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Abbreviations

4Fe-4S	iron–sulfur clusters
AMP	antimicrobial peptide
ATSDR	Agency for Toxic Substances and Disease Registry
BPI	bacterial permeability increasing protein
CAT	catalase
CCF	coelomic cytolytic factor
CD14	cluster of differentiation 15
CF	coelomic fluid
CFU	colony forming units
COI	cytochrome c oxidase
CRT	calreticulin
*Ea*IRP	*E. andrei* earthworm IRP
*Ea*LBP/BPI	*E. andrei* earthworm LBP/BPI
EALys	*E. andrei* earthworm lysozyme
*Ea*TLR	*E. andrei* earthworm TLR
EFAF	*Eisenia fetida andrei* factors
GlcNAc	*N*-acetylglucosamin
GNBP	Gram-negative bacteria-binding protein
*Hm*TLR1	*H. medicinalis* TLR2
IARC	International Agency for Research on Cancer
IRE	iron-responsive element
IRP	iron regulatory protein
ISO	International Organization for Standardization
LBP	lipopolysaccharide (LPS)-binding protein
L-DOPA	L-β-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
LRR	leucin rich region
LTAs	lipoteichoic acids
MAMPs	microbe-associated molecular pattern
MyD88	Myeloid differentiation primary response gene 89
NAM	*N*-acetylmuramic acid

NLRs	NOD-like receptors
OECD	organisation for economic co-operation and development
PAMPs	pathogen associated molecular patterns
PCDD/Fs	polychlorinated dibenzofurans
PCDD/PCDF	polychlorinated dibenzo-*p*-dioxins/dibenzofurans
PCR	polymerase chain reaction
PGRPs	peptidoglycan recognition proteins
PLFA	phospholipid-derived fatty acids
PO	phenoloxidase
proPO	prophenoloxidase
PRRs	pattern recognition receptors
RNI	reactive nitrogen
ROI	reactive oxygen
RT	reverse transcription
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophore
SOD	superoxide dismutase
SPR	surface plasmon resonance
TCDD	2,3,7,8-tetrachlordibenzo-*p*-dioxin
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFRs	tumor necrosis factor receptor
UTR	untranslated region

Abstract

Survival of earthworms in the environment depends on their ability to recognize and eliminate potential pathogens. Two closely related earthworm species *Eisenia andrei* and *Eisenia fetida* inhabit different environment with specific microbiota. Both species can be reliably determined using of species-specific primers for cytochrome c oxidase I (COI) and stringent PCR conditions. Whereas, we did not observed any substantial differences in the expression and activity of CCF and lysozyme upon microbial challenge, the expression as well as the hemolytic activity of fetidin/lysenins was considerably higher in *E. andrei* as compared to *E. fetida*. Genomic DNA analyses revealed significantly higher level of fetidin/lysenins in *E. andrei* compared to *E. fetida* suggesting hypothetical gene duplication.

Earthworms live in permanent close contact with microbial environment. Coelom cavity as well as the gut of *E. andrei* earthvworm differs in the number of bacteria. The number of bacteria in the gut is more than six time higher than in coelomic fluid. High microbial load of *E. coli* O55, *B. subtilis* W23, and *S. cerevisiae* S288 in the earthworm environment, resulted in an increase of microorganisms in both, the coelom and the gut. The changes in mRNA levels of defense molecules (pattern recognition receptors CCF, *Ea*TLR, *Ea*LBP/BPI and antimicrobial molecules lysozyme and fetidin/lysenins) in the coelomocytes and gut tissue were determined by quantitative PCR. The immune response at a cellular level was determined in histological sections, and the expression of CCF and *Ea*LBP/BPI was localized using *in situ* hybridization. The immune response in gut tissue is less affected by microbial stimulation because the epithelial cells of the gut exhibit basically high mRNA synthesis of *ccf* and *eatlr* as a defense against the continuous microbial load in the gut lumen. This defense is also supported by variability and number of TLRs in the gut and increased gut enzyme activities as protease, laminarinase, and glucosaminidase, which are important for the release and recognition of molecular patterns by pattern-recognition molecules.

The cellular immune response in the coelomic cavity is mediated by coelomocytes released from the mesenchymal lining. Coelomocytes respond to the presence of bacteria by increasing the mRNA levels of pattern recognition receptors, especially CCF, *Ea*LBP/BPI, *Ea*TLR and an important iron storage molecule ferritin. *Ea*LBP/BPI as one of the LPS-binding molecules is constitutively expressed in coelomocytes and seminal vesicles.

The exposition of *E. andrei* earthworms to dioxin-contaminated soil caused damage of intestinal wall and adjacent chloragogenous tissue. It was also shown high gene expression of oxidative stress molecules calreticulin (CRT), Hsp70 and defense molecule CCF. But higher expression of CCF was probably caused by the effect of microbial biomass than the pollutant itself. These results indicate that immune-related molecules can be useful for monitoring of soil contamination but the microbiota cannot be overlooked in the evaluation of the results.

1. Introduction

Invertebrates represent more than 95% of around 1,500,000 total number recent animal species. They are organisms with a broad diversity from unicellular protozoans to much more complex echinoderms and protochordates. Despite lacking of an adaptive immunity mechanisms such as antibodies production and clonal expansion of lymphocytes, invertebrates live very often in very hostile environments. Invertebrates have developed a variety of defense mechanisms to recognize and efficiently respond to non-self substances. The invertebrate cells possess special pattern recognition receptors (PRRs) recognizing pathogen associated molecular patterns (PAMPs) or better, microbe-associated molecular patterns (MAMPs). These patterns are well-conserved molecular structures expressed by various pathogens (viruses, bacteria, fungi, protozoans, helminths). There are many prominent PRR representatives within this group, such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) or scavenger receptors. Recognition process of foreign substances is often followed by production of antimicrobial peptides (AMPs), toxic oxygen and nitrogen metabolites or by melanization pathway.

Knowledge of the less complex invertebrate defense strategies may contribute to understanding more sophisticated vertebrate immune system, and lead us to identify new molecules with possible therapeutic use.

Since the pioneering works of Ellie Metchnikoff at the end of the 19th century (Mechnikoff 1887) were done, invertebrate immunology has become a proper topic of study. However, it is only the last three decades when detailed analyses of the invertebrate immune reactions and their molecular basis have been emerged.

Earthworms belonging to oligochaete annelids became a model for comparative immunologists in the early 1960s with the publication of results from transplantation experiments. For their crucial role in soil fertilization, they are used in monitoring of environmental pollution.

Moreover, earthworms might be considered as a source of biologically active compounds with potential industrial or medical use. Earthworm powder has been used in traditional medicine as a drug for treatment of various diseases in the Far East already several thousand years ago. Currently, the therapeutic effect of earthworm active factors is re-evaluated by modern scientific approaches. For instance, a potent fibrinolytic

enzyme extracted from *Lumbricus rubellus* is in a clinical-trial phase as a possible antithrombotic drug (Mihara *et al.* 1991, Nakajima *et al.* 1996, Nakajima *et al.* 1999).

2. Earthworm biology

Annelida is a group commonly referred to segmented worms. They are found worldwide, through the deepest marine sediments to the soils. Originally, Annelida were split up into three major groups; Polychaeta, Oligochaeta and Hirudinea.
New cladistics system now classifies Oligochaeta (earthworms etc.) and Hirudinea (leeches), in one clade that should be referred to as Oligochaeta (Siddall et al. 2001). Moreover, it is possible that this group may well belong inside Polychaeta, thus making Polychaeta synonymous with Annelida (Westheide et al. 1999).

In terms of taxonomy, earthworms are the largest family of the class Oligochaeta, phylum Annelida. Members of this class inhabit terrestrial ecosystems (Edwards and Shipitalo 2004) and represent the major macrofauna in many soils (Edwards and Bohlen 1996). There are more than 3000 different earthworm species worldwide known, in range size from several millimeters to over one meter.

Earthworms are protostomian animals with bilateral symmetry and true coelomic cavity of mesenchymal origin. The earthworm body is metameric and the segments are separated by transversal septa. Each segment contains a complete set of excretory organs and nerve center. Generally, Annelids have a closed circulatory system, consisting of the dorsal and ventral vessels and connecting capillaries. The earthworms are hermaphroditic, but two worms are required to mate and reproduce. Reproductive organs are in the clitellum, where the cocoons are formed to protect developing eggs.
The alimentary canal of earthworms begins with the mouth; continues as pharynx, esophagus, crop, gizzard and intestine; and finally ends at anus. Salivary glands in the pharynx release amylase- and protease-containing mucus to facilitate food movement through the alimentary canal. The esophagus carries calciferous glands secreting mucus containing calcium carbonate for pH regulation in gut and coelomic fluid. The crop is responsible for storing food. The gizzard is a muscular grinder crushing the ingested material. Digestive enzymes (e.g., cellulases, chitinases, lipases) are secreted into the intestine by worm to digest microorganisms (Urbasek and Pizl 1991). The digested food is absorbed by the intestinal villi and typhlosole (Fig. 1). The typhlosole is surrounded by the intestinal lumen, which increases the effective surface area of gut. The outside

part of the intestine and majority interior of the typhlosole is a specialized tissue composed of chloragogen cells. These cells fulfill metabolic functions including fat and hemoglobin synthesis. The intestine can be anatomically divided into three parts: foregut, midgut and hindgut. The digested food is passed into the blood stream for distribution. Undigested food and soil are eliminated in the form of worm castings through the anus.

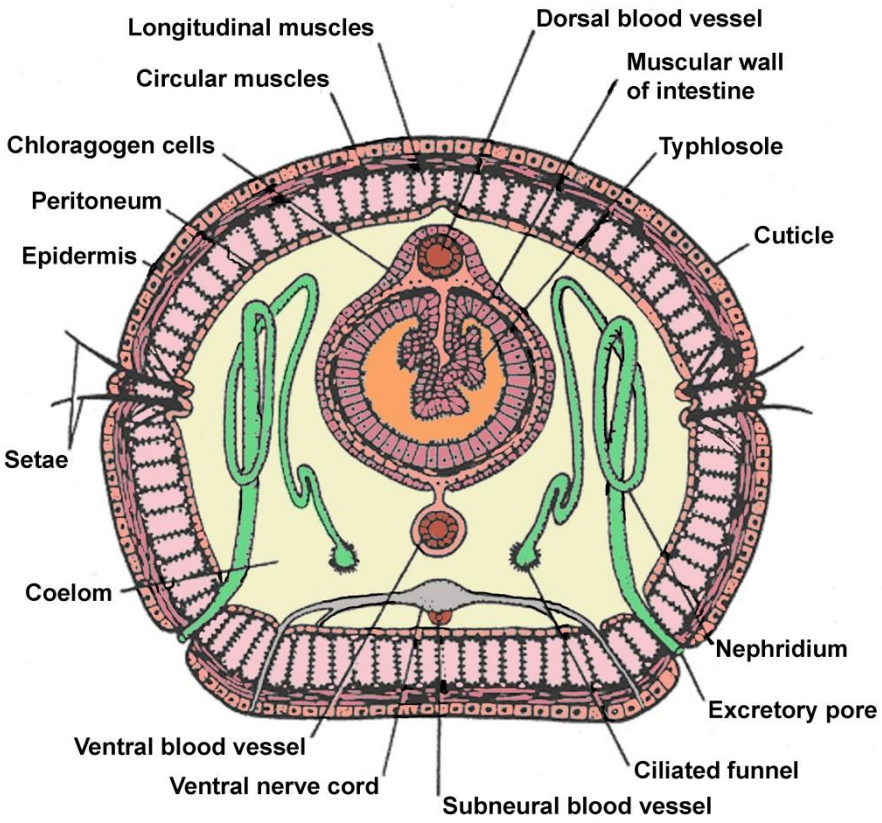

Fig.1: Earthworm cross section (adopted from Biology of the Invertebrates, (Pechenik 1996)).

2.1. Earthworm ecology

Earthworms are classified into three main ecophysiological categories depending on their location and feeding strategies (Fig. 2): Epigeic worms (e.g. *Eisenia andrei, Eisenia fetida, Lumbricus rubellus*) live above the mineral soil, rarely form borrows and

feed preferentially on leaf litter. These small deeply pigmented worms are often used in vermicomposting systems. Endogeic worms (e.g. *Aporrectodea caliginosa*, *Allolobophora chlorotica*) are small to large sized, with weakly pigmented body and form extensive horizontal burrows. They ingest high amounts of mineral soil and particle of organic matter. Anecic worms (e.g. *Aporrectodea longa*, *Lumbricus terrestris*) are larger, dorsally pigmented and they build permanent vertical burrows from the surface soil through the mineral soil layer. The anecic species ingest soil and feed on litter, which they drag into their burrows.

Earthworms live in permanent close contact with soil particles and soil microorganisms via both their highly permeable skin and alimentary tracts (Jager *et al.* 2003). Therefore, they are significantly affected by the soil contaminants, such as organic pollutants (Jager *et al.* 2005), heavy metals (Nahmani *et al.* 2007) or nanoparticles (Canesi and Procházková 2014).

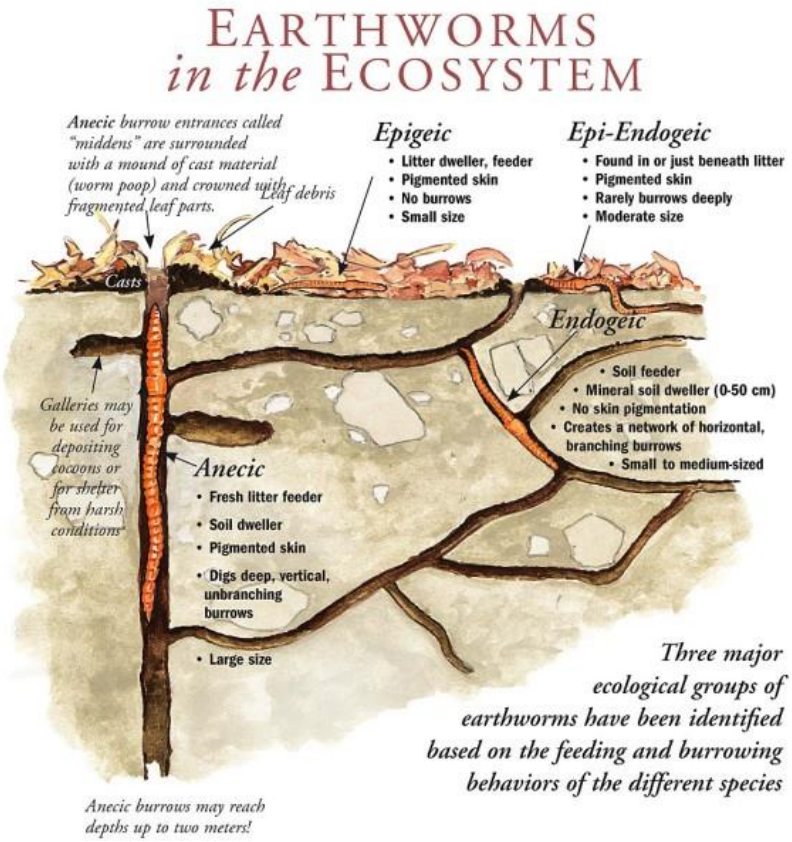

Fig. 2: Earthworms in the ecosystem. Adopted from Biodiversity "Earthworms." Aboutbiodiversity.org. Bay and Paul Foundation, n.d. Web. 29 Nov. 2011. http://aboutbiodiversity.org/soil/earthworms.htm

Earthworms represent an important part of the terrestrial food chain and for that reason are eligible for the monitoring of soil contamination.

Two closely related earthworm species inhabit significantly different habitats, *Eisenia andrei* (Bouché 1972) and *Eisenia fetida* (Savigny, 1826). While *E. andrei* lives in a compost and manure, *E. fetida* can be found in the litter layer in forests. These two species were first described as different morphotypes of *E. fetida* according to differences in the body pigmentation (André 1963), and subsequently established as subspecies (Bouché 1972) named *Eisenia fetida andrei* and *Eisenia fetida fetida*. Recently, they are considered as two independent species, *E. andrei* and *E. fetida* (Dominguez *et al.* 2005). However, a reliable discrimination of these two species is rather difficult due to the variability of morphological and anatomical characteristics.

These two epigeic species *E. fetida* and *E. andrei*, have been used for many years to monitor ecotoxicity (Ribera *et al.* 2001, Zheng *et al.* 2013). The choice of suitable biomarkers for study of the molecular and cellular response of earthworms to contaminants is crucial. Convenient biomarkers include DNA damage, enzyme activities (acetylcholinesterase, metallothioneins, biotransformation and antioxidant enzymes), gene expression of defense molecules and lysosomal membrane stability (Lionetto *et al.* 2016).

There are two sets of protocols for monitoring of environmental pollution, one published by the Organization for Economic Cooperation and Development (OECD) and the second one by the International Organization for Standardization (ISO). These guidelines describe methods for the assessment of the ecological risk of contaminated soil, the determination of the acute toxicity of chemicals on earthworms (OECD 1984, ISO 1993), and the effect on their reproduction (ISO 1998, OECD 2004).

2.2. Microbial environment and earthworms

Soil is probably the most complex microbial habitat on the Earth (Tate 1997). It is estimated that the soil microbial population varies from 10^4 to 10^8 CFU per gram of dry weight of soil (Edwards and Shipitalo 2004). The most common bacterial species in the soil are representatives of genus *Bacillus*, *Pseudomonas* and *Streptomyces*.

Earthworms, as the most common terrestrial animals, live in the permanent contact with soil. The communication with the outer environment is conducted by dorsal pores, pairs of nephridia and through the alimentary system.

The earthworm coelomic fluid is not aseptic and always contains naturally occurring bacteria, protozoans and fungi entering from the outer environment mainly through the dorsal pores. The growth of bacteria is kept under the control mainly by phagocytic cells and different antimicrobial proteins.

Earthworm gut provides a unique and mobile anaerobic environment in the aerated soil ecosystem (Drake and Horn 2007). The microbial communities are able to growth under anaerobic conditions, acetogens (Drake *et al.* 2006) and methanogens (Whitman *et al.* 2006), are more abundant in the gut than in the aerated soil, which earthworms obtained from (Drake and Horn 2007).

Composition of microbial community associated with earthworm intestine depends on an ecological group (epigeic, endogeic and anecic) and habitat earthworms live in (Thakuria *et al.* 2010).

Earthworms, as well as their gut microbes contribute to nutrients decomposition in the soil. Gut microbes are responsible for cellulase and mannose activities (Munnoli *et al.* 2010). The gut organic matter decomposition brings the benefit to both earthworm and gut microbes, called a mutualistic digestive system (Drake and Horn 2007). Certain groups of microorganisms as well as, protozoa, fungi and some microfungi were found to be significant compounds in the earthworm diet. Gram-positive bacteria *Bacillus cereus* var. *myceides* were decreased during gut passage while Gram-negative (*E. coli* and *Serracia marcessens*) were completely eliminated (Edwards and Fletcher 1988). Fischer and colleagues demonstrated that passage by *L. terrestris* enhanced the germination of *Bacillus megaterium* spores (Fischer *et al.* 1997).

The existence of specific interactions of bacterial genus with earthworms nephridia vertically transferred from parenteral *E. fetida* to their offspring was reported. These microbes belong to the *Acidovorax sp.* and its presence is probably important for nephridia development (Davidson and Stahl 2006).

2.3. Defense mechanisms of the earthworms

Earthworms often occupy very hostile environments. The first protective barrier is represented by skin, covering the entire body. The skin consists of the epidermis and thin cuticle containing mucopolysaccharides with antimicrobial role (Rahemtulla and Lovtrup 1974, Rahemtulla and Lovtrup 1975). The epidermis is formed by a single layer of epithelium supporting cells, basal cells and secretory cells. The basal cells play

an important role during the process of wound healing and graft rejection and often exhibit phagocytic activity.

In earthworms, cellular immune functions are maintained by different types of mesodermal origin cells, generally named coelomocytes floating in the coelomic fluid that fills the coelomic cavity (Vetvicka and Sima 2009). The nomenclature of coelomocytes is based mainly on their morphological characteristics and cytochemical criteria. Three main populations of earthworm cells were described: hyaline coelomocytes, granular coelomocytes or amoebocytes, and eleocytes (free-floating chloragocytes) (Sima 1994, Cooper 1996). The existence of three different subgroups of coelomocytes was also proved by flow cytometry (Engelmann *et al.* 2002, Vernile *et al.* 2007). Hyaline or granular amoebocytes represent effector immunocytes with strong phagocytic and encapsulation activity. However, this activity differs in both types of amoebocytes - granular amoebocytes engulf less foreign particles than hyaline cells. The third type, eleocytes, is usually very abundant subpopulation in the coelomic cavity and most authors assume that they were derived from the chloragogen tissue (Jamieson 1981). Eleocytes possess nutritive functions such as glycogens and lipids production and were also described as producers of bioactive molecules such as haemoglobin or metal-sequestering cysteine-rich proteins (Fischer 1993, Morgan *et al.* 2004). Regarding their role in immune mechanisms, they do not have phagocytic activity but are considered as a potential source of antimicrobial molecules (Cooper *et al.* 2002).

2.3.1. Cellular defense mechanisms

2.3.1.1. The elimination of foreign objects

Microorganisms can enter the coelomic cavity mainly by the dorsal pores (Fig. 1 and 3). Nevertheless, they can be eliminated in different ways: expelled by dorsal pores, excreted by nephridia or engulfed by the cells of nephrostome and middle tube (Cameron 1932, Villaro *et al.* 1985). The invading microorganisms can be also eliminated by phagocytic cells (see chapter phagocytosis) in combination with humoral antibacterial substances. Subsequently, exhausted phagocytic cells can be expelled through dorsal pores (Cameron 1932).

Large particles e.g. agglutinated bacteria or parasites, are eliminated by encapsulation (Ratcliffe *et al.* 1985) that leads to formation of the fibrous capsule called brown body (Valembois *et al.* 1992). Brown bodies contain two important pigments,

lipofuscin and melanin, final products of prophenoloxidase cascade (proPO), (see chapter prophenoloxidase cascade). The presence of pigment might probably inactivate the effect of free radicals released to segregate unwanted particles (Valembois *et al.* 1994). When the brown bodies are about 1-2 mm in diameter, they migrate towards the posterior segments of the coelomic cavity. There are often eliminated by autotomy of caudal segments followed by wound healing (Keilin 1925, Alonsobedate and Sequeros 1983, Alonsobedate and Sequeros 1985). The process of autotomy enables the elimination of highly toxic organic (Paris-Palacios *et al.* 2010) and inorganic (Mendez-Fernandez *et al.* 2013) residues. Autotomy process seems to be under neurohormonal control (Alonsobedate and Sequeros 1985).

This assumption was later proved by experiments, where the autotomy was decreased or even inhibited/abolished after nicotine pretreatment (Kocinski *et al.* 2016).

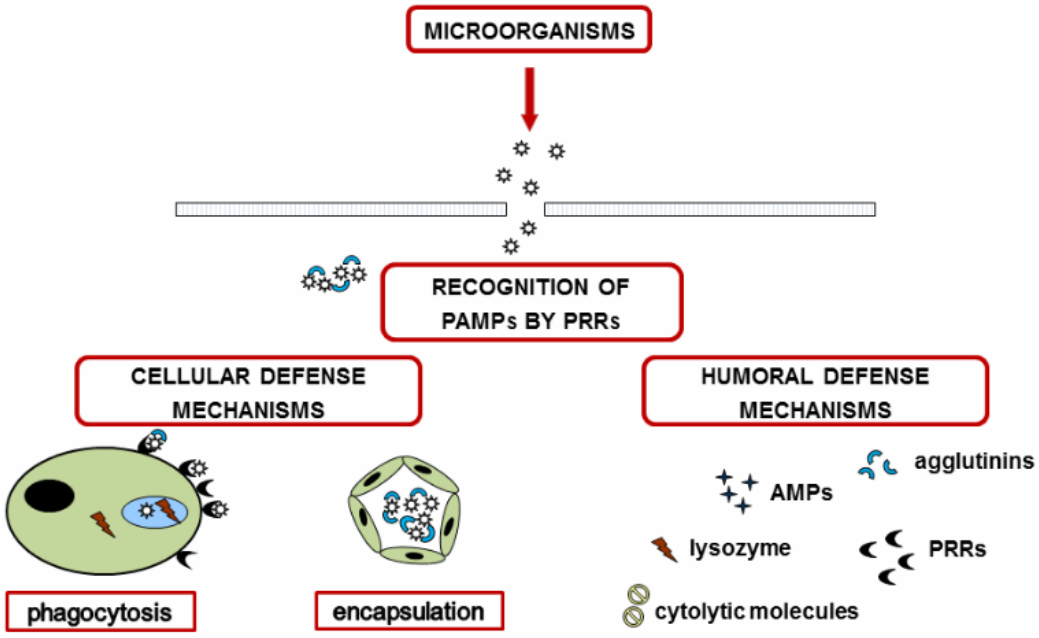

Fig. 3: The general scheme of the innate defense mechanisms in earthworms. The first protective barrier of earthworms is the skin in combination with the secreted mucus that contains various antimicrobial factors. Invading microorganisms are recognized by both soluble and membrane-bound pattern recognition 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.

2.3.1.2. Phagocytosis

Phagocytosis represents a phylogenetically ancient defense mechanism. This process was discovered by Metchnikoff in 1887 while studying the origin of the digestive organs in the starfish floating larvae (Mechnikoff 1887). The first detailed description of this process in earthworms was reported by Cameron (1932). He showed inert particles, foreign cells and bacteria as well, being phagocytized both *in vitro* and *in vivo* (Cameron 1932) by all types of coelomocytes with the exception of free chloragogen cells (eleocytes). The phagocytic activity differed in the amount of engulfed particles and in the type of ingested material. Cameron´s results were subsequently confirmed in reports describing uptake of yeast (Stein and Cooper 1981), erythrocytes (Laulan *et al.* 1988), bacteria (Dales and Kalac 1992) and inert particles (Bilej *et al.* 1990a, Bilej *et al.* 1991).

Phagocytosis begins with recognition of non-self, followed by engulfment and destruction of particles. Phagocytosis by coelomocytes, similarly to that of vertebrates, can be modulated by humoral components, opsonins, which coat the engulfed particle. The opsonins are than recognized by receptors on the phagocytic cells and thus promote the phagocytosis. The presence of opsonins possesses enhanced effect on phagocytosis in the coelomic fluid, which was proven by preincubation of both, yeast and sheep red blood cells with, IgG or C3b complement fragment (Stein and Cooper 1981, Laulan *et al.* 1988). On the other hand, this effect was not found out after application of IgM and C3d fragment. The phagocytosis was also enhanced after opsonization of synthetic particles in the coelomic fluid (Bilej *et al.* 1990a, Bilej *et al.* 1990b).

Engulfed material can be also eliminated beside mechanical ways by intracellular degradation by proteolytic enzymes, lysosomal enzymes or by an oxidative burst. The oxidative burst involves in production of highly reactive oxygen radicals. The oxygen radicals were detected both in the coelomic fluid and in chloragosomes of chloragogen cells (Valembois *et al.* 1991).

2.3.1.3. Transplantation immunity

The ability to recognize and respond to allografts as well as xenografts and, on the other hand, not to destroy and accept autografts, was observed in several annelid species (Cooper 1970). The beginning of this process is similar to the reaction to injury or wound healing. The first major change, occurring after the wound healing, regardless

the transplanted graft origin, is accumulation of the coelomocytes and their infiltration near the graft sites and subsequently into the matrix. Strong response to the xenografts leads to complete rejection of the graft and subsequent destruction by encapsulation (Parry 1978). Maximal response to xenograft was observed 3 to 4 days on and the final destruction was completed approximately after 17 days. If the second graft is transplanted at this time then accelerated rejection occurs within 6 or 7 days. Moreover, the number of invading coelomocytes is 20 – 30% higher, most likely due to an increased proliferation activity of mesenchymal lining of the coelomic cavity and the septa. The maximum number of coelomocytes surrounding allografts was detected within 24 hours, returning to normal level in 72 hours. If the second allografts from same donor to same recipient were transplanted less than 10 days after first allografts, the accelerated rejection was observed. In contradiction, any accelerated rejection was not observed while second allograft was transplanted at later intervals, after 20 - 90 days (Cooper and Roch 1986). These data suggest the existence of short-term "memory", based strictly on cells in the earthworm species (Bailey *et al.* 1971, Hostetter and Cooper 1973). Autografts elicit weaker coelomocyte response. Hostetter and Cooper observed markedly lower number of invading coelomocytes in the autograft site, even though the reaction seems to be rapid and the autologous transplants are easily accepted (Cooper 1970, Hostetter and Cooper 1973, Cooper and Roch 1994).

2.3.2. Humoral defense mechanisms

Humoral components play a crucial role in the process of recognition and elimination of invading microbial pathogens and supplement the cellular compartment of the innate immunity. The coelomic fluid of annelids contains various antimicrobial factors like lysozyme (Cotuk and Dales 1984) and number of antimicrobial peptides (Fig. 3) (Cho *et al.* 1998, Wang *et al.* 2003, Liu *et al.* 2004, Tasiemski *et al.* 2007, Ovchinnikova *et al.* 2008). Particular attention has also been devoted to antimicrobial factors with hemolytic activity secreted by coelomocytes into the coelomic cavity. These proteins were originally described as hemolytic factors acting against different vertebrate erythrocytes (Du Pasquier and Duprat 1968). Later on, it was observed that coelomic fluid lyses other eukaryotic cells, erythrocytes, namely chicken fibroblasts, guinea-pig polymorphonuclear leukocytes, and insect hemocytes (Kauschke and Mohrig 1987).

2.3.2.1. Lysozyme

Lysozyme is widely distributed enzyme within the animal and plant kingdoms (for review (Jolles 1996)). Lysozyme is a bacteriolytic enzyme that cleaves β-1,4-glycosidic bonds between *N*-acetylglucosamin (GlcNAc) and *N*-acetylmuramic acid (NAM) of the peptidoglycan present in bacterial cell walls, which leads to cell death (Ellison and Giehl 1991). Lysozyme represents an efficient defense mechanism against Gram-positive bacteria, but less efficient against Gram-negative bacteria (Cunningham *et al.* 1991).

Based on differences in their structure, catalytic character and original source, lysozymes are classified into six groups. The most studied is chicken-type of lysozyme (c-lysozyme) present in many vertebrates and insects. Goose-type of lysozyme (g-lysozyme) was identified in many vertebrates including mammals, birds and fish (Prager and Jolles 1996). Besides the above mentioned groups, another plant lysozyme (Beintema and Terwisscha van Scheltinga 1996), bacterial lysozyme (Holtje 1996), phage lysozyme (Fastrez 1996) and cysteine–rich invertebrate type lysozyme (i-lysozyme) were described.

In 1960, Jolles reported a lysozyme-like activity in coleomic fluid of the earthworm *Nephtys* (Jolles and Zuili 1960). Later on, lysozyme-like activity was described in the coelomocytes extract and in the coelomic fluid of *E. fetida* (Cotuk and Dales 1984). Purified lysozyme has a molecular weight of 13 kDa and shows a considerable homology with a marine bivalve, a marine conch and a starfish, but the similarity with other known types of lysozyme was negligible (Ito *et al.* 1999). Later, a full-length cDNA of *E. andrei* earthworm lysozyme (EALys) was assembled with an open reading frame coding 160 amino acids. Subsequent analysis of predicted amino acid sequence revealed a high content of cysteine residues, which occurrence is typical for lysozyme family (Joskova *et al.* 2009). These residues are essential for proper protein folding and molecule stability. Besides the conserved cysteine residues, the molecule also contains three amino acid residues potentially important for the lysozyme activity – Glu14, Asp26 and Ser29. The recombinant *E. andrei* lysozyme (rEALys) possesses isopeptidase activity, in addition to its lysozyme activity. Subsequently Joskova *et al*. studied effect of pH, pI and temperature on the both activities of rEALys. They proved the involvement of EALys in protection against infections caused not only by Gram-positive, but also by Gram-negative bacteria (Joskova *et al.* 2009).

2.3.2.2. Hemolytic molecules with antimicrobial activity

The coelomic fluid of *E. fetida* earthworms contains antimicrobial proteins with hemolytic activity. These proteins form heterogeneous group without clear relations. The first isolated proteins with hemolytic activity were called *Eisenia fetida andrei* factors (EFAF) (Du Pasquier and Duprat 1968). EFAFs are two glycoprotein isoforms secreted by eleocytes (Du Pasquier and Duprat 1968, Roch 1979, Roch *et al.* 1981). The 45 kDa protein is encoded by single nonpolymorphic gene and has pI of 6.0, while the 40 kDa protein is encoded by gene having several alleles, each representing one isoform with pI of 6.3, 6.2, 5.95 and 5.9. Each individual earthworm possesses a 45 kDa protein and either two or three isoforms of the 40 kDa molecule (Roch 1979, Roch *et al.* 1987). Besides the hemolytic activity, EFAFs are able to agglutinate red blood cells (Valembois *et al.* 1984) and participate in cytotoxic activity of the coelomic fluid (Kauschke and Mohrig 1987). Importantly, EFAFs also show the antibacterial activity against both Gram-positive and Gram-negative bacteria (Hirigoyenberry *et al.* 1990), participate in coelomic fluid clotting process (Valembois *et al.* 1988) and opsonization (Sinkora *et al.* 1993). Upon binding to sphingomyelin (a major lipid constituent of plasma membranes of most mammalian cells), these proteins polymerize and form 10 nm channels through the lipid bilayer (Roch *et al.* 1989). Both glycoprotein isoforms display peroxidase activity. The 40 kDa isoform was characterized at the molecular level. The putative amino acid sequence containing *N*-glycosylation site and peroxidase motif was found (Lassegues *et al.* 1997, Milochau *et al.* 1997). Later, EFAFs proteins were named fetidins.

Independently, a 41 kDa protein, with ability to cause contraction of rat vascular smooth muscle, was identified. This protein is secreted by coelomocytes of *E. fetida* and was called lysenin (Sekizawa *et al.* 1996). Later on, two 42 kDa lysenin-related proteins with weak contraction activity were identified (Sekizawa *et al.* 1997) and another member of this lysenin-like multi-gene family was cloned and called lysenin-related protein 3 (Bruhn *et al.* 2006). Lysenin was expressed only in large coelomocytes and in free chloragocytes present in the lumen of typhlosole but the expression was not detected either in the intestine, or any other tissues (Sekizawa *et al.* 1997, Ohta *et al.* 2000). Lysenin shows strong hemolytic activity, which correlates with relative level of sphingomyelin in membranes. After binding sphingomyelin, oligomer and subsequently pores about 3 nm in diameter are formed (Yamaji *et al.* 1998, Yamaji-Hasegawa *et al.*

2003). The primary function of this cytolytic system is probably to destroy membranes of foreign cells. Due to these observations, it has been proposed lysenin use as a valuable probe for sphingomyelin detection in sphingomyelin storage diseases, particularly in the cells of Niemann-Pick A patients (Yamaji *et al.* 1998). Lysenins and fetidins display a great homology in amino acid sequence suggesting a close relation between these hemolytic molecules.

Another 38 kDa hemolytic protein also identified in the coelomic fluid of *E. fetida* earthworm was named Eiseniapore. It was found out that Eiseniapore functionally requires sphingomyelin or galactosylceramide to bind and lyse erythrocytes (Lange *et al.* 1997). Similarly, fetidin, lysenin and Eiseniapore lytic activity is enhanced by cholesterol. Six monomers form ring-shaped hydrophilic pore with an outer diameter of 10 nm and an inner diameter of 3 nm on the target membrane (Lassalle *et al.* 1993, Lange *et al.* 1997). Eiseniapore seems to be associated with a natural inhibitor called Eiseniapore-regulating protein (Lange *et al.* 1997) in the coelomic fluid.

Furthermore, three hemolytic proteins H_1 (46 kDa), H_2 (43 kDa) and H_3 (40 kDa) were isolated from the coelomic fluid of *E. fetida.* Each protein consists of various isoforms of pI between 5.1 and 6.2 mainly further structural similarities. Despite the cross-reactivity of monospecific antisera, hemolysins are functionally different. H3 is a bifunctional protein – besides hemolytic activities common to all hemolysins, it also possesses agglutinating activity (Fig. 3). Eue and Koenig isolated and characterized two hemolytic proteins from cell lysate (CL39 and CL41) and three hemolytic proteins from coelomic fluid (H_1, H_2 and H_3) of *E. fetida*. By using mass spectrometry, the identity of CL_{39} with fetidin and CL_{41} with lysenin was demonstrated. Moreover, H1-3 proteins and fetidins display sequence homology however fetidins seem to be glycosylated (Eue *et al.* 1998, Koenig *et al.* 2003).

The *E. fetida* hemolytic molecules share similar lytic function but display interindividual heterogenity. Eiseniapore is quite different from lysenin and possibly from fetidins, because it does not only bind sphingomyelin but also galactosylceramide.
In 2006, Prochazkova and colleagues compared more than thirty sequences of *E. fetida* hemolytic molecules and showed that fetidin and CL_{39} belong to fetidin/CL_{39} group and lysenin and CL_{41} to lysenin/CL_{41} group. These hemolytic molecules are encoded by two individual genes with high homology and their expression level differs from individual to individual (Prochazkova *et al.* 2006).

2.3.2.3. Pattern recognition receptors (PRRs)

In 1989 Charles Janeway, Jr., published a general concept based on the existence of pattern recognition receptors (PRRs) on the immune cells that recognize and bind conserved molecular structures of microorganisms known as pathogen-associated molecular patterns (PAMPs) and trigger the immune response to potential pathogens (Janeway 1989). The PAMPs include lipopolysaccharide (LPS), bacterial peptides (flagellin), peptidoglycans and lipoteichoic acids, nucleic acids (such as bacterial or viral DNA or RNA), *N*-formylmethionine, and fungal glucans. The PRRs were described in a wide range of animals, plants and invertebrates, suggesting their ancient role.

2.3.2.3.1. Coelomolytic cytolytic factor (CCF)

Coelomic fluid of earthworm *E. fetida* contains a 42 kDa pattern recognition receptor named coelomic cytolytic factor (CCF) (Bilej *et al.* 1995, Beschin *et al.* 1998). Originally, CCF was described as a protein lytic for TNF-sensitive tumor L929 cell line (Bilej *et al.* 1995) that shares functional analogies with the mammalian cytokine tumor necrosis factor (TNF). Indeed, similarly to TNF, CCF is secreted by macrophage-like coelomocytes upon lipopolysaccharide stimulation, exhibits opsonizing properties and has trypanolytic activity (Bilej *et al.* 1998), while TNF is produced by macrophages (Aggarwal *et al.* 1985). Further, it was shown that CCF agglutinates both Gram-positive and Gram-negative bacteria (Beschin *et al.* 1998) and contributes to the opsonizing properties of the coelomic fluid.

When CCF was cloned, striking homology to the other PRRs was revealed (Beschin *et al.* 1998). This PRR is able to bind, via lectin-saccharide interactions, Gram-negative bacteria (O-antigen of lipopolysaccharide), yeast (β-1,3-glucans and *N,N´*-diacetylchitobiose) and cell wall components of Gram-positive bacteria (the peptidoglycan constituents muramic acid and muramyl dipeptide). Upon binding these microbial-associated molecular patterns, CCF triggers the activation of the prophenoloxidase cascade (proPO) in earthworm coelomic fluid, which results in production of melanin (Johansson and Soderhall 1996, Beschin *et al.* 1998, Cerenius and Söderhäll 2004). The broad specificity of CCF is based on the presence of two pattern recognition domains (Bilej *et al.* 2001) (Fig. 4).

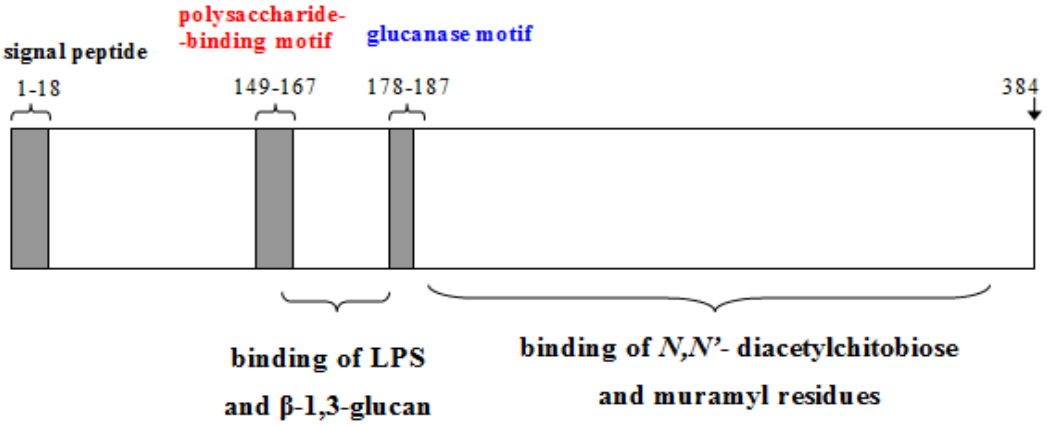

Fig. 4: Structure of *E. fetida* CCF.

First domain is located in the central part of CCF molecule and displays amino acid homology with bacterial and animal β-1,3-glucanases and invertebrate defense molecules (Yamamoto *et al.* 1993, Kozhemyako *et al.* 2004), but the glucanase activity was not proved. The domain-mediated interaction of CCF molecule with lipopolysaccharide and β-1,3-glucans. The C-terminal tryptophan-rich domain, binds *N,N´*-diacetylchitobiose and peptide constituents, namely muramyl dipeptide and muramic acid (Beschin *et al.* 1998, Bilej *et al.* 2001).

The process of opsonization facilitates the phagocytosis, a very important defense reaction in earthworm (Bilej *et al.* 1995). Moreover, CCF contributes to the cell-mediated cytotoxic reactions (Bilej *et al.* 1998).

Comparative analysis of CCF molecules from six various Lumbricidae species (*Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestis*) revealed the unique ability of CCF molecule of *E. fetida* to activate the prophenoloxidase cascade in the presence of *N,N'*-diacetylchitobiose and β-1,3-glucan laminarin, in contrast to the others CCF molecules, which activate this pathway only in the presence of laminarin (Silerova *et al.* 2006). Cytolytic or trypanolytic activity of the coelomic fluid of other earthworm species except that of *E. fetida* was not detected (Silerova *et al.* 2006). This broad recognition repertoire of *E. fetida* CCF probably reflects a particular microbial environment this species lives in.

Interestingly, the lectin-like domain of TNF is involved in killing of bloodstream forms of African and American trypanosomes (Lucas *et al.* 1994, Magez *et al.* 1997,

Fontt *et al.* 1998) as well as the purified CCF and coelomic fluid of *E. fetida* earthworms (Fontt *et al.* 2002). In both cases, CCF and TNF probably bind N-linked *N,N´*-diacetylchitobiose core of variant specific glycoprotein (VSG) covering trypanosome surface.

Lytic effect of coelomic fluid or CCF can be efficiently inhibited in the case of African *Trypanosoma brucei brucei* by anti-CCF monoclonal antibodies but also *N,N´*-diacetylchitobiose and anti-TNF antibodies (Beschin *et al.* 1999). In the case of American *Trypanosoma cruzi,* lytic activity is only partially inhibited (Fontt *et al.* 2002).

Furthermore, especially/particularly TNF, its *N,N´*-diacetylchitobiose lectin-like domain, induces the increase of pH-dependent membrane conductance of lung endothelial cells and peritoneal macrophages (van der Goot *et al.* 1999) and, causes membrane depolarization (Hribar *et al.* 1999). This effect is not dependent on the presence of TNF receptors, because similar activity in cells isolated from mice deficient in both TNF receptors (TNFR1, TNFR2) was observed. It was suggested that the membrane depolarization is due to the TNF interaction with amiloride-sensitive ion channels, most likely sodium ion channels (van der Goot *et al.* 1999, Fukuda *et al.* 2001). In contrast to TNF, CCF does not require acidification of a lysosomal compartment for activation of amiloride-sensitive ion channels and works also in a TNF receptor (TNFR) independent manner (Bloc *et al.* 2002). Membrane depolarization is inhibited in case of CCF as well as TNF by amiloride or *N,N´*-diacetylchitobiose. This effect suggests lectin-like saccharide interaction of CCF with a sodium channel or with associated structure (Bilej *et al.* 2006).

2.3.2.3.2. Prophenoloxidase cascade (proPO)

The prophenoloxidase activation cascade (proPO; Fig. 5) is one of the most important defense mechanism in many invertebrates, allowing a rapid response to pathogen infection.

The cascade (Söderhäll and Cerenius 1998, Cerenius and Söderhäll 2004) is triggered upon binding of PRRs to microbial structures known as PAMPs, such as lipopolysaccharide, peptidoglycan or β-1,3-glucans, resulting in proteolytic cleavage by serine proteinases of inactive zymogen prophenoloxidase (proPO) to its active form, phenoloxidase (PO). The active form of PO catalyses both the *o*-hydroxylation of monophenols and the oxidation of diphenol to quinones. Consequently, the quinones

non-enzymatically polymerize to melanin (Ashida and Yamazaki 1990, Söderhäll *et al.* 1994).

Melanin has bacteriostatic, fungistatic and antiviral properties and together with the cytotoxic quinones, reactive oxygen (ROI) or nitrogen (RNI) intermediates is involved in the innate immune response of certain invertebrates, especially arthropods. Melanin also takes part in wound healing and is important for encapsulation of foreign materials.

The molecular weight of proPO as well as PO varies between 70 and 90 kDa. The active site of all enzymes contain a canonical di-nuclear copper center, with each copper ion coordinated by three conserved His residues. Glu395 of the subunit-2 may act as a general base to deprotonate monophenols, a key step in the *o*-hydroxylation of tyrosine by PO (Rosenzweig and Sazinsky 2006).

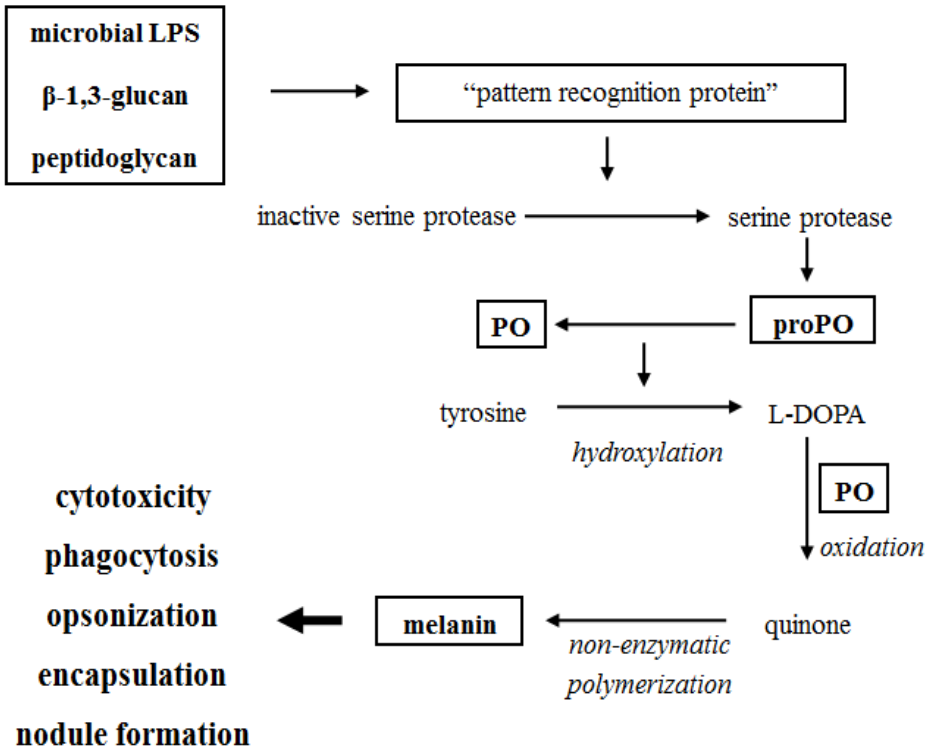

Fig. 5: Prophenoloxidase activating cascade. The recognition of PAMPs leads to the cleavage of inactive prophenoloxidase to its active state phenoloxidase. Active enzyme catalyzes both hydroxylation and oxidation of phenols to quinones, which are subsequently polymerized to melanin. Melanin exhibits various biological activities important in defense reactions.

Another phenoloxidase conserved peptide motif GCGEQNM (Armstrong and Qiuigley 1999) is also present in α-2-macroglobulins in both invertebrates and vertebrates (Spycher *et al.* 1987, Hall *et al.* 1989) and in the vertebrate complement proteins C3 and C4 (Dodds and Law 1998).

The proPO cascade activation must be strictly regulated and localized only in the place of injury or infection, because of the production of toxic intermediates. This control is partially achieved by synthesizing the enzyme as an inactive zymogen (Johansson and Söderhäll 1985, Sritunyalucksana *et al.* 1999) and also by the presence of several inhibitors of PO and serine proteinases (Daquinag *et al.* 1999).

The PO activity found in the coelomic fluid of *E. fetida* suggested the presence of proPO cascade in annelids (Seymour *et al.* 1992, Beschin *et al.* 1998, Prochazkova *et al.* 2006). It was documented that proPO cascade of *E. fetida* is directly activated by Gram-negative bacteria and yeast, while Gram-positive bacteria needs to be pretreated with lysozyme to activate the cascade (Bilej *et al.* 2001). The recognition of microbial PAMPs activates proPO cascade, triggered by the presence of CCF molecules in CF of *E. fetida* was documented. (Beschin *et al.* 1998, Bilej *et al.* 2001).

2.3.2.3.3. Toll and Toll-like receptors (TLRs)

Originally, Toll was identified as a molecule playing the role in embryonal development in fruit fly *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus 1980). For this pioneering work, Christiane Nüsslein-Volhard was awarded the Nobel Prize. Later on, Toll was found by Jules A. Hoffman and his colleagues to have an essential role in the fly's immunity to fungal infection (Lemaitre *et al.* 1996) that is achieved by activating the synthesis of antimicrobial peptides. For this work Jules A. Hoffman was awarded the Nobel Prize in 2011 making Toll the only protein awarded the Nobel Prize repeatedly.

Soon after the characterization of the immune function of Toll and its ligand S*pätzle*, a family of Toll-like receptors was described. The Toll-like receptors (TLRs) are membrane pattern recognition receptors (PRRs) recognizing specific conservative molecules expressed by bacteria, fungi or viruses (so-called pathogen-associated molecular patterns-PAMPs).

The Toll-like receptor family is conserved throughout the evolution from plants (Gassmann *et al.* 1999) to animals (Coscia *et al.* 2011, Satake and Sekiguchi 2012). Toll and Toll-like receptors (TLRs) are type I transmembrane glycoproteins composed of

intracellular Toll/Interleukin-1 receptor homology domain (TIR), transmembrane and extracellular leucine-rich repeats (LRRs) containing domain (Medzhitov *et al.* 1997). Based on the structure of TLR extracellular domain, they can be categorized into two major types. The first "vertebrate-like" type (V-type) includes all described deuterostomian and a minority of insect TLRs. The second group, here referred as "protostome-like" type (P-type), comprises nearly all TLRs genes found in insects - and other protostomes. Both receptor groups differ in structure of their transmembrane domain (Hibino *et al.* 2006).

The Toll or TLRs receptors were discovered almost in all living organisms, but have not yet been identified in the earthworms (Cooper *et al.* 2006). Only recently genomic surveys revealed high diversity in the number of TLRs among invertebrate species. For example, in annelids 105 TLRs were described in marine polychaete *Capitella capitate*, but only 16 in leech *Helobdella robusta* (Davidson *et al.* 2008) and one *Hm*TLR1in the nerve cord of *Hirudo medicinalis* (Cuvillier-Hot *et al.* 2011).

2.3.2.3.4. Lipopolysaccharide binding protein/Bacterial permeability increasing protein (LBP/BPI)

Lipopolysaccharide (LPS)-binding proteins (LBPs) and bacterial permeability increasing proteins (BPIs) are closely related proteins involved in innate immunity.
When LPS occurs in the blood system of mammals, its lipid A part is recognized by both lipopolysacharide binding protein (LBP) and bacterial permeability increasing protein (BPI).

Both LBP and BPI are pattern recognition molecules that play an important role in organism protection against Gram-negative bacteria. Although these proteins have similar structure, they have antagonistic biological functions. LBP is produced by hepatocytes and it is secreted into the bloodstream. BPI can be found in cytoplasmic granules of neutrophils and has anti-inflammatory and antimicrobial effects. Whereas LBP mediates the inflammatory response, transporting LPS monomers onto CD14 and together with TLR4 receptor and MD2 adaptor mediates the inflammatory response (Fenton and Golenbock 1998), BPI has anti-inflammatory and antimicrobial effects (Elsbach and Weiss 1998). Both molecules are also members of LBP/BPI family. LBP and BPI were also identified and described also in other animals, mainly in different invertebrate species, in fishes or birds. But, in invertebrates and non-mammalian vertebrates, the distinction between LBP and BPI has not been established to date.

LBP/BPI-related genes have been identified in non-mammalian vertebrates and in invertebrates. For example *lbp/bpi* related genes in some fish and birds (*Gadus morhua*, *Cyprinus carpio*, *Oncorhynchus mykiss*, *Gallus gallus*) (for review see (Imler and Hoffmann 2002)) or in some invertebrates (*Biomphalaria glabrata*, *Crassostrea gigas*, *Euprymna scolopes*) were identified (Gonzalez *et al.* 2007, Baron *et al.* 2013).

Studies of non-mammalian and invertebrate systems reveals features that are evolutionarily conserved across the animal kingdom and can provide insight into the essential functional features of proteins, such as LBP and BPI. Analogous contributions to the study of Toll-like receptors (TLRs) in humans were resulting from the discovery and characterization of these proteins in the fruit fly *Drosophila melanogaster*.

LBP/BPI proteins are predicted to have the basic "boomerang" two domain fold, similar to human BPI and LBP molecules (Krasity *et al.* 2011). The members of this family also contain some conserved amino acids for example: Cys135 and Cys175 form disulfide bond necessary for their biological activity (Beamer *et al.* 1998). It is believed that the ancestor of LBP and BPI was a single-domain protein, which was cis-duplicated during the evolution (Beamer *et al.* 1998). Some invertebrates, for example arthropods, completely omitted these molecules. For instance, *D. melanogaster* uses for detection of Gram-negative bacteria PGRPs (peptidoglycan recognition proteins) and GNBP (Gram-negative bacteria-binding protein) (Gottar *et al.* 2002, Choe *et al.* 2002).

2.4. Regulation of cellular iron homeostasis

Iron is an essential element for all living organisms constituting a central component of hem groups of iron–sulfur cluster-containing proteins and some enzymes involved in mitochondrial respiration and DNA synthesis. Ferritins play the key role in iron storage and keep it in a non-toxic state in cells. Ferritins are ubiquitous (animals, plants, fungi and bacteria) and spherically symmetrical proteins, characterized by a remarkably high stability to temperature and extreme pH values. Its expression is iron dependent but it could be also induced by oxidative stress or during infection (Krivoruchko and Storey 2010). Most ferritins have similar structure, consisting of 24 subunits capable of storing up to 4500 iron atoms as a ferric complex (Aisen *et al.* 1999) with the presence of the ferroxidase center (Andrews *et al.* 1992). Ferroxidase center compose from two metal-binding sites localized in a close distance. They are conserved in both vertebrates and invertebrates.

Ferritins from various organisms differ in size, distribution and way of regulation. Despite these differences, ferritins share common features in their sequence and structures. Vertebrates ferritin consists of two types of polypeptides, heavy (H) and light (L) chain, which are encoded by different genes (Harrison and Arosio 1996) but their sequences are 50% homologous (Theil 1987). Interestingly, Amphibians have an additional ("M") type of ferritin (Andrews *et al.* 1992). The single ferritin of plants and bacteria most closely resembles the vertebrate H-type (Andrews *et al.* 1992). The majority of invertebrate ferritins is similar to vertebrate H-type subunit, while insect ferritins are more closely related to the vertebrates L- type subunit (Nichol *et al.* 2002). Ferritins play also an important role for blood sucking arthropod. *Since* ticks and malaria mosquito females consume an enormous quantity of blood relative to their body size, it is assumed that ferritins represent important molecules for detoxification of the iron excess. This storage protein could be used as a potential antigen for an anti-tick vaccine (Hajdusek *et al.* 2010).

Iron exists in two oxidative states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) in the nature. Ferritin internalizes Fe^{2+}, which migrates to the ferroxidase center where is subsequently oxidized to Fe^{3+} forming co-ordination complex together with phosphate and hydroxide ions (Chasteen and Harrison 1999). Iron is released from ferritin particles by the proteosomal or autophagic degradation (Asano *et al.* 2011), main pathway of iron recycling.

The expression of ferritin is regulated at the post-transcriptional level and involves binding between iron regulatory protein (IRP) and iron-responsive element (IRE) in the 5´-untranslated region (UTR) of ferritin mRNA (Hentze and Kuhn 1996). Two IRPs (IRP1 and IRP2) have been known to play a crucial role in cellular iron homeostasis in mammals (Hentze and Kuhn 1996). These proteins regulate the expression of several proteins involved in iron transport, storage or iron utilization at the posttranscriptional level. IRP1 and IRP2 bind to iron regulatory elements (IREs) present in 5′ or 3′ untranslated regions (UTRs) of the mRNAs encoding these proteins, whereas only IRP1 as a bifunctional molecule can either binds an IRE site of ferritin mRNA or has function as a cytosolic form of aconitase (Guo *et al.* 1994, Hentze and Preiss 2010).

The conversion of IRP1 between an IRP-binding protein and aconitase is regulated by iron concentration through the conformation changes of [4Fe-4S] cluster (Theil 1994). At low iron levels, IRP1 binds to the IRE and thus, the IRE/IRP1 complex

blocks translation of ferritin (Gray and Hentze 1994). Once, the intracellular iron level is sufficient, a [4Fe-4S] cluster is formed and as a consequence, IRP1 lost the ability to bind ferritins mRNA and adopted aconitase activity. Aconitases are iron-sulfur enzymes that interconvert citrate to isocitrate (Eisenstein *et al.* 1993). This enzymatic reaction requires binding of the substrate to [4Fe-4S] cluster (Walden 2002).

IREs are evolutionary conserved hairpin structures of ~30 nucleotides (Piccinelli and Samuelsson 2007) forming a "CAGUGN" stem-loop and an unpaired C residue or asymmetric UGC/C bulge/loop (Henderson *et al.* 1994).

IRE structures have been found in many vertebrates and invertebrates, but some of them, have shown certain distinctions. Based on a computer model of the secondary structure of *E. andrei* ferritin IRE, no conventional bulge is created regardless whether a cytosine is present five nucleotides upstream of the CAGUGN loop. Instead, a bulged uracil is formed as an optimal secondary conformation (Prochazkova *et al.* 2011). Some distinctions were also found in the crayfish ferritin RNA stem-loop structure containing a bulge of guanine instead of cytosine at its expected position, but it can still bind IRP1 *in vitro*. Moreover, an IRP1-like protein isolated from a crayfish hepatopancreas can bind to the IRE site of crayfish ferritin mRNA (Huang *et al.* 1999). Furthermore, the crustacean *Litopanaeus vannamei* (Hsieh *et al.* 2006) and another member of Annelida, *Periserrula leucophryna*, have guanine bulges instead of cytosine bulges in IRE sequences of their ferritin (Jeong *et al.* 2006). The only metazoan species, in which IREs have not been identified and IRPs failed to bind to the ferritin mRNAs is *Caenorhabditis elegans* (Schussler *et al.* 1996) and *Schistosoma mansoni* (Thompson and Kavaliers 1994).

3. Aims

The main aim of the presented thesis was focused on the screening and description of the antimicrobial immune response of known factors in *E. andrei* earthworms. The special emphasis was aimed on the role and function of respective PRRs.

1. To study the impact of microbial environment on two closely related earthworm *E. andrei* and *E. fetida* species and to determinate the difference between their immunological profiles.
    - To develop reliable PCR method for discrimination between both earthworm species based on a polymorphism of mitochondrial gene coding for cytochrome c oxidase I (COI).
    - To compare the differences in numbers of culturable compost and forest microbiota and the identification based on the 16S rRNA sequence analysis of isolates.
    - To study the impact of cross-colonization of microbiota (from the forest soil and from the compost) on the expression of selected defense molecules (CCF, lysozyme and fetidin/lysenin).

2. To study the correlation between immunological response of chosen PRRs and enzyme activities in the gut of *E. andrei* earthworm after microbial challenge.
    - To compare the numbers of culturable bacteria in the coelomic fluid and in the gut after microbial challenge.
    - To study the changes of gene expression of CCF, lysozyme and fetidin/lysenin after microbial administration in the coelomocytes and in the gut.
    - To assess the tissue expression of CCF by using *in situ* hybridization in the intestinal epithelial cells. To study the enzyme activities (protease, laminarinase, cellobiase, chitobiosidase and glucosaminidase) in the gut after microbial challenge.

3. To identify and characterize new PRRs involved in the innate immunity at the molecular level. Molecular characterization of these molecules provides a new tool for monitoring of the innate immunity in earthworms.
    - To characterize the TLR and LBP/BPI of *E. andrei* earthworm both structurally and functionally.
    - To study the tissue expression and also the impact of the microbial challenge on the both PRRs expression in coelomocytes and gut.
    - To assess the tissue expression of both PRRs by *in situ* hybridization in the intestinal epithelial cells.

4. To identify ferritin as an iron storage protein and to study the impact of the microbial challenge on its expression. To identify main iron regulatory molecule IRP and study its role in iron metabolism.
    - To characterize the ferritin of *E. andrei* earthworm both structurally and functionally and assess its potential immune function.
    - To characterize IRP of *E. andrei* earthworm and ascertain binding capacity of both molecules.

5. To use known earthworm immunological molecules in ecotoxicology studies. To investigate the impact of PCDD/Fs polluted soils on the earthworm *E. andrei*,
    - To study the CCF, lysozyme, fetidin/lysenin and ferritin gene expression changes after microbial administration in the coelomocytes and in the gut.
    - To analyze the differences in microbial abundance and composition in artificial and dioxin-contaminated soil.
    - To study the expression of some molecules which play role in stress such as calreticulin (CRT), heat shock protein (Hsp70), superoxide dismutase (SOD) and catalase (CAT).
    - The histological studies of damage in both the intestinal epithelial layer and the adjacent chloragogen tissue in PCDD/Fs-polluted soil.

4. List of publications

This thesis consists of the following papers:

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). IF=2,341

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). IF=2,601

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). IF=3,705

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*. Nov 1; 8(11) (2013). IF=3,534

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*. 2014 Oct; 193:22-8. doi: 10.1016/j.envpol.2014.05.026. Epub 2014 Jul 1 (2014). IF=4,143

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*. 2014 Oct 3;9(10):e109900. doi: 10.1371/journal.pone.0109900. eCollection (2014). IF=3,234

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). IF=0,929

Škanta F., Procházková P., Roubalová R., Dvořák J., Bilej M.: LBP/BPI homologue in *Eisenia andrei* earthworms. *Dev. Comp. Immunol* 54: 1-6 (2016). IF=2,815

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). IF=2,815

Rui, Z., K. Petrickova, **F. Škanta,** S. Pospisil, Y. L. Yang, C. Y. Chen, S. F. Tsai, H. G. Floss, M. Petricek and T. W. Yu. Biochemical and Genetic Insights into Asukamycin Biosynthesis. Journal of Biological Chemistry 285(32): 24915-24924 (2010). IF=5,328

5. Methods

Material and methods are described in detail in the particular publication.

Methods used during the experiments:

- RNA isolation
- DNA isolation
- cDNA synthesis using reverse transcriptase (RT-PCR)
- synthesis of the terminal gene parts using 3´RACE and 5´RACE
- cloning of amplification products and bacterial transformation
- plasmid isolation and DNA sequencing
- quantitative PCR (qPCR)
- isolation of coelomic fluid
- determination of protein concentration
- SDS-PAGE
- native PAGE and substrate gel electrophoresis
- cytolytic assay
- hemolytic assay
- lysoplate assay
- protease assay
- *in situ* hybridization
- hematoxylin/eosin staining
- toluidine blue staining
- expression, purification and folding of recombinant IRP
- *in vitro* transcription
- electromobility shift assay
- phospholipid fatty acid (PLFA) analysis

6. Results

6.1. Antimicrobial defense in the earthworm

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*. Nov 1; 8(11) (2013). IF=3,534

Earthworms live in permanent contact with microorganisms that affect their survival strategies. The coelomic cavity of the earthworms is not aseptic and always contains microorganisms from the outer environment. The coelomic fluid exerts numerous biological activities exhibits effective defense mechanisms against invaders. In the previous studies, various microbial factors such as lysozyme (Joskova *et al.* 2009), antimicrobial peptides (Cho *et al.* 1998, Wang *et al.* 2003) and several antimicrobial factors with hemolytic activity, (e.g. fetidin, lysenin, H1-H3 proteins and Eiseniapore) were described in the coelomic fluid (Roch 1979, Valembois *et al.* 1982, Roch *et al.* 1991). Coelomic fluid of *E. fetida* earthworm also causes lysis of broad spectrum of cell types including chicken fibroblasts, insect hemocytes (Kauschke and Mohrig 1987). Cytolytic protein named CCF was found to be responsible for the activity against tumor cells (Bilej *et al.* 1995). Moreover, CCF acts as a pattern recognition receptor with ability to bind microbial pathogen associated molecular patterns, and to trigger prophenoloxidase cascade (Beschin *et al.* 1998).

E. fetida is the only species living in mold, an environment with strong antigenic pressure that can result in the broader saccharide recognition capacity of CCF (Silerova *et al.* 2006). More variable and potent binding capacity of *Eisenia* CCF assumes a better tool for the recognition of potential pathogenic bacteria. Heterogeneity of microbiota represents a higher pressure to the immune system of earthworms.

Based on this assumption we demonstrate cross-colonization experiments of compost and forest-soil microbiota on the immune mechanisms of "Californian" earthworm (i.e. *E. andrei*)" and "European" earthworm (i.e. *E. fetida*).

The taxonomy of *E. andrei*/*E. fetida* is complicated since the most of current literature uses indiscriminately the term *E. fetida* and often it is not clear, which of the two species is being referred to. It was previously published that nucleotide sequences of COI differ between *E. andrei* and *E. fetida* species (Perez-Losada *et al.* 2005). Based

on these published sequences, pairs of primers specific for both *E. andrei* and *E. fetida* COI were designed. By using primers specific for *E. andrei* COI, we could detect PCR products only in reactions containing *E. andrei* cDNA while no PCR product was detected if *E. fetida* cDNA was used as a template. Conversely, primers specific for *E. fetida* COI binds solely to *E. fetida* cDNA and not to *E. andrei* cDNA. Therefore, these primers can be used as a reliable tool for the differentiation of these two species.

As was described previously, the coelomic fluid exhibits many biological activities involved in the innate defense of earthworms. Whereas, CCF and lysozyme showed only slight differences in their expression and activity, fetidins/lysenins expression as well as the hemolytic activity was considerably higher in *E. andrei* as compared to *E. fetida*. The expression of fetidins/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 fetidins/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.

6.2. Cross-talk between digestive enzymes and pattern recognition receptors in the gut of *Eisenia andrei* earthworms after microbial challenge

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). IF=2,601

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). IF=2,815

According to the published estimations, a single earthworm swallows daily approximately a mass of a soil substrate equal to more than 50% of its weight. It means that up to 10^8 bacteria pass through the gut, interacting with and affecting the mucosal surface of the gut. From this point of view, the cross-talk between enzyme activities, pattern-recognition process, and general defense strategies seems to be important.

The activity of some PRR molecules often depends on the previous cleavage of the recognized molecules by protease. This cleavage is often necessary to uncover hidden PAMPs, which are then accessible for humoral factors as PRRs. For example, a lysozyme predigestion of peptidoglycan is essential for the recognition of peptidoglycan constituents by CCF (Bilej *et al.* 2001).

We showed the changes in the expression of related immune molecules (CCF, fetidin/lysenin and lysozyme) in coelomocyte and in the gut of *E. andrei* earthworms after microbial challenge and correlated these changes with enzyme activities.

The numbers of bacteria in the coelomic fluid and gut homogenate of non-stimulated earthworms were estimated. The number of bacteria in gut homogenate was more than six times higher than in the coelomic fluid.

We observed increased number of microorganism in both the coelom and the gut after the high microbial stimulation. The relative fold change of CFU in the coelomic fluid reached a maximum (150-, 330-, 6,5-fold increases, compare to the naïve worms) on the third day after the administration of *E. coli* O55, *S. cerevisiae* S288 and *B. subtilis* W23. An increased relative fold change of CFU in the gut was detected also after three days after the microbial challenge. The range of change varies only between twenty to thirty-five-fold in comparison with naïve worms.

The protease, laminarinase, and glucosaminidase activities were increased in parallel to up-regulated CCF and lysozyme expression. 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 the 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 microbial rich environment of soil. These data suggest that enzyme activities are 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.

6.3. Pattern recognition receptors in *E. andrei* earthworm

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). IF=3,705

Škanta F., Procházková P., Roubalová R., Dvořák J., Bilej M.: LBP/BPI homologue in *Eisenia andrei* earthworms. *Dev. Comp. Immunol* 54: 1-6 (2016). IF=2,815

Toll-like receptors (TLRs) were characterized as mammalian orthologs of the fruit fly *Drosophila melanogaster* transmembrane protein Toll, which plays important role in the development of embryonic polarity (Nusslein-Volhard and Wieschaus 1980) and later in the adulthood plays are involved in antifungal and Gram-positive bacteria protection (Lemaitre *et al.* 1996). TLRs are membrane PRRs which play an important role in defense responses in both plants (Gassmann *et al.* 1999) and animals (for review (Coscia *et al.* 2011, Satake and Sekiguchi 2012).

Based on the structure of TLR extracellular domain, they can be categorized into two major types ("vertebrate-like" type and "protostome-like" type) (Hibino *et al.* 2006). Toll and TLRs are activated upon microbial challenge. Whereas the activation of Toll receptor is mediated by a ligand *spätzle* (Weber *et al.* 2003), TLRs interact directly with PAMPs.

The first evidence of the presence of TLRs in annelids has been recently described in the context of two genome sequencing projects of polychaete *Capitella* and leech *Helobdella* (Davidson *et al.* 2008). Later, the molecule of *Hm*TLR1 of the leech *Hirudo medicinalis* was described) (Cuvillier-Hot *et al.* 2011). The existence of the TLR or Toll in the earthworms was predicted but they were not characterized until now (Cooper *et al.* 2006).

Here we characterize the first TLR isolated from an oligochaete annelid, namely, *Eisenia andrei* (*Ea*TLR) and show its expression pattern. The full-length *Ea*TLR 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

high 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 *Ea*TLR with polychaete annelid, *Capitella teleta* and TLRs of mollusks and echinoderms. Finally, the highest constitutive expression of *Ea*TLR was observed in the digestive tract. Gene expression was significantly increased in coelomocytes of *E. andrei* after the challenge with Gram-positive bacteria.

LBP/BPIs are pattern recognition receptors that are often present in vertebrates and in invertebrates. In mammals, LBP/BPI molecules exist as two individual molecules LBP and BPI that play an important defense role against Gram-negative bacteria. Although these proteins have similar structure, they have antagonistic biological functions. Whereas LBP mediates the inflammatory response (Fenton and Golenbock 1998), BPI has anti-inflammatory and antimicrobial effect (Elsbach and Weiss 1998). Members of LBP/BPI protein family are predicted to have the basic "boomerang" two domain fold, similar to human BPI and LBP molecules (Krasity et al., 1998). It is believed that the ancestor of LBP and BPI was a single-domain protein, which was cis-duplicated during evolution (Beamer *et al.* 1998). Some invertebrates, for example arthropods, completely omitted these molecules.

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 (*Ea*LBP/BPI). Sequence analysis of *Ea*LBP/BPI proved the existence of two conserved domains with the potential ability to bind LPS. The predicted molecular mass of the *Ea*LBP/BPI protein is 53.5 kDa, and its high basicity (pI 9.8) is due to 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.

To sum up, the earthworm *E. andrei* possesses genes coding for at least two PRRs recognizing LPS, *Ealbp/bpi* and *ccf* that differ in their tissue expression. The up-regulation of mRNA level of *Ealbp/bpi* after bacterial infection suggests its significant role in earthworm defense.

6.4. Regulation of cellular iron homeostasis in the earthworm *E. andrei*

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). IF=2,341

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*. 2014 Oct 3;9(10):e109900. doi: 10.1371/journal.pone.0109900. eCollection (2014). IF=3,234

Iron is an essential element for all living organisms. The key role in iron storage and its homeostasis play ferritin proteins. Ferritins are ubiquitously present in animals, plants, fungi and bacteria. Moreover, these molecules play a significant immune role in invertebrates; as acute phase reaction proteins (Torti and Torti 2002).

The expression of ferritin is regulated at the transcriptional level by the interactions between an iron regulatory protein (IRP) and iron-responsive element (IRE) in the 5´-untranslated region (UTR) of ferritin mRNA (Hentze and Kuhn 1996). Two IRPs have been described in vertebrates so far. IRP1 as a bifunctional molecule can either bind an IRE site or have function as a cytosolic form of aconitase, while IRP2 has only the IRE-binding activity (Guo *et al.* 1994). The conversion of IRP1 between an IRP-binding protein and aconitase is regulated by iron concentration (Theil 1994).

At low iron levels, IRP1 binds to the IRE and thus, the IRE/IRP1 complex blocks translation of ferritin (Gray and Hentze 1994). Once the intracellular iron level is sufficient, IRP1 loses the ability to bind ferritins mRNA and receives aconitase activity. Aconitases are iron-sulfur enzymes that interconvert citrate to isocitrate.

IREs are evolutionary conserved hairpin structures of ~30 nucleotides (Piccinelli and Samuelsson 2007) forming stem-loop. This loop can be localized at both 5´- and 3´-UTRs of messenger mRNA which encode proteins playing important role in iron storage, iron transport or iron utilization.

In this study, we report on the sequence characterization of a ferritin-coding cDNA and a new regulatory protein belonging to the family of IRPs in the earthworm *Eisenia andrei* (*Ea*IRP). Ferritin-coding cDNA was 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. Instead, a bulged uracil is present as an optimal secondary conformation.

The presence of ferritin in the coelomic fluid of *E. andrei* was proven by iron staining assay. Prepared recombinant *Ea*IRP and proteins from mammalian liver extracts are able to bind both mammalian and *Eisenia* IRE structures of ferritin mRNA, although the affinity of the r*Ea*IRP/*Eisenia* IRE structure is rather low. When IRP is supplemented with a Fe-S cluster, it can function as a cytosolic aconitase. Aconitase activity in the coelomic fluid was assessed by aconitase assay, suggesting the presence of an iron regulatory protein. Cellular cytosolic and mitochondrial fractions, as well as recombinant *Ea*IRP, exhibit aconitase activity, that can be abolished by the action of oxygen radicals.

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. Ferritin is also induced by oxidative stress or during infection (Krivoruchko and Storey 2010).

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 *Ea*IRP is most likely more relevant.

6.5. The effect of dibenzo-*p*-dioxin- and dinezofuran-contaminated soil on the earthworm *Eisenia andrei*

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*. 2014 Oct; 193:22-8. doi: 10.1016/j.envpol.2014.05.026. Epub 2014 Jul 1 (2014). IF=4,143

Because the earthworms can mediate pollutant transfer from soil to a range of predators, including birds, they can be used in the monitoring of soil contamination (Nahmani *et al.* 2007).

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans represent environmental contaminants that cause severe impacts to health such as hepatotoxicity, suppression of the immune system, reproductive and developmental abnormalities, carcinogenesis, skin defects and diverse effects on hormones and growth factors (Van den Berg *et al.* 2006, White and Birnbaum 2009). The most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was classified as carcinogenic to humans (IARC 1997).

PCDD/Fs belong to the group of persistent organic pollutants with tendency to be associated with ash, soil, or any surface with high organic content; in biota, they concentrate in fatty tissues (ATSDR 1998).

In this study, we examined with historically PCDD/Fs-contaminated soil from northern Sweden, which has arisen as an unintentional by-product of wood impregnation performed some 60 to 80 years ago in this area.

Earthworms 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 chloragogen tissue was observed as a consequence of contaminant exposure. Further, the up-regulation of expression of several genes was detected. Based on 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 the protection against oxidant toxicity, calreticulin and Hsp70. It was also observed a significantly greater amount of both aerobic and anaerobic microbial biomass in dioxin polluted soil, which suggests that increased CCF expression was more likely affected by microbial

composition than by the dioxins themselves. There were not detected any significant changes in the expression of fetidins and lysenin (antimicrobial proteins with hemolytic activity), the antimicrobial protein lysozyme and the expression of ferritin in the dioxin polluted soil.

7. Discussion

Microbiota plays an important role in various ecosystems and it is presumed that can modulate the defense mechanisms of the host. In previous studies of our group we compared binding specificity and biological activity of CCF as PRR in epigeic, endogeic and anecic earthworms. It was shown that epigeic *Eisenia* earthworms possess a broader saccharide-binding specificity as compared to other earthworm species. It was suggested that the immune capacity can be affected by the microbial environment (Silerova *et al.* 2006). Based on this assumption we focused on a comparison of defense mechanism of two closely related epigeic earthworms *E. andrei* and *E. fetida* living in rather different environment. *E. andrei* lives in compost and manure rich in microorganism, whereas *E. fetida* earthworm lives in the litter layer of moist forests that are considerably less abundant in a number of microorganisms (Dvorak *et al.* 2013).
As was mention above, the taxonomy of *E. andrei*/*E. fetida* is complicated since the most of current literature uses indiscriminately the term *E. fetida* and often it is not clear, which of the two species is being referred to.

Peréz-Losada et al. have determined these two species based on mitochondrial and nuclear DNA sequences using conserved primers amplifying COI fragments of most species (Perez-Losada *et al.* 2005), while we designed and used discrimination primers specific only for one species. Differences in COI sequences of both species are distributed in the entire length of obtained sequences, therefore we were able to design suitable sets of primer pairs. The main advantage of such species-specific primer pairs is the possibility to quickly discriminate *E. andrei* and *E. fetida* without the requirement of sequencing.

The comparison of the amino acid sequences of both CCF and lysozyme of *E. andrei* and *E. fetida* showed high level of homology. Similarly, we did not observe any significant differences in proteolytic activity of the coelomic fluid of both species that could affect proper prophenoloxidase cascade activation (Beschin *et al.* 1998, Prochazkova *et al.* 2006) or other immunodefense pathways (Kauschke *et al.* 1997). On the other hand, our results revealed much higher hemolytic activity of *E. andrei* coelomic fluid as compared to the *E. fetida*. Quantitative PCR confirmed differences between hemolytic molecules, fetidin and lysenin, at genomic DNA as well as mRNA level. We found out two times higher number of fetidin and lysenin gene copies in the

genomic DNA. Based on this result we can hypothesize that one or more of these genes were duplicated/multiplicated in the genome of *E. andrei*.

In 1968, Du Pasquier and Duprat described for first time *E. fetida* hemolytic proteins (Du Pasquier and Duprat 1968). Later on, they were named EFAF (*Eisenia fetida andrei* factors), and characterized as two glycoproteins of 40 and 45 kDa and that constitute a polymorphic system (Roch 1979). Roch identified four different protein products corresponding to alleles of the second gene with a pI ranging from 5.9 to 6.3. Coelomic fluid of all earthworms contains either two or three isoforms, with one isoform invariably present (pI 6). In European population of *E. fetida andrei*, three proteins of pI 5.9, 5.95 and 6.3 are encoded by the same gene that expresses three allelic forms. The combination of three alles (*a*, *b* and *c*) provides the possibility of six genotypes (*aa*, *bb*, *cc*, *ab*, *ac* and *bc*) corresponding to six phenotypes (A, B, C, D, E, F). In American populations that originated from Californian ancestors, the fourth allele *d* is present but it has never been detected in the European population. Valembois *et al*. (1986) explained the occurrence of allele *d*, originally very rare in ancestral European worms (i.e. *E. fetida*), by more favorable conditions for its expression after migration of European ancestors into a new biotope during the 15th century. This opinion can be supported by the fact that even in "Californian" earthworms (i.e. *E. andrei*), the homozygous genotype *dd* has not been proved. The significant antibacterial effect was found mainly in the most frequent phenotype B (*bb* genotype) and in phenotype K, whose frequency is considered as intermediate. Because of this fact and that bacteriostatic effect of other frequently occurring phenotypes are relatively low, perhaps alternative mechanisms are involved in antibacterial humoral defense (Valembois *et al.* 1986).

We isolated bacterial strains from both compost and forest soil, cultured them and the mixtures were used for the stimulation in cross-colonization experiments to show, how the defense system responds to microbial challenge. After microbial cross-colonization challenge, the expression of fetidin/lysenins was significantly upregulated in *E. andrei*, whereas non-significant changes were found in *E. fetida* challenged with compost microbiota. The absence of detectable reaction *E. fetida* to compost microbiota can be explained either by the 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. The expression of CCF and lysozyme showed only slight up-regulation in both *E. andrei* and *E. fetida*. *E. andrei* colonizing compost as a new habitat acquired an

evolutionary selection advantage resulting in a higher expression of antimicrobial proteins (Dvorak *et al.* 2013).

Earthworms live in permanent close contact with soil particles. A single earthworm swallows daily approximately a mass of a soil substrate equal to more than 50% of its weight. It means that up to 10^8 bacteria pass through the gut, interacting with and affecting the mucosal surface of the gut. Microorganisms are also an important parts of the earthworms diet. Small fraction of bacteria is permanently associated with gut wall (Singleton *et al.* 2003). However, the symbiotic microorganisms seem to be less important, since the intestinal microbiota is similar and related to soil microbiota (Drake *et al.* 2006). As it has been mentioned above, coelomic cavity is not aseptic, it contains bacteria entering the coelomic cavity via dorsal pores and nephridia. The number of bacteria in coelom is kept under control by various mechanisms such as antimicrobial factors (Lassegues *et al.* 1989, Prochazkova *et al.* 2006, Joskova *et al.* 2009) and phagocytic cells (Bilej *et al.* 1991, Dales and Kalac 1992). The number of bacteria in coelomic fluid is more than six times lower than in the gut. High microbial load of *E. coli* O55, *B. subtilis* W23, and *S. cerevisiae* S288 in the earthworm environment, resulted in increase of microorganisms in both, the coelom and the gut. The increase in the number of microorganisms in the gut is much lower in comparison with the coelomic fluid. We have assessed the levels of mRNA for different immune related molecules in the coelomocytes and in the gut, upon the microbial challenge by quantitative PCR and i*n situ* hybridization. The coelomocytes respond to bacteria present in the coelomic cavity by increasing expression of defense molecules, especially CCF. Interestingly, the immune response in the gut is less affected by microbial stimulation because the epithelial cells of the gut exhibit strong mRNA synthesis of *ccf* as a defense against the continuous microbial load in the gut lumen.

On the other hand, the midgut exhibits the highest constitutive expression of CCF in the earthworm body. Nevertheless we suggest that the experimental microbial challenge stimulates the immune response in the gut. This response triggers the increased release of phagocytic coelomocytes from the mesenchymal lining of the coelom, and thus increases the defense reaction in the coelomic cavity of earthworms.

The mRNA levels of fetidin/lysenins were observed to be significantly increased in all three types of microbial stimulations (Gram-negative, Gram-positive bacteria and yeast). It was previously reported that the microbial environment affects the mRNA levels of fetidin and lysenins in the coelomocytes of *E. andrei* earthworms (Dvorak *et*

al. 2013). Moreover, the injection of bacteria into the coelomic cavity has resulted in increased hemolytic activity (Köhlerova *et al.* 2004). Similarly, the highest lysozyme mRNA levels were detected after one day of the *B. subtilis* challenge, which is consistent with the results of Joskova *et al*. (Joskova *et al.* 2009). From this point of view, the cross-talk between enzyme activities, pattern-recognition process, and general defense strategies seems to be important (Prochazkova *et al.* 2013).

CCF and lysozyme expression correlates with enzyme activities in the gut of *E. andrei* earthworms following a microbial challenge. The enzyme activities are 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.

Based on these results, we propose a model of the earthworm defense against infection. The increased number of microorganisms is sensed by pattern recognition receptors in the gut, and activation signal is transferred into the adjacent mesenchymal lining representing the precursor tissue of free coelomocytes and the site of their release. 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 (Sato *et al.* 2000).

To enlarge the panel of defense molecules, we have aimed to describe another PRRs, LBP/BPI and TLR, in this thesis. These molecules are involved in earthworm antimicrobial defense. The occurrence of the LBP/BPI molecules was proved in vertebrates (Krasity *et al.* 2011), invertebrates (Gonzalez *et al.* 2007, Baron *et al.* 2013) and in some plants (Sato *et al.* 2000), which suggests that these molecules are evolutionarily conserved.

*Ea*LBP/BPI found in the genome of *E. andrei* earthworm shares homology with the LBP/BPI family (Skanta *et al.* 2016). Sequence analysis of *Ea*LBP/BPI proved the existence of two conserved domains with the potential ability to bind LPS. *Ea*LBP/BPI contains a high amount of basic amino acids (arginine mostly), another recently described members of the LBP/BPI family contain a higher amount of lysine e.g. *Crassostrea gigas* (Gonzalez *et al.* 2007), *Amphimedon queenslandica* (Gauthier *et al.* 2010), *Euprymna scolopes* (Krasity *et al.* 2011) and, *Biomphalaria glabrata* (Baron *et al.* 2013).

We aimed to investigate the difference between the binding activity of recombinant LBP/BPI and CCF molecule with LPS and also experimentally confirm the potential existence of antimicrobial activity of LBP/BPI against Gram-negative bacteria. But we did not succeed to express recombinant LBP/BPI in any the following expression system *E.coli* BL21StarDE3LysE, *Pichia pastoris* or *Drosophila* Schneider S2 cells.

The antibacterial activity of the LBP/BPI molecules is probably not determined by the dominant basic amino acid, but the total charge value of the molecule (Schultz and Weiss 2007). The charge of the earthworm *Ea*LBP/BPI molecule is highly positive, in contrary to sponge *Amphimedon queenslandica* LBP/BPI molecules showing a negative or neutral charge, which most likely indicates the lack of antibacterial activity (Gauthier *et al.* 2010).

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 anterior part of the intestine. These findings correspond to the study of murine BPI (Lennartsson *et al.* 2005). Another study showed a high abundance of OCX-36 protein with the homology to the LBP/BPI family in the chicken reproductive tract and also a high concentration of this protein in the eggshell membrane (Gautron *et al.* 2007), suggesting its protective role against pathogens during development of gametes or immature offspring. In contrary to *Ea*LBP/BPI another earthworm LPS-binding molecule CCF was expressed only in the intestine and coelomocytes. CCF participates in the activation of the prophenoloxidase cascade through the recognition of the yeast β-1,3-glucans, *N,N′*-diacetylchitobiose and Gram-positive bacteria cell wall components (Bilej *et al.* 2001).

In *E. andrei* coelomocytes, the transcription of *Ealbp/bpi* gene was up-regulated in response to bacterial stimulation. The faster and higher expression was reached upon stimulation with *Gram-positive bacteria* than *Gram-negative* bacteria.

This response can be explained by the potential ability of *Ea*LBP/BPI to recognize lipoteichoic acids (LTAs) that are integrated in the cell wall of Gram-positive bacteria. It was shown that human LBP, in addition to LPS, also recognizes the LTA of Gram-positive bacteria as well as structurally similar spirochaetal glycolipids (Schroder *et al.* 2004). Moreover, Weber and colleagues (Weber *et al.* 2003) proved the ability of human LBP specifically bind to pneumococcal cell wall multimers. Dentener and

colleagues (Dentener *et al.* 1996) found a higher expression of BPI after stimulating human polymorfonuclear leukocytes with LTAs.

In invertebrates, lacking adaptive immunity, other defense molecules such as, TLRs play an essential role in immune reactions. TLR is a transmembrane PRR responsible for the recognition of various PAMPs. TLRs have been recently retrieved from *in silico* analyses of whole genomes in invertebrates, but only a few were functionally characterized (Hibino *et al.* 2006, Davidson *et al.* 2008).

The *Ea*TLR described by our group in the genome of *E. andrei* earthworm (Skanta *et al.* 2013) is structurally similar to the other reported TLRs with the highest homology to polychaete *Capitella teleta* (Davidson et al. 2008).

Studying *Ea*TLR genome variability is very complicated due to high number of genes encoding for TLRs. The high number of TLRs probably correlates with microbe-rich environment these earthworms live in. The changes in mRNA level of *Ea*TLR in the coelomocytes of earthworms stimulated with Gram-positive *B. subtilis* and Gram-negative *E. coli* bacteria coincidently correspond with the data concerning signaling pathways in *Drosophila*, where the recognition of Gram-positive peptidoglycan (Lys-type) results in the activation of Toll-pathway, whereas recognition of Gram-negative bacteria peptidoglycan (DAP-type) is mostly Toll-independent (Michel *et al.* 2001, Gottar *et al.* 2002, Choe *et al.* 2002, Ramet *et al.* 2002, Leulier *et al.* 2003). The highest constitutive expression of *Ea*TLR was observed in the digestive tract. Finally, gene expression was significantly increased in coelomocytes of *E. andrei* after the challenge with Gram-positive bacteria. From our findings we are not able to answer if *Ea*TLR is able directly bind to the PAMPs or the Toll pathway activation need the participation of any ligand analogous to Spätzle. In the genome of *E. andrei* another TLR molecule with the highest expression in the seminal vesicles was also identified (to be published). The putative protein sequence revealed the domain organization homologous to "protostomial type of TLR". In contrary to the *Ea*TLR this molecule probably exists only in one copy in the genome (data was not shown). We can summarize that *E. andrei* earthworm possess two type of TLRs. In the future studies we would like to check the binding ability between TIR domain of P-TLR and *Ea*TLR with TIR domain of adaptive protein MyD88. For this task the recombinant proteins were prepared and the bond will be check by method SPR (Surface plasmon resonance).

Earthworms represent an important part of the terrestrial food chain and for that reason are eligible for the monitoring of soil contamination. *E.andrei* earthworms living

in dioxin-contaminated soils demonstrated damages of the intestinal wall and adjacent chloragogen tissue, serving as a center of the excretion of the waste products and xenobiotics (Morgan and Morgan 1989, Sturzenbaum *et al.* 2004). Thirty percent of free coelomocytes present in the coelomic fluid originate from chloragogen tissue (Adamowicz and Wojtaszek 2001). The significant decrease in coelomocyte yield after dioxin exposure was described (Belmeskine *et al.* 2012) explained by the reduction in chloragogen tissue. Our results correspond with Sforzini and collegues, who immunohistochemically demonstrated the localization of TCDD (Tetrachlorodibenzo-*p*-dioxin) in the chloragogen tissue and intestine (Sforzini *et al.* 2014). Dioxins accumulating in the lipophilic structures cause an increase in production of 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. It was shown, that CCF is localized in the chloragogen tissue and in free large coelomocytes (Bilej *et al.* 1998).

Ferritin is a storage protein that plays a key role in iron metabolism. The presence of both ferritin and iron regulatory protein in the coelomic fluid of *E. andrei* was proven. Surprisingly, although the IRE secondary structure of earthworm ferritin mRNA differs from the conventional bulge, its mRNA binds mammalian as well as rIRP. The iron overload causes higher activity of IRP, but did not make any significant changes in ferritin expression. Cytosolic and mitochondrial fractions of *E. andrei* cells as well as r*Ea*IRP possess aconitase activity, which can be abolished by H_2O_2 treatment, due to the destruction of the Fe-S cluster. Because the aconitase activity of rIRP can be reduced substantially, we suppose that *Ea*IRP predominantly acts as aconitase rather than as an iron regulatory protein.

The highest expression of *Ea*IRP was revealed in the digestive tract, the expression was also detected in other tissues and the cells. This is in agreement with the fact that *Ea*IRP is an important enzyme affecting many basic cellular biochemical processes.

Ferritin has also been described as an acute phase protein responding to a nonlethal injury of organism (Beck *et al.* 2002). Accordingly in our experiments, ferritin expression was induced by the stimulation of coelomocytes by bacteria, suggesting its involvement in the protective defense against infectious agents. This increased levels of

fetidin mRNA suggested essential role of ferritin in antimicrobial innate immune defense (Dunkov and Georgieva 2006).

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 *Ea*IRP is most likely more relevant.

We believe that the data about iron regulatory proteins in earthworms can enrich knowledge concerning proteins similarities in other animals.

8. Conclusions

1. We compared the expression and function of immune related molecules of two closely related earthworms *E. fetida* and *E. andrei* living in different microbial environment. Based on these results we can hypothesize that *E. andrei* colonizing compost as a new habitat, acquired an evolutionary selection advantage resulting in a higher expression of antimicrobial proteins. Furthermore we developed the reliable method to discriminate *E. fetida* and *E. andrei* by PCR using specific COI primers.
2. We described the effect of microbial challenge on defense mechanisms of earthworms and demonstrate the cross-talk between digestive enzymes and innate defense of the earthworms. These results allowed us to suggest a model of systematic of antimicrobial respond in earthworms.
3. We identified and characterized on the molecular level two novel PRRs in oligochaetes namely polymorphic "vertebrate type" *Ea*TLR and *Ea*LPB/BPI.
4. We characterized ferritin as an iron storage protein and showed its up-regulation in coelomocytes upon bacterial challenge suggesting a role of ferritin in earthworm defense. Furthermore we proved the aconitase activity suggesting the presence of an iron regulatory protein. 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 *Ea*IRP is most likely more relevant.
5. We applied the panel of immune related molecules in eco-toxicological study to follow the impact of pollutants on the innate immunity of earthworms. Importantly we showed that microbial biomass modulates the expression of immune related molecules besides the direct effect of organ-pollutants.

9. References

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