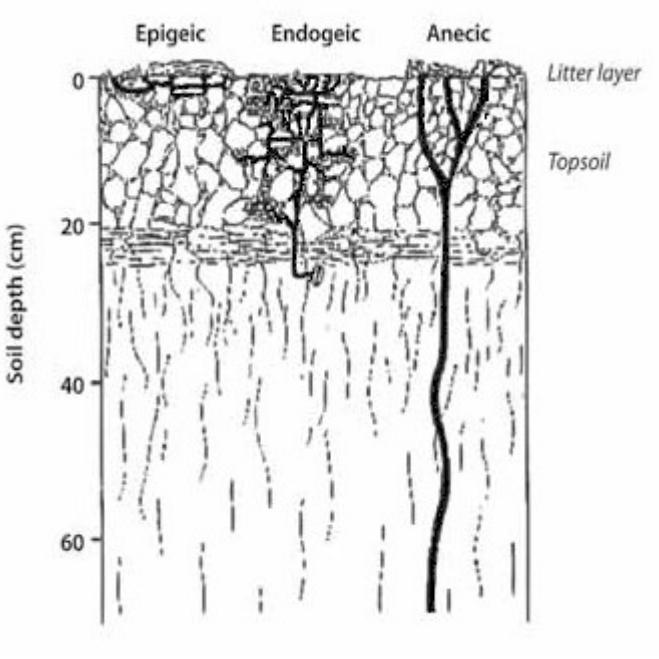

Fig. 4. Schematic representation of three ecological groups of earthworms, adapted from (Fraser and Boag 1998)

Humans produce many chemicals that can be accumulated in soils, such as heavy metals, organic residues as polyaromatic hydrocarbons (PAH), organophosphates, insecticides, benzo(a)pyrene and so on (Panagos et al. 2013). There is the necessity to monitor the impact of this pollution to soil ecosystems. Earthworms, as an important part of soil ecosystems, can be used as a tool for monitoring of pollution. Epithelial surfaces of earthworms are in close contact with soil particles (see above under 2.1.), therefore various chemicals reaching the soil system can affect the epithelia (Jager et al. 2003, Morgan et al. 2004). This makes earthworms a suitable model for studying an impact of various soil pollutants on their fitness and on physiological processes. Accordingly, various protocols based on the measurement of different aspects of earthworm's life were developed. These include for example a reproduction toxicity tests (Vangestel et al. 1989, Kula and Larink 1997) and avoidance tests (Yeardley et al. 1996). Cellular and biochemical approaches were also used for monitoring soil contamination, such as the measurement of lysosomal membrane stability using neutral red retention assay (Weeks and Svendsen 1996), detection of effects on cellular immune reactions (Giggleman et al. 1998), the comet assay measuring DNA strand breaks following

exposure to genotoxins (Salagovic et al. 1996), assessment of sperm number and morphology (Cikutovic et al. 1993, Reinecke and Reinecke 1997), measurement of activity of different enzymes involved in various repair/detoxification mechanisms or oxidative stress response: gluthation S transferase (Hans et al. 1993), cytochrome P450 enzymes (Achazi et al. 1998), superoxide dismutase, catalase and peroxidase (Saint-Denis et al. 1999), metallothioneins (Sturzenbaum et al. 2001) and heat shock proteins (Marino et al. 1999). Different earthworm species were used in these tests. Two species *E. fetida* and *E. andrei* have been used for many years in monitoring ecotoxicity. There are two guidelines for assessment of ecological risk of contaminated soil reported by both the Organisation for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO) determining the acute toxicity of chemicals on earthworms (OECD 1984, ISO 1993) and the effect on their reproduction (ISO 1998, OECD 2004). These species are easily cultured under the laboratory conditions and have high rate of reproduction (Tomlin and Miller 1989).

2.3. Soil microorganisms and earthworms

Soil is a vibrant environment for huge spectrum of microorganisms including bacteria, protists, algae and fungi. They continually interrelate with each other and with the soil and therefore generate ever changing conditions. For that reason, soils are considered the most microbially diverse environments on earth (Daniel 2005). Number and diversity of microorganisms is not homogeneous across the soil profiles. Abundance and composition of microbial communities is significantly decreased with soil depth (Ekelund et al. 2001, Taylor et al. 2002). Concurrently the proportion of Gram-negative to Gram-positive bacteria is declining in deeper strata compared to surface soil (Blume et al. 2002, Fierer et al. 2003). Therefore, earthworms belonging to different ecotypes are in contact with different populations of microorganisms.

Functional domain of soil which is affected by activities of earthworms is referred to as drilosphere (Bouche 1975, Lavelle 1988, Brown et al. 2000). It includes internal micro-environment of earthworm gut, the earthworm surface in contact with soil and structures produced by earthworms (casts and burrows).

During the feeding process, earthworms dramatically modify the structure and properties of soils (Barois and Lavelle 1986, McLean et al. 2006). Earthworms are able to ingest between 2 and 30 times of soil as much as they weight per day (Lee 1985). One gram of soil can contain up to 10^9 microbial cells (Torsvik and Ovreas 2002). It has been demonstrated that soil microbiota can serve as a part of earthworm diet (Moody et al. 1995, Zhang et al. 2000). Moreover it was shown that physicochemical modifications during digestion processes increase the respiratory activity of the soil microflora in the gut of earthworms (Barois and Lavelle 1986). Therefore intestine of earthworms can act as a kind of bioreactor where microbial activity and biomass are increased due to favorable conditions (Lavelle and Gilot 1994). It was also proved that enzymatic machinery of gut microbiota contribute to digestive processes in the gut of epigeic earthworms (Curry and Schmidt 2007). This indicates that interactions between earthworms and microorganisms are very complex and beneficial for both. This kind of mutualism must be very well balanced and thus it is necessary to possess a complex system of defense mechanisms, which are responsible for maintaining of such equilibrium.

2.4. Defense system of earthworms

Fundamental functions of immune system consist in securing of cellular integrity, maintaining of homeostasis and survival of host. Elementary principles enabling these functions are based on abilities to recognize "self" from "non-self" followed by the elimination of "non-self". The mechanism of differentiation between host structures (self) and invader structures (non-self) is mediated by cellular receptors. These receptors have been termed “pattern recognition receptors” (PRRs) that serve to recognition of invader structures called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). PAMPs are small molecular motifs conserved within a class of microbes which are not present in the bodies of host organism. Important PRR named Toll was identified in *Drosophila.* It was proved that the absence of a functional Toll protein is responsible for the susceptibility of *Drosophila* to fungal infections (Lemaitre et al. 1996). Recognition of non-self structures via PPRs is widespread among all Metazoan (Dzik 2010, Messier-Solek et al. 2010). Most primitive principle of elimination is based on

absorption of foreign particles by host cells. This phenomenon is called phagocytosis and it is evolutionary very old mechanism. First appearance of phagocytosis can be traced up to the origin of first unicellular eukaryotes (Cosson and Soldati 2008). Another complex of mechanisms based on non-cellular principles of elimination is collectively called humoral immune system. It includes a broad spectrum of extracellular molecules with different mechanisms of action such as antimicrobial peptides, agglutinins, hemolysins, opsonins, cytolytic proteins, lysozymes and so on. Some of these molecules are able to trigger a biochemical cascades that then can eliminate potential pathogens. This set of defense mechanisms (cellular and non-cellular) is called innate immune system. The innate immune system is an evolutionarily old defense strategy, and it is the dominant immune system found in lower organisms including all invertebrates (Janeway et al. 2001).

Invertebrates were used as a model in immunological research in some fundamental studies. For example, process of phagocytosis was first described by Élie Metchnikoff (Metchnikoff 1893) on a model of floating larvae starfish. Among invertebrates, earthworms (in comparison with other invertebrate models like *Drosophila* or *C. elegans*) do not a play central role in immunological research nowadays. Nevertheless, some important findings were achieved using this model. Earthworms were used in the experiments with rejections of grafts and thus significantly contributed to the knowledge of immunological tolerance and transplantation immunology (Cooper 1969). The progress in the development of new high-throughput technologies of molecular biology methods have allowed to reveal the microbial complexity of some environments like is a soil (Roesch et al. 2007). Earthworms can contribute to the knowledge of evolutionary significant mechanisms, which are able to strengthen their immune system for surviving in this microbially abundant environment.

The skin of earthworms represents the first protective barrier against outer environment of soil. It consists of single layer of epidermis covered by thin cuticle. The cuticle contains mucopolysaccharides that act as an antimicrobial barrier (Rahemtulla and Lovtrup 1974). The skin is not absolutely impermeable, but it contains dorsal pores and nephridiopores that represent a connection between

outer environment and coelomic cavity. As a consequence, coelomic fluid of earthworms naturally contains a number of microorganisms (Marks and Cooper 1977). The coelom is lined with mesenchymal layer, which is considered to be a source of free coelomocytes (Cameron 1932, Liebmann 1942). Coelomocytes can be classified according to different criteria. Most general classification divided the cells to two subgroups: amoebocytes and eleocytes (Stein and Cooper 1983). They are present in various proportions in different species (Kurek et al. 2002). Origin, function and classification of these cell subpopulations are still not sufficiently resolved. Generally it is believed, that eleocytes are responsible for nutrition and amoebocytes for defense (Sima 1994). However, eleocytes of *E. foetida* (*E. andrei*) are capable of phagocytosis (Duprat and Bouc-Lassalle 1967), contrary to eleocytes of *Lumbricus terrestris* (Stein et al. 1977). Therefore, all coelomocytes can be considered as a part of both cellular and humoral immunological response.

Coelomocytes play critical role in controlling numbers of microorganisms in coelomic cavity. Their quantity exceeds more than ten times the number of naturally occurring bacteria in the coelom (Dales and Kalac 1992) suggesting that microorganisms can be effectively eliminated by phagocytosis. Coelomocytes acts simultaneously as phagocytes and producers of various molecules involved in bacterial recognition and killing via non-cellular processes. One of these molecules involved in process of recognition is named coelomic cytolytic factor (CCF). This molecule was isolated from a coelomic fluid of E*. foetida (andrei)* earthworm and identified as a cytolytic protein (Bilej et al. 1995). CCF was later found to be expressed in free coelomocytes (Bilej et al. 1998). Further studies revealed that this protein acts as PRR with binding affinity to ß-1,3-glucan and lipopolysaccharide (LPS) (Beschin et al. 1998). Detailed characterization of CCF resulted in identification of two distinct carbohydrate domains binding cell wall components of Gram-positive bacteria (peptidoglycan constituents muramic acid and muramyl dipeptide), Gram-negative bacteria (O-antigen of lipopolysaccharide) and yeast (ß-1,3-glucan and *N, N'*-diacetylchitobiose) (Bilej et al. 2001). One domain is located in the central part of CCF molecule and interacts with LPS and ß-1,3-glucan. The C-terminal tryptophan-rich domain mediates interactions of CCF with *N, N'*-diacetylchitobiose and peptidoglycan constituents. CCF represents the first PRR

which has been described in earthworms. Comparative analysis of CCF molecules from seven lumbricid species revealed the differences of binding specificity in C-terminal domain. Only CCF from *E. fetida (andrei)* has broader saccharide-binding specificity, being the only one recognizing *N, N'*-diacetylchitobiose (Silerova et al. 2006). This species lives in compost, which is microbially abundant environment. *E. fetida (andrei)* must therefore resist to broader microbial load, which is probabily reflected in the broader CCF pattern recognition repertoire.

Large foreign particles that cannot be engulfed by a single phagocyte, such as agglutinated bacteria or parasites, are eliminated by the process of encapsulation. Encapsulation starts with the capsule formation consisting of coelomocytes, which recognize and surround foreign particles. Foreign bodies are subsequently destroyed in the process of melanization and generation of free radical byproducts. The process of melanization starts with the activation of prophenoloxidase cascade, which is a basic defense mechanism in many invertebrate species (Cerenius and Söderhäll 2004). Pattern recognition receptors play crucial role in this activation and it was shown, that CCF triggers the prophenoloxidase cascade in earthworms (Beschin et al. 1998). Protein fraction, which participates in prophenoloxidase cascade, was later detected in coleomic fluid of *E. fetida* earthworm (Prochazkova et al. 2006b). Final product of encapsulation is called brown body. Brown bodies of earthworms usually contain tissue waste, agglutinated bacteria or parasites such as gregarines and nematodes (Valembois et al. 1992, Valembois et al. 1994). Brown bodies are transported to posterior segments where they can be eliminated by caudal autotomy. In earthworms, both amoebocytes and elocytes participate in the process of encapsulation and brown bodies formation (Cooper 1996).

Coelomocytes synthesize and release a broad spectrum of extracellular proteins with various defense effects. This includes production of factors with antibacterial, lytic and agglutination activities that act against "non-self". Together they create a complex system of non-cellular humoral immunity.

The coelomic fluid of *E. fetida* earthworms exhibits numerous biological activities including hemolytic activity. The majority of the proteins with hemolytic activity identified so far show bactericidal and/or bacteriostatic activities against

pathogenic soil bacteria (Valembois et al. 1986, Roch et al. 1991). Therefore, the biological relevance of the *E. fetida* cytolytic and agglutinating system consists partly in growth inhibition of the potential pathogens living in manure and possessing antigens common with red blood cells. For example, proteins with hemagglutination activity against rabbit and rat erythrocytes were revealed in coelomic fluid of earthworms (Stein et al. 1982). Factors responsible for agglutination of bacteria were detected also in coelomic fluid of *Lumbricus terrestris* (Stein et al. 1986). Some of these molecules were characterized in detail and their roles in defense mechanisms were elucidated. First hemolytic proteins were described in late 1960s (Du Pasquier and Duprat 1968) and later on they were named *Eisenia fetida andrei* factors (EFAF) and characterized as two glycoproteins with molecular masses of 40 and 45 kDa (Roch et al. 1981). Polymorphism on genetic level was revealed among these proteins (Roch et al. 1987). Moreover these proteins participate in the cytotoxic activity of coelomic fluid (Kauschke and Mohrig 1987) and exhibit antibacterial activity against Gram-positive and Gram-negative bacteria (Lassegues et al. 1989). EFAF were characterized at molecular level. They were sequenced and named fetidins (Lassegues et al. 1997, Milochau et al. 1997). Independently, a similar protein secreted by coelomocytes that causes contraction of rat vascular smooth muscles was purified from coelomic fluid and named lysenin (Sekizawa et al. 1996, Sekizawa et al. 1997). Simultaneously, two 42 - kDa lysenin-related proteins with weak contraction activity were identified. More recently, new member of this gene family was described and named lysenin-related protein 3 (Bruhn et al. 2006). Analysis of all these proteins showed that they share high sequence homology. It was proved that fetidin and lysenin are encoded by two distinct genes and their expression differs in individual earthworms (Prochazkova et al. 2006a).

Lysozyme is a ubiquitous enzyme widely distributed within the animal and plant kingdoms. It possesses the hydrolytic activity to specifically cleave β- 1,4-glycosidic bonds between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (NAM) of the peptidoglycan present in the bacterial cell walls and thus efficiently protects the host against infections caused by Gram-positive bacteria (Jolles 1996). The lysozyme activity was documented also in coelomic fluid of *E. fetida* (Cotuk and

Dales 1984). Later on, partial protein sequence of earthworm lysozyme was obtained and showed its high homology with other known invertebrate lysozymes (Ito et al. 1999). Finally cDNA sequence of earthworm lysozyme was characterized and recombinant protein was prepared (Joskova et al. 2009). Moreover it was shown that lysozyme expression can be up-regulated after a microbial challenge.

General scheme of immunological processes in coelomic cavity is viewed in Fig. 5. To sum up, excessive amount of phagocytic cells and powerful antibacterial humoral mechanisms are combined into efficient system which prevents the multiplication of microorganisms in coelomic cavity.

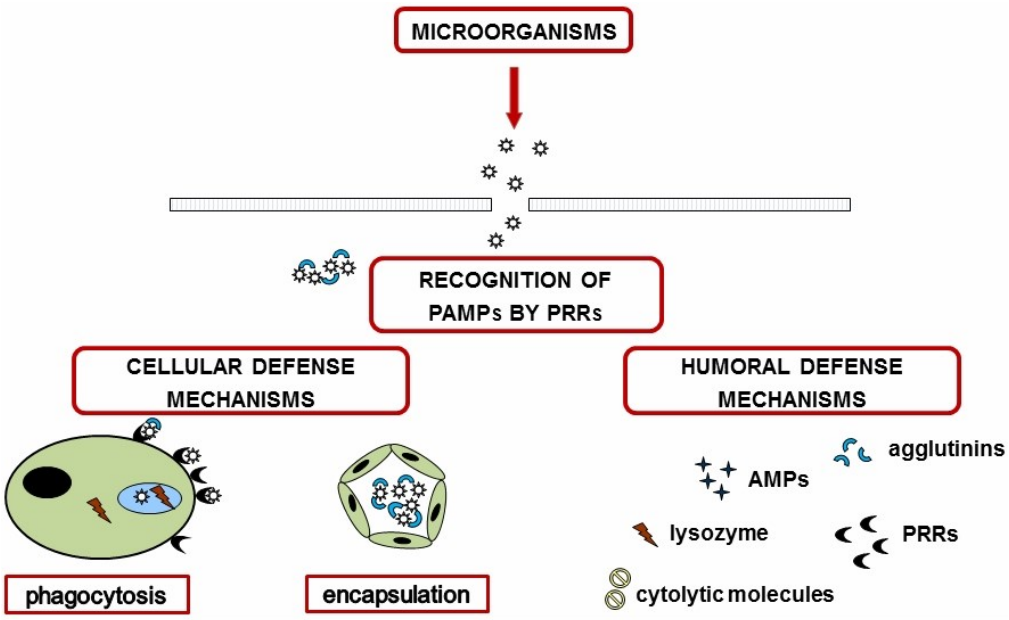

Fig. 5. The general scheme of the innate defense mechanisms in earthworms. The first protective barrier of earthworms is the skin in combination with the secreted mucus that contains various antimicrobial factors. Invading microorganisms are recognized by both soluble and membrane-bound pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs). On the basis of this recognition, microorganisms are phagocytized by coelomocytes or agglutinated and subsequently encapsulated. Moreover, genes encoding various humoral factors involved in the elimination of invaders are expressed, such as antimicrobial peptides (AMPs), cytolytic molecules, agglutinins, lysozyme and various soluble PRRs that trigger the activation of the prophenoloxidase cascade (Roubalova et al. 2015).

Mucosal immune processes in gastrointestinal tract of earthworms are not well characterized. Therefore basic mechanisms of innate intestinal immunity will be explained more generally. Multicellular organisms are metaorganisms composed of the macroscopic host and synergistically interdependent both prokaryotic and eukaryotic microorganisms (Bosch and McFall-Ngai 2011). Ecological communities of these commensal, symbiotic and pathogenic microorganisms found in and on all multicellular organisms are collectively called microbiota (Peterson et al. 2009). The lines between the body of multicellular organism and microbiota are referred to as mucous membranes. These membranes surround the internal organs and consist of one or more layers of epithelial cells. The origin and development of mucosal surfaces represent a major evolutionary step that supported metazoan life (Mestecky et al. 2015). Mucous membranes represent a physical barrier and participate in a key part of the immune system, which is called mucosal immunity. Microbiota and host mucosal immune system are connected with each other by exchanging of components based on molecular recognition. Microbiota modulates immune system and immune system can change microbiota composition. Key components of mucosal immunity that mediate interaction between host and microbiota are pattern recognition receptors (Fukata and Arditi 2013). Two contradictory principles should be equilibrated for maintaining of homeostasis in the environment of the gut. Potential pathogens must be recognized and eliminated. Constant higher level of inflammation against commensal bacteria should be avoided. Complex interactions of commensal communities of microbiota residing on epithelial surfaces of host's organisms are crucial for normal development and maintenance of immunological integrity (Hooper and Gordon 2001). For example microflora-activated innate immunity can enhance the integrity and barrier functions of intestinal epithelial cells (Cario et al. 2007). Signaling via TLR can contribute to homeostatic functions of the gut (Fukata and Abreu 2007) and it was shown that TLR mediated activation of innate immunity has resulted in the secretion of anti-microbial peptides, but not proinflammatory response (Uehara et al. 2007). Taken together, activation of innate immunity at the host-microbial interface of intestinal tract has adapted from inducing inflammation to healing and restitution (Raz 2007). This tissue adaptation can be explained by the ability of

enterocytes to undergo polarization, which modifies inflammatory TLR activation (Lee et al. 2008). In conclusion, protective effect of mucosal immunity on the mucosal surfaces is essential for maintaining epithelial integrity and thus proper metabolic function of gastrointestinal tract.

2.5. Iron homeostasis and immune processes

Immunological processes can be influenced by the concentration of some essential elements. Free iron is one example and its concentration must be under control. It is essential element for all living organisms that acts as a cofactor in fundamental biochemical processes in cells. On the other hand, high concentration of free iron is toxic and it catalyzes the formation of oxidative radicals that can damage cells (Albretsen 2006). Thus, both iron deficiency and iron excess can have adverse effects on basic life functions.

Ferritins are major iron-storage proteins (Harrison 1986). They play a key role in iron homeostasis of all organisms (Theil 1987). Molecule of ferritin consists of 24 subunits forming a nanocage capable of storing up to 4500 iron atoms (Aisen et al. 1999). These subunits are able to incorporate iron due to the presence of ferroxidase centers (Andrews et al. 1992). The expression of ferritin is regulated at the posttranscriptional level by the interactions between the iron regulatory protein (IRP) and the iron-responsive element (IRE) in the 5'-untranslated region (UTR) of ferritin mRNA (Hentze et al. 1989). Two IRPs have been described in vertebrates (Leibold and Munro 1988, Rouault et al. 1988). IRP1 can either bind an IRE site or function as a cytosolic isoform of aconitase (Haile et al. 1992). Three-dimensional structure of IRP1 is dependent on iron concentration and it switches between IRE-binding protein form and aconitase form (Rouault et al. 1991, Kennedy et al. 1992). At low iron concentration, IRP binds to the IRE and thus blocks the translation of ferritin due to impossibility of ribosomes to bind 5' UTR region of ferritin mRNA (Gray and Hentze 1994). High concentration of iron leads to assembling of 4Fe-4S cluster in IRP1 and this provokes the change of this protein into aconitase form which does not possess a function of mRNA binding protein. IRP2 has only the IRE binding activity (Guo et al. 1994). IRE is evolutionary conserved structure recognized by regulatory proteins (Piccinelli and Samuelsson 2007). It is a short stem-loop

containing typical CAGUN motif in the loop and unpaired C residue five nucleotide upstream from this motif. This arrangement appears to be critical for the function of IREs (Henderson et al. 1994). It was shown that ferritin played a role in immune response as an acute phase reaction protein in process of infection (Torti and Torti 2002). The role of ferritin in immune reactions of invertebrates was documented in echinoderms (Beck et al. 2002) and crustaceans (Ruan et al. 2010).

3. List of publications

I. **Dvořák J.**, Mančíková V., Pižl V., Elhottová D., Šilerová M., Roubalová R., Škanta F., Procházková P., Bilej M.: Microbial environment affects innate immunity in two closely related earthworm species *Eisenia andrei* and *Eisenia fetida*. *PLoS One* 8(11) (2013).

II. Škanta F., Roubalová R., **Dvořák J.**, Procházková P., Bilej M.: Molecular cloning and expression of TLR in the *Eisenia andrei* earthworm. *Dev. Comp. Immunol*. 41: 694-702 (2013).

III. Škanta F., Procházková P., Roubalová R., **Dvořák J.**, Bilej M.: LBP/BPI homologue in *Eisenia andrei* earthworms. *Dev. Comp. Immunol.* 54: 1-6 (2016).

IV. **Dvořák J.**, Roubalová R., Procházková P., Rossmann P., Škanta F., Bilej M.: Sensing microorganisms in the gut triggers the immune response in *Eisenia andrei* earthworms. *Dev. Comp. Immunol.* 57: 67-74 (2016).

V. Procházková P., Šustr V., **Dvořák J.**, Roubalová R, Škanta F., Pižl V., Bilej M.: Correlation between the activity of digestive enzymes and nonself recognition in the gut of *Eisenia andrei* earthworms. *J. Invertebr. Pathol.* 114: 217-221 (2013).

VI. Procházková P., **Dvořák J.**, Šilerová M., Roubalová R., Škanta F., Halada P., Bilej M.: Molecular characterization of the iron binding protein ferritin in *Eisenia andrei* earthworms. *Gene* 485: 73-80 (2011).

VII. Procházková P., Škanta F., Roubalová R., Šilerová M., **Dvořák J.**, Bilej M.: Involvement of the iron regulatory protein from *Eisenia andrei* earthworms in the regulation of cellular iron homeostasis. *Plos One* 9(10) (2014).

VIII. Roubalová R., **Dvořák J.**, Procházková P., Elhottová D., Rossmann P., Škanta F., Bilej M.: The effect of dibenzo-p-dioxin- and dibenzofuran-contaminated soil on the earthworm *Eisenia andrei. Environ. Pollut*. 193:22-8 (2014).

REVIEW:

IX. Roubalová R., Procházková P., **Dvořák, J.**, Škanta F., Bilej M.: The role of earthworm defense mechanisms in ecotoxicity studies. *Invert. Surv. Journal* 12: 203-213 (2015).

Paper I

Dvořák J., Mančíková V., Pižl V., Elhottová D., Šilerová M., Roubalová R., Škanta F., Procházková P., Bilej M.: Microbial environment affects innate immunity in two closely related earthworm species *Eisenia andrei* and *Eisenia fetida*. *PLoS One* 8(11) (2013)

Survival of earthworms in the environment depends on their ability to recognize and eliminate potential pathogens. This work is aimed to compare the innate defense mechanisms of two closely related earthworm species, *Eisenia andrei* and *Eisenia fetida*, that inhabit substantially different ecological niches. While *E. andrei* lives in a compost and manure, *E. fetida* can be found in the litter layer in forests. Therefore, the influence of environment-specific microbiota on the immune response of both species was followed. Firstly, a reliable method to discern between *E. andrei* and *E. fetida* based on species-specific primers for cytochrome c oxidase I (COI) and stringent PCR conditions was developed. Secondly, to analyze the immunological profile in both earthworm species, the activity and expression of lysozyme, pattern recognition protein CCF, and antimicrobial proteins with hemolytic function, fetidin and lysenins, have been assessed. Whereas, CCF and lysozyme showed only slight differences in the expression and activity, fetidin/lysenins expression as well as the hemolytic activity was considerably higher in *E. andrei* as compared to *E. fetida*. The expression of fetidin/lysenins in *E. fetida* was not affected upon the challenge with compost microbiota, suggesting more substantial changes in the regulation of the gene expression. Genomic DNA analyses revealed significantly higher level of fetidin/lysenins (determined using universal primer pairs) in *E. andrei* compared to *E. fetida*. It can be hypothesized that *E. andrei* colonizing compost as a new habitat acquired an evolutionary selection advantage resulting in a higher expression of antimicrobial proteins.

Paper II

Škanta F., Roubalová R., **Dvořák J.**, Procházková P., Bilej M.: Molecular cloning and expression of TLR in the *Eisenia andrei* earthworm. *Dev. Comp. Immunol*. 41: 694-702 (2013)

Toll-like receptors (TLRs) play an important role in defense responses to pathogens in invertebrates. Here we characterize the first TLR isolated from an oligochaete annelid, namely, *Eisenia andrei* (EaTLR) and show its expression pattern. The full-length EaTLR cDNA consists of 2615 bp encoding a putative protein of 675 amino acids. The predicted amino acid sequence comprises of an extracellular domain containing 31 amino acid signal peptide and seven leucine-rich repeats (LRR), capped with cysteine-rich N- and C terminal LRRs followed by a transmembrane domain and cytoplasmic Toll/IL-1R domain (TIR). TIR domains of twenty individual earthworms were sequenced and the variability suggesting the presence of a high number of TLR genes in the genome of *E. andrei* was observed. Phylogenetic analysis revealed the highest similarity of EaTLR with polychaete annelid, *Capitella teleta* and TLRs of mollusks and echinoderms. Finally, the highest constitutive expression of EaTLR was observed in the digestive tract. Gene expression was significantly increased in coelomocytes of *E. andrei* after the challenge with Gram-positive bacteria.

Paper III

Škanta F., Procházková P., Roubalová R., **Dvořák J.**, Bilej M.: LBP/BPI homologue in *Eisenia andrei* earthworms. *Dev. Comp. Immunol.* 54: 1-6 (2016)

LBP/BPIs are pattern recognition receptors that are often present in vertebrates and in invertebrates, and they play a defense role against pathogens. We have identified 1698 bp cDNA sequence from the *Eisenia andrei* earthworm with predicted amino acid sequence that shares homology with the LBP/BPI family (EaLBP/BPI). Sequence analysis of EaLBP/BPI proved the existence of two conserved domains with the potential ability to bind LPS. The predicted molecular mass of the EaLBP/BPI protein is 53.5 kDa, and its high basicity (pI 9.8) is caused by its high arginine content. Constitutive transcription of the Ealbp/bpi gene was shown in all tested tissues, with the highest level in coelomocytes and seminal vesicles; the lowest level was detected in the intestine. On the contrary, another earthworm LPS-binding molecule CCF (coelomic cytolytic factor) was expressed only in the intestine and coelomocytes. In *E. andrei* coelomocytes, the transcription of Ealbp/bpi gene was up-regulated in response to bacterial stimulation, reaching a maximum at 8 and 16 h post stimulation with *Bacillus subtilis* and *Escherichia coli*, respectively.

Paper IV

Dvořák J., Roubalová R., Procházková P., Rossmann P., Škanta F., Bilej M.: Sensing microorganisms in the gut triggers the immune response in *Eisenia andrei* earthworms. *Dev. Comp. Immunol.* 57: 67-74 (2016)

The tube-within-tube body plan of earthworms is appropriate for studying the interactions of microorganisms with the immune system of body cavities such as the digestive tract and coelom. This study aims to describe the immune response on the molecular and cellular level in the coelomic cavity and the gut of the earthworm *Eisenia andrei* after experimental microbial challenge by administering two bacterial strains (*Escherichia coli* and *Bacillus subtilis*) or yeast *Saccharomyces cerevisiae* to the environment. The changes in mRNA levels of defense molecules (pattern recognition receptor CCF, lysozyme, fetidin/lysenins) in the coelomocytes and gut tissue were determined by quantitative PCR. The immune response at a cellular level was captured in histological sections, and the expression of CCF was localized using in situ hybridization. Coelomocytes respond to the presence of bacteria in the coelomic cavity by increasing the mRNA levels of defense molecules, especially CCF. The immune response in gut tissue is less affected by microbial stimulation because the epithelial cells of gut exhibit basically strong mRNA synthesis of ccf as a defense against the continuous microbial load in the gut lumen. The cellular immune response is mediated by coelomocytes released from the mesenchymal lining of the coelomic cavity. These combined immune mechanisms are necessary for the survival of earthworms in the microbially rich environment of soil.

Paper V

Procházková P., Šustr V., **Dvořák J.**, Roubalová R, Škanta F., Pižl V., Bilej M.: Correlation between the activity of digestive enzymes and nonself recognition in the gut of *Eisenia andrei* earthworms. *J. Invertebr. Pathol.* 114: 217-221 (2013)

Earthworms *Eisenia andrei*, similarly to other invertebrates, rely on innate defense mechanisms based on the capability to recognize and respond to nonself. Here, we show a correlation between the expression of CCF, a crucial pattern-recognition receptor, and lysozyme, with enzyme activities in the gut of *E. andrei* earthworms following a microbial challenge. These data suggest that enzyme activities important for the release and recognition of molecular patterns by pattern-recognition molecules, as well as enzymes involved in effector pathways, are modulated during the microbial challenge. In particular, protease, laminarinase, and glucosaminidase activities were increased in parallel to up-regulated CCF and lysozyme expression.

Paper VI

Procházková P., **Dvořák J.**, Šilerová M., Roubalová R., Škanta F., Halada P., Bilej M.: Molecular characterization of the iron binding protein ferritin in *Eisenia andrei* earthworms. *Gene* 485: 73-80 (2011)

Ferritin is a storage protein that plays a key role in iron metabolism. In this study, we report on the sequence characterization of a ferritin-coding cDNA in *Eisenia andrei* earthworms isolated by RT-PCR using degenerated primers, and we suggest the presence of a putative IRE in the 5'-UTR of ferritin mRNA. The obtained ferritin sequence was compared with those of other animals showing sequence and structure homology in consensus sites, including the iron-responsive element (IRE) and ferroxidase centers. Despite the sequence homology in the *E. andrei* mRNA of ferritin with the sequences of other animals in consensus IRE sites, the presented cytosine in the IRE of *E. andrei* ferritin in the expected position does not form a conventional bulge. The presence of ferritin in the coelomic fluid of *E. andrei* was proven by iron staining assay. Moreover, aconitase activity in the coelomic fluid was assessed by aconitase assay, suggesting the presence of an iron regulatory protein. Quantitative analysis revealed changes in the gene expression levels of ferritin in coelomocytes in response to bacterial challenge, reaching the maximum level 8 h after the stimulation with both Gram-positive and Gram-negative bacteria.

Paper VII

Procházková P., Škanta F., Roubalová R., Šilerová M., **Dvořák J.**, Bilej M.: Involvement of the iron regulatory protein from *Eisenia andrei* earthworms in the regulation of cellular iron homeostasis. *PlosOne* 9(10) (2014)

Iron homeostasis in cells is regulated by iron regulatory proteins (IRPs) that exist in different organisms. IRPs are cytosolic proteins that bind to iron-responsive elements (IREs) of the 59- or 39-untranslated regions (UTR) of mRNAs that encode many proteins involved in iron metabolism. In this study, we have cloned and described a new regulatory protein belonging to the family of IRPs from the earthworm *Eisenia andrei* (EaIRP). The earthworm IRE site in 59-UTR of ferritin mRNA most likely folds into a secondary structure that differs from the conventional IRE structures of ferritin due to the absence of a typically unpaired cytosine that participates in protein binding. Prepared recombinant EaIRP and proteins from mammalian liver extracts are able to bind both mammalian and *Eisenia* IRE structures of ferritin mRNA, although the affinity of the rEaIRP/*Eisenia* IRE structure is rather low. This result suggests the possible contribution of a conventional IRE structure. When IRP is supplemented with a Fe-S cluster, it can function as a cytosolic aconitase. Cellular cytosolic and mitochondrial fractions, as well as recombinant EaIRP, exhibit aconitase activity that can be abolished by the action of oxygen radicals. The highest expression of EaIRP was detected in parts of the digestive tract. We can assume that earthworms may possess an IRE/IRP regulatory network as a potential mechanism for maintaining cellular iron homeostasis, although the aconitase function of EaIRP is most likely more relevant.

Paper VIII

Roubalová R., **Dvořák J.**, Procházková P., Elhottová D., Rossmann P., Škanta F., Bilej M.: The effect of dibenzo-p-dioxin- and dibenzofuran-contaminated soil on the earthworm *Eisenia andrei. Environ. Pollut*. 193:22-8 (2014)

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) belong to the group of persistent organic pollutants, highly toxic environmental pollutants that include hydrophobic compounds with the tendency to bioaccumulate. Earthworms (*Eisenia andrei*) were exposed to PCDD/Fs-contaminated soil, and changes in their lipophilic structures and the gene expression of their defense molecules were followed. Damage to the intestinal wall and adjacent chloragogenous tissue was observed. Further, the up-regulation of the expression of several genes was detected. On the basis of these results, the mechanism of the impact of PCDD/Fs on earthworms has been proposed. Dioxins that accumulate in the lipophilic structures cause an increase in reactive oxidative species that triggers oxidative stress followed by the gene expression of two molecules that play a role in protection against oxidant toxicity, calreticulin (CRT) and Hsp70. Moreover, the effect of microbial biomass on the expression of coelomic cytolytic factor (CCF), a pattern recognition receptor, was also observed.

Paper IX

Roubalová R., Procházková P., **Dvořák, J.**, Škanta F., Bilej M.: The role of earthworm defense mechanisms in ecotoxicity studies. *Invert. Surv. Journal* 12: 203-213 (2015)

Earthworms are important soil organisms that affect the soil structure by influencing organic and inorganic matter breakdown. Earthworms are in permanent contact with soil particles via their permeable skin and digestive tract and are thus strongly affected by pollutants present in the soil. Earthworms often live in very hostile environments with an abundant microflora and therefore have developed very potent defense mechanisms. These mechanisms have been described to be influenced by various types of organic and inorganic pollutants and also by the nanoparticles that reach the soil system. Reduced abilities of earthworms to protect themselves against pathogenic microorganisms result in lower reproduction rates and increased mortality. In this review, a summary of the up-to-date data describing the effects of contaminants on the natural defense barriers and immune system of earthworms is presented.

4. Methods

Aconitase assay (paper VI, VII)

Analysis of microbial communities (paper I, VIII)

Computational molecular modeling (paper VI, VII)

Cultivation of microorganisms (paper I, IV)

Cytolytic, hemolytic and protease assay (paper I)

Electromobility shift assay (paper VII)

Enzyme activity assessment (paper V)

Expression of recombinant proteins (paper VII)

Histological staining techniques (paper IV, VIII)

In situ hybridization (paper III, IV)

In vitro transcription (paper VII)

Isolation of nuclei acids (paper I, II, III, IV, V, VI, VII, VIII)

Lysoplate assay (paper I)

Mass spectrometry (paper VI)

PCR (paper I, II, III, VI, VII)

Phylogenetic analyses (paper II, VII)

Polyacrylamide gel electrophoresis (paper VI)

Quantitative real-time PCR (paper I, II, III, IV, V, VI, VII, VIII)

Rapid amplification of cDNA ends (paper I, II, III, VI, VII)

Reverse transcription reaction (paper I, II, III, IV, V, VI, VII, VIII)

Sequencing (paper I, II, III, VI, VII)

5. Results and Discussion

Various earthworm species were used as a model of immunological research during years. Among them, *Eisenia andrei* plays a major role. Unfortunately, name of this earthworm was often changed during the time and moreover this species was often erroneously listed as *Eisenia fetida*. In many of papers is not clear, which of the two species is being referred to. For that reason, a brief history about the naming of these two earthworms should be clarified. These two species were first described as different morphotypes of *E. fetida* according to differences in the body pigmentation (Andre 1963), and subsequently established as subspecies (Bouche 1972) named *Eisenia fetida andrei* and *Eisenia fetida fetida*. Nowadays, they are considered as two independent species, *Eisenia andrei* (Bouche 1972) (NCBI Taxonomy: 168636) and *Eisenia fetida* (Savigny 1826) (NCBI Taxonomy: 6396) belonging to Lumbricidae family (Dominguez et al. 2015). They are both epigeic earthworms, but their natural environment substantially varies. Whereas *E. andrei* lives in compost and manure rich in potential pathogens, non-synanthropic indigenous populations of *E. fetida* earthworm can be found in the litter layer of moist forests that are considerably less abundant in a number of microorganisms (Pižl 2002). Therefore, it was of interest to inquire how the natural environment and its microbiota affect various defense mechanisms of these two earthworm species.

First, a reliable discrimination method was developed for distinction among these two species. Different approaches were used for differentiation of *E. andrei* and *E. fetida* in the past including biochemical approach (Roch et al. 1980), biological approach (Dominguez et al. 2005) and metabolic profiling approach (Bundy et al. 2002). Molecular taxonomy allowed an implementation of new procedures for classification of species based on comparison of differences in gene sequences. Mitochondrial cytochrome c oxidase (COI) gene is widely used as a DNA barcode for identification of animal samples. Peréz-Losada and colleagues have determined *E. andrei* and *E. fetida* based on mitochondrial and nuclear DNA sequences using conserved primers amplifying COI fragments of most animal species (Perez-Losada et al. 2005). Contrary to, we designed and used discrimination primers specific only for one species (Dvorak et al. 2013). The main

advantage of such approach is the possibility to quickly discriminate *E. andrei* and *E. fetida* without the requirement of sequencing.

Natural biological activities of coelomic fluids were compared between these two earthworms. Specifically, cytolytic, hemolytic, proteolytic and lysozyme activities were determined. Significant differences were observed in the case of hemolytic activities. Coelomic fluid of *E. andrei* manifested much higher hemolytic activity compared to that of *E. fetida*. Natural polymorphism was described among hemolytic protein families of earthworms (Valembois et al. 1986). Moreover, high variability of hemolytic patterns and differences in the expression of fetidin and lysenins in *Eisenia* were previously observed (Prochazkova et al. 2006a). For that reason we designed a universal primer pair for the detection of all known fetidin- and lysenins-related molecules with the hemolytic activity. Quantitative real-time PCR confirmed that hemolytic molecules are more abundant in *E. andei* on the level of genomic DNA as well as mRNA (Dvorak et al. 2013). Previously it was described, that the hemolytic activity of earthworms can be stimulated by the microbial challenge (Köhlerova et al. 2004). In order to monitor the possible adaptation of earthworms to foreign microbiota, the cross-colonization experiments were performed. We isolated and cultured bacterial strains from both compost and forest soil. The obtained mixtures were used for the earthworm stimulation in the cross-colonization experiments. The mRNA levels of CCF, lysozyme and fetidin/lysenins in coelomocytes of both species were determined. While the expression of fetidin/lysenins was significantly upregulated in *E. andrei*, biologically nonsignificant changes were found in *E. fetida* challenged with compost bacteria (Fig. 6). The absence of detectable reaction of *E. fetida* to compost microbiota can be explained either by lower number of gene copies coding for fetidin/lysenins as compared to *E. andrei* or by unknown difference in the gene expression regulation in both species. Heterogeneity of microbiota represents a higher pressure to the immune system of earthworms. It can be hypothesized that *E. andrei* colonizing compost (habitat rich in the complexity of microbiota) acquired an evolutionary selection advantage resulting in higher expression of antimicrobial proteins. Therefore, defense strategies of this species were analyzed in broader context.

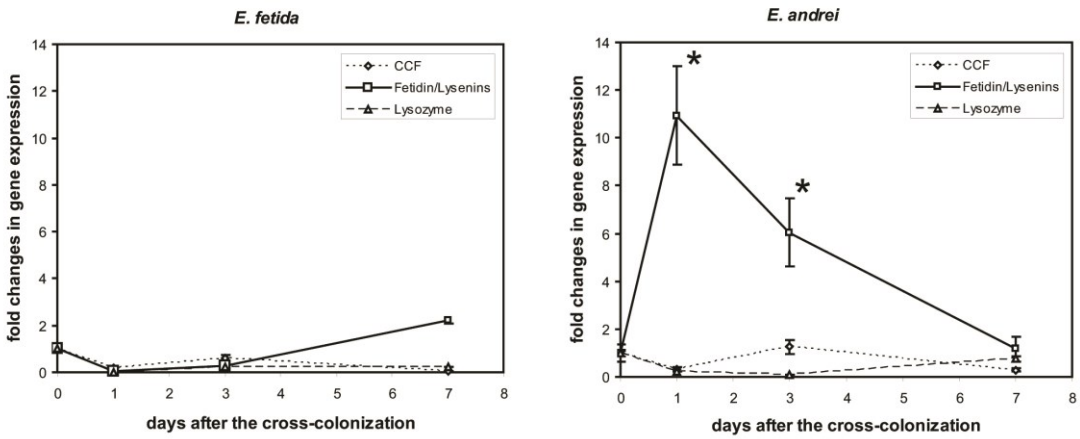

Fig. 6. Comparison of gene expression levels in *E. andrei* and *E. fetida*. Gene expression levels of selected genes (CCF, lysozyme, fetidin/lysenins genes) in *E. andrei* and *E. fetida* earthworms upon bacterial cross-colonization determined by real-time PCR and normalized for the reference gene RPL17 (ribosomal protein L17). Fold change in the gene expression are relative to the expression in earthworms maintained with bacteria isolated from their natural environment. The values are the means of three independent experiments (± SD) performed in duplicates (* $P < 0.05$) (Dvorak et al. 2013).

Until recently, CCF molecule was the only representative of PPRs described in earthworms (Beschin et al. 1998). Toll-like receptors (TLRs) represent very important PRRs, which play a key role in the mechanisms of innate immunity. TLRs are transmembrane proteins consisiting of an intracellular signaling Toll/Interleukin-1 receptor homology (TIR) domain and an extracellular ligand recognition domain containing leucine-rich repeats (LLR) (Bell et al. 2003, O'Neill and Bowie 2007). Some TLRs were previously described in annelids (Coscia et al. 2011). In our study, we sequenced the TLR of *E. andrei* (EaTLR). The deduced amino acid sequence revealed standard organization of protein domains. It consists of extracellular domain with seven leucine rich repeats followed by transmembrane domain and cytoplasmic TIR domain. According to the organization of extracellular domain, TLR receptors can be divided into two groups: vertebrate-like type (V-type) and protostome-like type (P-type) of TLRs (Hibino et al. 2006). Analysis of ectodomain structure classified EaTLR as V-type (Skanta et al. 2013). Both V-type and P-type of

TLRs were identified in genomes of invertebrates (Hibino et al. 2006, Davidson et al. 2008). Finding of V-type TLRs in invertebrates is not surprising since this type occurred after the divergence of Cnidaria and Bilateria (Wu et al. 2012). Detailed analyses demonstrated a high intraspecific variability in amino acid sequences of this molecule suggesting that high number of TLRs is encoded within the genome. This is in accordance with findings of multiple TLRs genes present in genomes of other annelids (Davidson et al. 2008). In our experiments with microbial stimulation, the increased levels of EaTLR mRNA after stimulation with Gram-positive bacteria confirmed its role in defense reactions. Similar results were previously described in *Drosophila*, where the recognition of Gram-positive Lys-type of peptidoglycan results in activation of Toll-pathway (Michel et al. 2001).

Another earthworm PRR, which is originally documented in this work, belongs to the LBP/BPI/CETP protein family. The two main members of this family are lipopolysaccharide binding protein (LBP) (Schumann et al. 1990) and bactericidal/permeability-increasing protein (BPI) (Gray et al. 1989). These two closely related proteins act as antagonists of inflammatory response to LPS in mammals. LBP mediates inflammatory response (Fenton and Golenbock 1998). BPI plays an anti-inflammatory role (Elsbach and Weiss 1998). In invertebrates, the distinction between LBP and BPI has not been established to date. We described protein, which shares homology with LBP/BPI protein family. Predicted amino acid sequence revealed two BPI domain (N- and C-terminal) interconnected by a proline-rich central domain (Skanta et al. 2016). Identical arrangements of domains were previously described in other LBP/BPI proteins of invertebrates (Gonzalez et al. 2007, Baron et al. 2013, Mao et al. 2013). The mRNA level of LBP/BPI in different tissue was determined. The highest gene expression was detected in coelomocytes and seminal vesicles. High expression of LBP/BPI proteins in reproductive system was described also in other animals. Lennartsson et al. proved the highest expression of BPI in Sertoli cells of murine testis (Lennartsson et al. 2005). Massive occurrence of LBP/BPI protein was evidenced in the eggs of freshwater snail *B. glabrata* (Baron et al. 2013). This may suggest that LBP/BPI proteins play a role in protection of gametes against pathogens. To clarify the role of this protein in defense system, earthworms were stimulated by injection of bacterial suspension

into coelomic cavity. This resulted in upregulation of gene expression of LBP/BPI in coelomocytes and can imply the potential antimicrobial function. It was hypothesized that whole positive charge of these molecules plays a key role in their antimicrobial activity (Schultz and Weiss 2007). The positive charge of Ea LBP/BPI molecule is comparable with values of human BPI or BPI from *Crassostrea gigas*. Moreover antibacterial activity of BPI from *C. gigas* was proved (Gonzalez et al. 2007).

Both coelomic cavity and alimentary tract of earthworms contain naturally occurring microorganisms, but the immune response of these two body cavities differs. Multiplication of microorganisms in coelomic cavity is not desirable and must be strictly regulated. On the other hand, immune response of the gut must be balanced and excessive inflammatory responses against intestinal microbiota should be avoided. Hence next goal was to describe the differences in immune response of the gut and the coelom after microbial challenge.

Comparison in number of culturable bacteria (colony forming units CFU) in the gut and in coelomic fluid of naïve earthworms revealed approximately six times higher amount of bacteria in the gut than in coelom (Dvorak et al. 2016). As it was written above, the number of bacteria in coelom is kept under control by various immune mechanisms, such as antimicrobial factors (Lassegues et al. 1989, Prochazkova et al. 2006a, Joskova et al. 2009) and phagocytic cells (Dales and Kalac 1992). In earthworms subjected to high microbial load of Gram-negative bacteria *E. coli*, Gram-positive bacteria *B. subtilis* and yeast *S. cerevisiae*, the increased number of microorganisms was observed in both the coelom and the gut. However, microbial overload affects populations of microorganisms in the gut in lesser extent in comparison with the coelom. This suggests that the relations between immune system of the gut and microbiota are more complex. Partial populations of microorganisms in the digestive tract can be tightly associated with the intestinal wall of earthworms (Singleton et al. 2003). Microenvironment of the earthworm gut can provide ideal living conditions for some group of bacteria as are *Clostridiaceae* and *Enterobacteriaceae* (Wust et al. 2011). The evidences of long-term symbiosis between earthworms and bacteria were documented within excretory organs (nephridia), where stable populations of Acidovorax-like bacteria were found

(Schramm et al. 2003). The question of real gut symbiosis in earthworms remains unresolved (Curry and Schmidt 2007), although bacteria associated with the gut wall of earthworms have been described as determined mainly by the ecological group (Thakuria et al. 2010). All these findings imply that there exist favorably conditions for maintaining stable populations of bacteria in the gut of earthworms.

Increasing number of bacteria in the body of earthworms is reflected in the higher immune response both in coelom and in the gut. We followed the changes in mRNA levels of pattern recognition receptor CCF and two antimicrobial molecules, fetidin/lysenins and lysozyme. We found that coelomocytes produce a significantly higher mRNA level of CCF compared to gut tissue upon microbial stimulation. The production of CCF in gut tissue is probabily not so affected by microbial challenge, because permanent close contact with the microorganisms present in ingested soil (Brown and Doube 2004, Koubova et al. 2015, Singh et al. 2015) can maintain higher basal transcription level of defense molecules in the gut epithelium. Moreover, PRRs play an important role in maintenance of host-microbial interactions in the intestinal mucosa (Fukata and Arditi 2013). Results from *in situ* experiments confirmed that CCF is expressed in epithelial cells of the foregut and midgut (Dvorak et al. 2016). In addition, transcription of CCF is exclusively localized in the gut tissue and coelomocytes (Skanta et al. 2016). Two others PRRs described in this work are also expressed in the alimentary tract of earthworms. EaTLR is highly expressed in midgut (Skanta et al. 2013). Contrary to, expression of LBP gene is localized in anterior part of digestive system (pharynx and esophagus) (Skanta et al. 2016). Gene expression profiles of CCF, EaTLR and LBP in different tissues are shown in Fig. 7. Balanced interactions of mucous immunity and microbiota are necessary for maintaining the integrity of mucosal surfaces. Molecules produced by intestinal bacteria may promote the integrity of the epithelial barrier and have been shown to regulate tight junctions (Ukena et al. 2007, Ewaschuk et al. 2008). Polysaccharide A is an example of the molecule that is produced by the human commensal microorganism *Bacteroides fragilis* and that can shape beneficial immune responses (Mazmanian et al. 2005). Stable production of PRRs in intestinal epithelia is also important for maintenance of non-pathological interactions. It was shown that loss

of TLR signaling can increase intestinal inflammation in case of damaged intestinal epithelium (Rakoff-Nahoum et al. 2004).

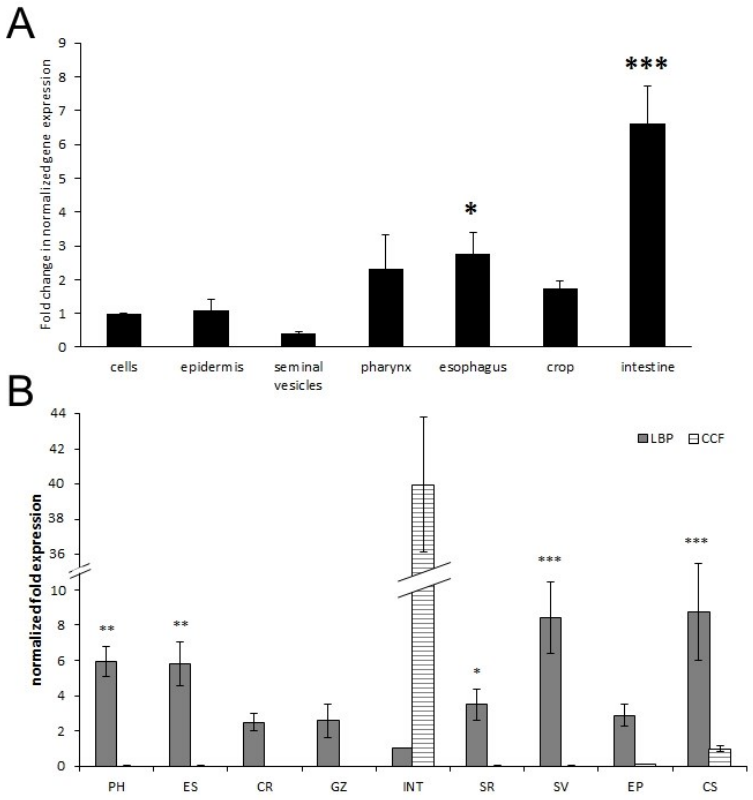

Fig. 7. **A** Gene expression levels of EaTLR determined by real-time PCR and normalized for the housekeeping gene RPL17. Fold changes in gene expression are relative to EaTLR expression in cells. The values are means (±SD) of three independent experiments performed in duplicate (* significant at $P < 0.05$, *** significant at $P < 0.001$) (Skanta et al. 2013)

B Transcription of Ealbp/bpi and ccf in different tissues normalized to two different housekeeping genes (PH pharynx, ES esophagus, CR crop, GZ gizzard, INT intestine, SR seminal receptacles, SV seminal vesicles, EP epidermis, CS coelomocytes). The expression level of Ealbp/bpi mRNA was related to the expression in the intestine where the level was the lowest. The expression of ccf mRNA was related to the expression in coelomocytes (N/D non-detectable). One-way ANOVA with Dunnett's post-test was performed, using GraphPad Prism software to evaluate the significance of the data. Differences were considered significant when $P < 0.05$*, 0.01** or 0.001***. The values are the means (±SD) of three independent experiments that were performed in duplicate (Skanta et al. 2016).

The increased number of microorganisms in the gut can also influence digestive processes, since they form a significant part of earthworm diet. It was proved that microbial stimulation has resulted in upregulation of laminarinase, glucosaminidase and protease activities in the intestine of earthworms (Prochazkova et al. 2013). Transcriptionally active genes involved in both digestive and immune processes were previously described in the gut of earthworms (Lee et al. 2005). Digestion is selective process for certain populations of bacteria. Part of them is digested inside the foregut and midgut, while some bacteria and actinomycetes can be documented in all parts of the digestive tract and their presence increases in the hindgut (Kristufek et al. 1994). Process of digestion results in releasing of pathogen-associated molecular patterns from bacteria and therefore it contributes to the mechanisms of recognition by PRRs. As it was documented previously, CCF requires a lysozyme pre-treatment for an efficient recognition of peptidoglycan constituents (Bilej et al. 2001). In addition, digestive processes can contribute to the production of secondary metabolites such as short-chain fatty acids (SCFA). Bacteria synthesize these metabolites by fermentation of dietary fibers. Short-chain fatty acids are important modulators of immune processes including chemotaxis, proliferation and cytokine production (Meijer et al. 2010).

We hypothesize that an increased number of bacteria in the gut triggers signaling pathways that induce cell release and differentiation in the coelomic lining. Coelomocytes are released to the coelom, where they perform either nutritive (eleocytes) or immune (amoebocytes) functions according to their type (Sima 1994). The mechanism of coelomocyte release was captured in histological sections and is shown in Fig. 8 (Dvorak et al. 2016). Earthworm coelomocytes have been described to exhibit low proliferation activity. Therefore, the main source of these cells is their release from the mesenchymal coelomic lining. It was shown that precursor cells in the coelomic lining proliferate immediately after antigenic stimulation (Bilej et al. 1994). Experimental coelomocyte depletion was followed by extensive cell proliferation in the coelomic lining (Homa et al. 2008, Klimek et al. 2012). *In vivo* dermal exposure to different immunostimulants induced changes in the number, composition and activity of coelomocytes. Moreover, a reduction in the percentage of nutritive eleocytes and an increase in the percentage of immune

effector cells, amoebocytes, were observed in these earthworms (Homa et al. 2013). The released coelomocytes with immune function act as phagocytes and/or produce antimicrobial proteins and opsonins that reduce the number of microorganisms in the coelomic cavity, thus keeping the infection under control.

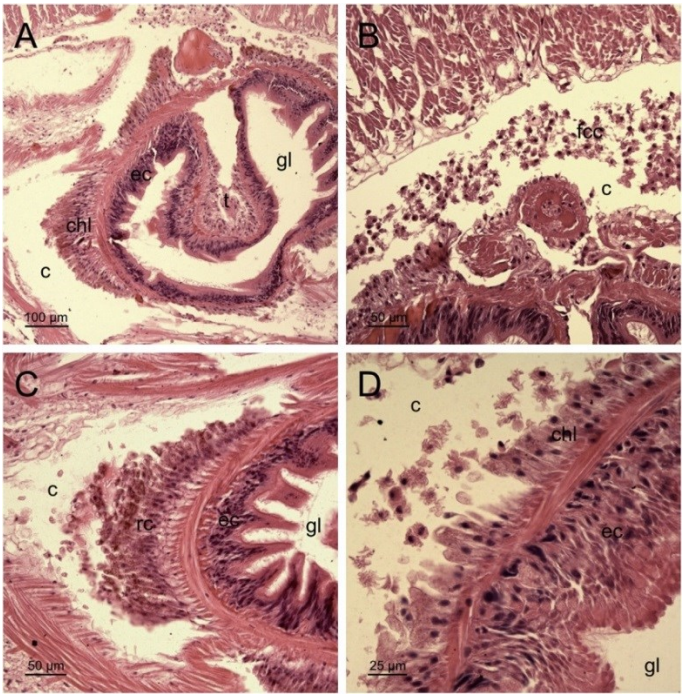

Fig. 8. Transversal sections representing *E. andrei* midgut stained with hematoxylin/eosin. A: General view of the structures of the gut. The gut lumen (gl) is lined with a layer of enterocytes (ec). The chloragogen layer (chl) surrounding the tube of the gut is oriented toward the coelom cavity (c). Typhlosolis (t) represents gut invagination that increases the surface for food absorption. B: Free-floating coelomocytes (fcc). C: Release of coelomocytes (rc) into the coelom from the mesenchymal layer; detail in D (Dvorak et al. 2016).

In bacteria, iron plays a crucial role in many physiological processes of central metabolism (Andreini et al. 2008). Therefore, host's strategy of iron limitation can act as a potent defense against infection (Weinberg 2009). Such tactic is commonly referred to as the iron-withholding strategy of innate immunity (Ong et al. 2006). It was proved that ferritins of invertebrates are involved in this mechanism of iron-withholding (Kong et al. 2010). Therefore, the mechanisms of iron homeostasis in earthworm *E. andrei* were elucidated. New ferritin molecule of this species was described and the mechanism of its regulation on the

posttranscriptional level was illustrated. First, cDNA molecule of ferritin was identified. On the basis of sequence analysis, the molecular mass of predicted protein was estimated to 19.7 kDa with the isoelectric point (pI) of 5.19 (Prochazkova et al. 2011). This is in the range of the molecular masses and pI of other known ferritins (Theil 1987). 5'UTR sequence of mRNA contains IRE binding site with typical CAGUN loop and cystosine 5 nucleotides upstream that seems to be important for the function of IREs. On the other hand, computer modeling of the *E. andrei* ferritin IRE secondary structure revealed nonstandard arrangements of nucleotides in the stem of loop compared to known IRE structures of vertebrates. IRE sequence of *E. andrei* do not have a conformation with bulged C, instead they have bulged U. This change is the result of site specific nucleotide substitution of A for G, located 5 nucleotides downstream to CAGUN. Similarly atypical IRE structures were described in mRNA ferritins of other invertebrates (Huang et al. 1996, Hsieh et al. 2006, Jeong et al. 2006). It seems that these alternate conformations do not have an impact on binding of IRP to IRE (Huang et al. 1999). Amino acid sequence of *E. andrei* ferritin is more similar to mammalian ferritin H-chains than to L-chains. Although *E. andrei* ferritin lacks tyrosine on position 30, which is specific for H ferritins of vertebrates (Andrews et al. 1992), other important amino acid residues are identical to those in H-type of ferritin. Presence of ferritin protein was also confirmed in coelomic fluid and its molecular mass was estimated to 400 kDa corresponding to 20 kDa per subunit. This is in good agreement with the calculated mass of 19.7 kDa. Ferritins were also isolated from the hemolymph of other invertebrates such as *Manduca sexta* (Winzerling et al. 1995), *Aedes aegypti* (Dunkov et al. 1995), *Musca domestica* (Capurro et al. 1996), *Drosophila melanogaster* (Charlesworth et al. 1997) and *Galleria mellonaella* (Kim et al. 2001). Earthworms were stimulated by Gram-negative and Gram-positive bacteria to elucidate the role of ferritin in defense system. The gene expression of ferritin in coelomocytes was determined by real-time PCR. Simultaneously the expression of ferritin was also measured after the stimulation of $FeCl_3$. The up-regulation of ferritin upon bacterial challenge suggests its role in the immune response against both Gram-positive and Gram-negative bacteria (Fig. 9A). Surprisingly, iron overload have no significant effect on the expression of ferritin. No effect of iron on the

expression of ferritin was also observed in the horseshoe crab *Carcinoscorpius rotundicauda* (Ong et al. 2005). On contrary, iron overload stimulates production of ferritin subunits in *Aedes aegypti* (Dunkov et al. 1995). The response to the changes of iron content in environment can be regulated on post-transcriptional level due to an iron regulatory protein (IRP). This is supported by the finding that stimulation with $FeCl_3$ results in the increase of the aconitase activity in coelomic fluid (Fig. 9B). Therefore we focused on the characterization of the iron regulatory protein from *Eisenia andrei* (EaIRP). cDNA sequence of EaIRP was obtained and the gene was consequently cloned to the expression vector. Recombinant EaIRP (rEaIRP) was used in the RNA electromobility shift assay (REMSA) to verify the binding capability of rEAIRP with IRE structures. It was shown that labeled RNA corresponding to the IRE within 5'UTR of earthworm ferritin mRNA can be bound by rEaIRP. Similarly to the earthworm IRE hairpin, the mammalian IRE site of ferritin was also bound by rEaIRP. Vice versa mammalian liver extract (containing mammalian IRP) can create complex with earthworm IRE structure (Prochazkova et al. 2014). Analogous organization of amino acids residues which is required for binding of human IRP1 to IRE structure (Walden et al. 2006) is present also in EaIRP. Therefore, both mammalian and earthworm IRPs can interact with both IRE structures. Increased concentration of iron results in the change of conformation of IRP. Formation of [4Fe-4S] cluster in the molecule of IRP switches its function to the aconitase enzyme. Two different aconitases can be found in two different cellular compartments. One form is localized in mitochondria (Walden 2002) and the second form is found in cytoplasm (Eanes and Kun 1971). Both cytosolic and mitochondrial fractions of *E. andrei* cells as well as rEaIRP exhibit aconitase activity. Reactive oxygen species induce oxidation of Fe-S cluster followed by gradual disassembly and its removal, which results in the loss of aconitase function (Cairo and Pietrangelo 2000). Aconitase activity of both cellular fractions and rEaIRP were decreased after the effect of H_2O_2 as a consequence of destruction of Fe-S cluster in the IRP molecule (Hentze and Kuhn 1996). Dual function of rEaIRP was demonstrated and it can be concluded that EaIRP plays a role in maintaining of iron homeostasis in *E. andrei* by mechanism of binding IRP to IRE sequence in 5'UTR region of ferritin mRNA.

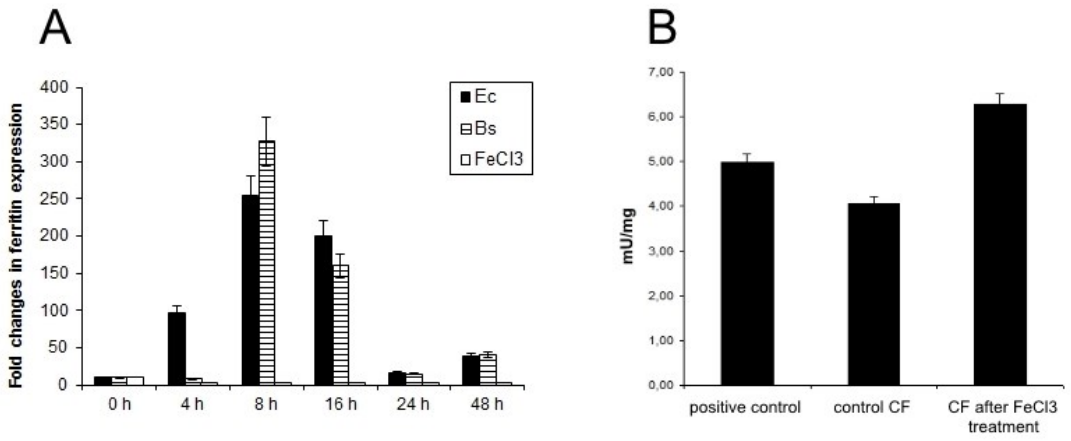

Fig. 9. **A** Gene expression levels of ferritin in coelomocytes in response to bacterial challenge and overload of $FeCl_3$ determined by quantitative PCR. Earthworms were stimulated with *E. coli*, *B. subtilis*, and $FeCl_3$, and samples were collected 4, 8, 16, 24, and 48 h after the stimulation. Values were normalized to the expression of 28S RNA. The values are the means of three experiments (±SD) performed in triplicate.
B Aconitase activity in the coelomic fluid of *E. andrei*. Activity is expressed in milliunits per mg of total extract protein. As a positive control, porcine heart aconitase was used. The activity was determined in the coelomic fluid of non-stimulated earthworms (control CF) and in the CF of earthworms incubated 12 h in an environment of 10mM $FeCl_3$ in LBSS. The values are the means of three experiments ± standard deviation (Prochazkova et al. 2011).

Integrity of mucosal surfaces can be disrupted by various toxic chemicals. A significant part of them is continuously accumulated in the soils. Earthworms and their mucosal surfaces are in permanent contact with soil particles and therefore they are able to absorb quantities of these chemicals. Dioxins represent an example of broad spectrum of contaminants which are accumulated in soils (ATSDR 1998). Destructive effects of dioxins on numerous systemic life functions including immune system were described (Van den Berg et al. 2006, White and Birnbaum 2009). Earthworms can be therefore used as a suitable model for studying the impact of soil pollutants on various life processes. Toxic effect of dioxins on cellular immune system of earthworms was documented (Belmeskine et al. 2012). In our study, we examined the impact of dibenzo-p-dioxins and dibenzofuran (PCDD/Fs)-polluted

soils on intestinal tract and the effect on production of immune and antioxidant molecules in coelomocytes (Roubalova et al. 2014). Exposure to dioxins is reflected in significant histological changes of the gut epithelium and adjacent chloragogen tissue (Fig. 10). Reduction of intestinal villi and chloragenous layer was observed. Harmful impact of dioxins to chloragogen tissue is expectable, because accumulation of diverse xenobiotics in this tissue was documented (Morgan and Morgan 1989, Sturzenbaum et al. 2004). Dioxin pollution also significantly alters the abundance and composition of soil bacteria. Significantly greater amount of both aerobic and anaerobic microbial biomass was determined in PCDD/Fs contaminated soils. The variation in abundance and composition of microbial communities of dioxin-polluted soil is reflected in higher expression of CCF in coelomocytes. Apart from that, mRNA levels of calreticulin and Hsp70 genes were upregulated. Calreticulin plays an important role in protection against oxidant toxicity and the increase of its expression represents a part of the cellular response to oxidative stress (Liu et al. 1998, Nunez et al. 2001). Hsp70 belongs to the heat shock protein family which contributes to the protection and repair of cells damaged due to various stressors (El Golli-Bennour and Bacha 2011, Kafel et al. 2012, Lauritano et al. 2012). Hsp70 can be recommended as a potential sensitive biomarker for environmental monitoring (Mukhopadhyay et al. 2003). In summary, dioxins enter the earthworm body through the lipophilic alimentary canal and consequently they have adverse effect both on the chloragogenous tissue and gut epithelium. We demonstrated that dioxins can affect mechanisms of oxidative response. No direct link between dioxins and gene expression of molecules participating in immune processes was observed. However, dioxin-pollution affects the composition and number of bacteria in the soil which is reflected in higher gene expression of CCF.

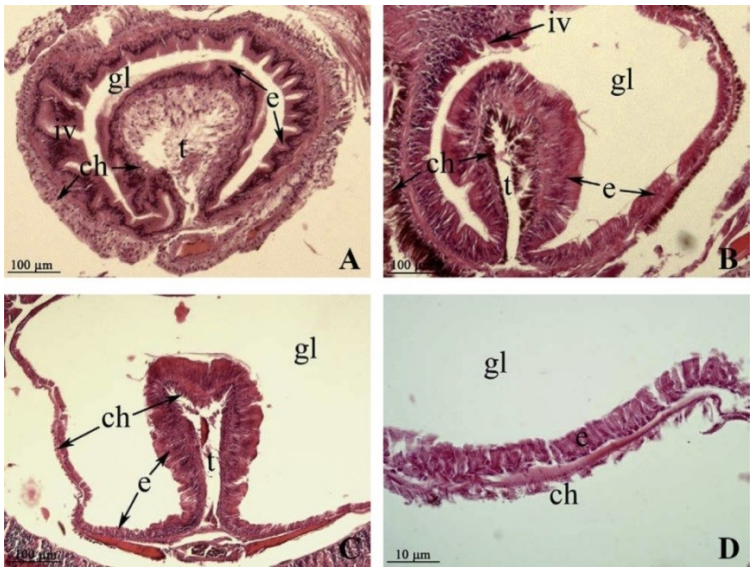

Fig. 10. Transversal sections representing an *E. andrei* post-clitellum, midgut body part (5 µm) stained with hematoxylin/eosin. The gut lumen (gl) is lined with a layer of epithelial cells (e), and the gut invagination is called typhlosolis (t). The intestine is surrounded by chloragogenous tissue (ch). The progressive reduction of intestinal villi (in A, B iv; not present in C and D) and chloragogenous tissue can be seen in earthworms kept in dioxin-polluted soil for 14 (B) and 21 days (C, in detail D) in comparison with earthworms maintained in artificial control soil (A) (Roubalova et al. 2014).

6. Conclusion

In my thesis I present new findings about interactions of microorganisms and immune system of earthworm *E. andrei* in coelom and in gut. *E. andrei* lives in the microbially abundant environment of compost, where the numbers of microorganisms significantly exceed average amount of microbiota in other types of soils. From that reason, orchestrated immune system enabling survival of this species in such microbially hostile environment had to be evolved.

Coelomic fluid of *E. andrei* exhibits higher hemolytic activity compared to another epigeic earthworm, *E. fetida*. The mRNA levels of hemolytic molecules with antibacterial activities produced by coelomocytes of *E. andrei* are upregulated upon microbial challenge and therefore can contribute to stronger defense reactions of this species.

Fundamental components of innate immune system that mediate the interactions with microorganisms are represented by pattern recognition receptors (PRR). Two new PRRs of *E. andrei* are described in this thesis, namely TLR and LBP/BPI. Both of these molecules are synthesized by coelomocytes and their gene expression is upregulated upon microbial challenge. Site specific expression of TLR and LBP/BPI molecules was documented in digestive tract of *E. andrei*. Expression of LBP/BPI is more localized in anterior part of digestive system, whereas TLR exerts highest expression in the intestine.

Experimental microbial overload in gut is also reflected in increased activities of digestive enzymes. Digestions of bacterial components can promote recognition of PAMPs by PRRs. Especially CCF exerts strong stable expression in epithelium of gut and can therefore contribute to the balanced interactions of intestine microbiota and immune system. We hypothesize that an increased number of bacteria in the gut triggers signaling pathways that induce cell release and differentiation in the coelomic lining. The released coelomocytes with immune function act as phagocytes and/or produce antimicrobial proteins and opsonins that reduce the number of microorganisms in the coelomic cavity.

Coelomocytes are also responsible for synthesis of ferritin, the main iron storage protein, which can influence its availability for bacteria. We showed that synthesis of ferritin mRNA is increased upon microbial challenge. Moreover regulation of ferritin expression on post-transcriptional level was described.

Pathological changes on chloragogen tissue and intestinal epithelium were observed after exposure to dioxins. Such disruption led to the activation of antioxidative and repair mechanisms. Dioxin pollution also alters the composition of bacteria in the soil which is reflected in higher expression level of CCF.

In summary, I submit in this thesis some new conclusions and hypotheses about immune system of *E. andrei* earthworm. These results can elucidate in detail their abilities to successfully survive in the microbially very rich environment. Innate immunity against pathogen infection is an evolutionarily conserved process among multicellular organisms. Therefore our results may shed a new light on the native immunity not only of invertebrates, but also of vertebrates.

7. References

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