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Antimicrobial Factors in Earthworms

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Motto

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Prague, July 2007 Marcela Šilerová

Contents

Abbreviations

12C9	monoclonal antibody against CCF
CCF	coelomic cytolytic factor
CHAPS	3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate
CPC	cetylpyridinium chloride
HEMA	2-hydroxyethylmetacrylate
LBSS	Lumbricus balanced salt solution
L-DOPA	L-β-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
MAPKK	mitogen-activated protein kinase kinase
NF-κB	nuclear factor-κB
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
PECs	peritoneal exudate cells
PO	phenoloxidase
proPO	prophenoloxidase
RT	reverse transcription
SDS	sodium dodecyl sulphate
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
UTR	untranslated region
VSG	variant specific glycoprotein

1. Introduction

Considering the fact that the majority of the immunologists all over the world are focused on the mammalian or particularly human immunology, it may sound surprising that invertebrates – and among others earthworms – have been an important experimental model since the very beginning of immunology. For example, phagocytosis, an important and evolutionarily conserved defense mechanism of innate immunity, was discovered in the late 1800s by a Nobel price winner Elie Mechnikoff while studying the origin of the digestive organs in the floating larvae of starfish.

From the total number of extant animal species, probably surpassing 2 millions, 95 % are included in the invertebrate taxa. Invertebrates have evolved for hundreds of millions of years, often surviving very hostile environments. Their successful survival strategies are likely based on short life span combined with numerous offspring. More importantly, all invertebrate species have developed a variety of defense mechanisms efficiently recognizing and responding to non-self substances. Like vertebrates, invertebrates possess various physical and chemical barriers including mucus surrounding the body of many coelenterates, annelids, molluscs and protochordates or exoskeleton of coelenterates, molluscs, arthropods, echinoderms and protochordates.

In contrast to adaptive immunity, which is a highly sophisticated system based on antigen-specific T and B cells and antibodies and which is observed in vertebrates only, many innate immunity mechanisms are conserved from invertebrates to vertebrates. Cellular mechanisms of invertebrate innate immunity include wound repair, clotting and coagulation responses, phagocytosis of invading microorganisms and encapsulation reactions. Apart from these cellular mechanisms, invertebrates possess a broad range of antimicrobial factors such as lysozyme-like proteins, proteases, cytolytic proteins, antimicrobial peptides and components of prophenoloxidase activation cascade; humoral defense also includes pattern recognition and lectin-like molecules that are designed to recognize few highly

conserved structures present in many different microorganisms. The majority of the above immune responses of invertebrates is non-adaptive with no ability either to "remember" or to respond more vigorously and effectively to repeated exposures to the same pathogens.

The earthworms are regarded as a model organism of comparative immunology since 1960s when transplantation experiments were performed (for rev. Cooper and Roch 1994) and cell-mediated short-term memory was observed (Bailey *et al.* 1971; Valembois 1971b). All these experiments proved the existence of self and non-self recognition in earthworms and initiated extensive studies of earthworm immune mechanisms. Moreover, earthworms were found useful in monitoring environmental pollution. The Organization for Economic Cooperation and Development (OECD) and the American Environmental Protection Agency accepted official protocols involving screening of earthworm immunological parameters as markers/indicators of impaired environmental conditions (OECD Guidelines for testing of Chemicals 1984; Green *et al.* 1989, for rev. Goven and Kennedy 1996). Furthermore, earthworms might be considered as a source of biologically active compounds with potential industrial or medical use. Actually, earthworm powder has been used as a traditional medicine in some South Asia countries for years to treat various diseases. Currently, the therapeutic effect of earthworm active factors is being evaluated by a modern scientific approach. Some therapeutics containing fibrinolytic enzymes from *Lumbricus rubellus* and *Eisenia fetida* earthworms (Mihara *et al.* 1991; Mihara *et al.* 1992; Nakajima *et al.* 1993; Nakajima *et al.* 1996) are already commercially available to support coagulation and fibrinolysis balance in the body and thus prevent or treat cardiac and cerebrovascular diseases (Boluoke® (lumbrokinase), Canada RNA Biochemical Inc.).

In terms of systematic biology, the earthworm family – Lumbricidae – is the largest member of the class Oligochaeta, phylum Annelida. There are over 3000 decribed earthworm species known worldwide and they have adapted to a wide range of soil habitats as well as freshwater lakes and streams. The earthworms are found

in leaf litter, manure, under stones and logs as well as some arid areas, but most species prefer wetter, more heavily vegetated regions. Earthworms range in size from two centimeters to over one meter.

The earthworms are protostomian animals possessing true coelomic cavity of mesenchymal origin. The coelomic cavity is filled with coelomic fluid containing free coelomocytes originating in the mesenchymal lining of the cavity. The coelomic cavity is metameric and the segments are separated by transversal septa. Transport of the coelomic fluid and coelomocytes between the segments is regulated by channels. Each segment of the coelomic cavity is opened to the outer environment by a pair of nephridia and by a dorsal pore (Fig. 1).

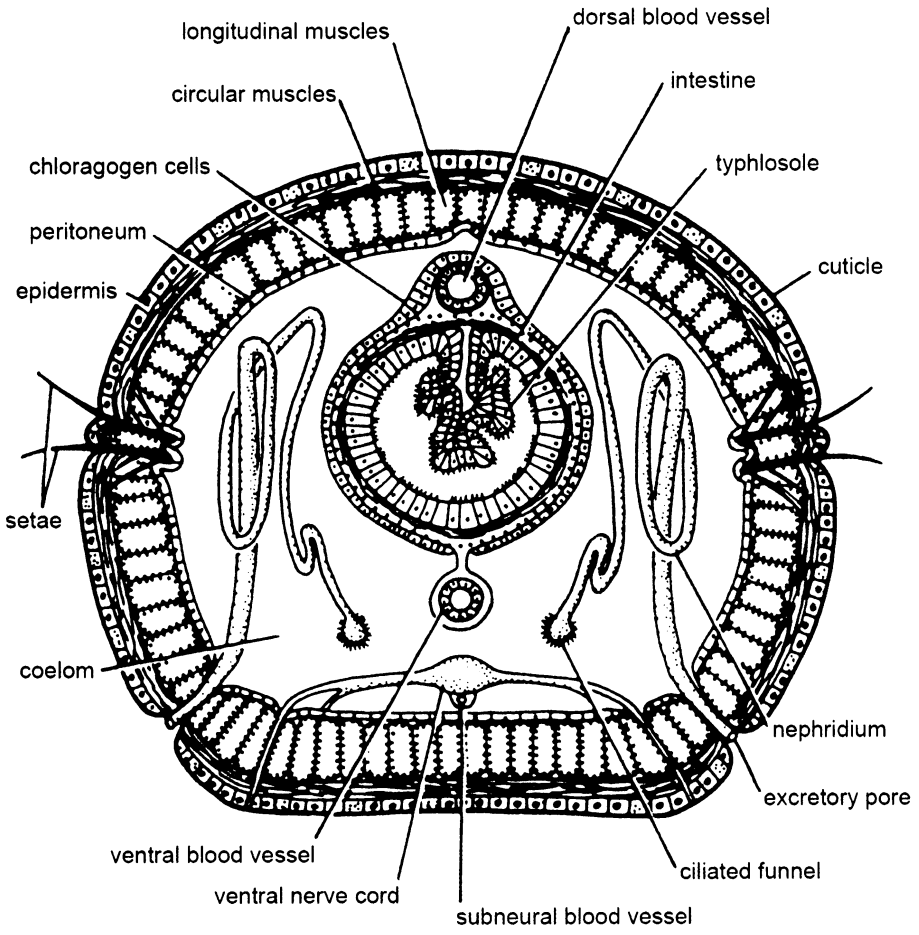

Fig. 1. Earthworm cross section (adopted from Biology of the Invertebrates, Pechenik, 1996).

2. Defense of the earthworms

2.1. Cellular defense mechanisms

2.1.1. Mechanisms of elimination of foreign objects

The first nonspecific barrier of earthworms is a skin covering their entire body. The skin consists of the epidermis and a thin cuticle, which contains mucopolysaccharides acting as an antimicrobial barrier (Rahemtulla and Lovtrup 1974, 1975). The epidermis is formed by a single layer epithelium of supporting cells, basal cells and secretory cells. The basal cells play an important role in wound healing and graft rejection, often exerting phagocytic activity (Chapron 1970; Valembois 1971a). Thus, these basal cells are sometimes considered not to be of epidermal origin, but rather homologous to coelomocytes (Burke 1974a, 1974b, 1974c).

However, this mechanical barrier is perforated by dorsal pores and therefore does not sufficiently prevent the microorganisms from entering the coelomic cavity. Consequently, the coelomic cavity is not aseptic and always contains bacteria, protozoans and fungi from the outer environment. Nevertheless, there are efficient mechanisms that keep the growth of microorganisms under control (Dales and Kalac 1992). It was reported that coelomic fluid contains 6 x 10^5/ml naturally occurring bacteria while the number of potentially phagocytic cells is more than ten times higher. These abundant phagocytes combined with the presence of various humoral factors can easily prevent the microorganisms from outgrowth.

The invading microorganisms can be eliminated by a number of ways. First, they can be excreted by nephridia (Cameron 1932) or engulfed by the cells of nephrostome or middle tube (Villaro *et al.* 1985). Second, as mentioned above, the microorganisms can be phagocytosed by certain coelomocytes and phagocytic cells that become exhausted are expelled through dorsal pores. The pores are equipped with muscular sphincters controlling intracoelomic pressure and the exchange of material between the outer and inner environments (Cameron 1932). Third, large foreign bodies,

e.g. agglutinated bacteria or parasites are eliminated by encapsulation (Ratcliffe *et al.* 1985). This process begins, similarly to phagocytosis, by the recognition of foreign material which, however, cannot be due to its size engulfed. Within the first day, the foreign body is surrounded by free coelomocytes and after several days a dense capsule (often called a brown body because of its melanin content) composed of flattened cells is formed. When the capsule is about 1-2 mm in diameter, its external cells lose their adhesiveness so that the capsule can migrate towards the posterior segments of the coelomic cavity where it is eliminated by autonomy followed by wound healing (Keilin 1925; Herlan-Meewis 1965; Alonso-Bedate and Sequeros 1983, 1985). It was documented in *E. fetida* earthworms that most brown bodies contain tissue wastes, agglutinated bacteria, gregarines or nematodes (Valembois *et al.* 1992).

2.1.2. Phagocytosis

As it was mentioned above, coelomic fluid contains different types of coelomocytes. Their nomenclature is based mainly on morphological and cytochemical criteria (for rev. Stein *et al.* 1977; Sima 1994). In general, there are three main cell types – eleocytes, free chloragogen cells with nutritive and accessory functions, and either hyaline or granular amoebocytes, both representing effectory immunocytes involved in a broad range of defense functions including phagocytosis.

Although all types of amoebocytes have phagocytic properties, their activity differs. In contrast to granular amoebocytes, the cytoplasm of hyaline amoebocytes is occasionally full of engulfed material (Stein *et al.* 1977). It should be mentioned that amoebocytes engulf all kinds of material including inert particles, microbial cell wall components as well as foreign cells. However, the phagocytosis of eukaryotic cells depends on the source of the cells. Unlike allologous cells, xenologous cells (both from different earthworm species and from non-invertebrate species) are rapidly phagocytosed (Cameron 1932; Stein and Cooper 1981).

Phagocytosis by coelomocytes, similarly to that of vertebrates, can be modulated by humoral components, opsonins, which coat the engulfed particle and thus promote its phagocytosis. It was proven that preincubation of both yeast and synthetic 2-hydroxyethylmethacrylate copolymer (HEMA) particles with the coelomic fluid significantly increased their phagocytosis (Bilej *et al.* 1990a; Bilej *et al.* 1990b; Bilej *et al.* 1991).

It is noteworthy that also mammalian opsonins, IgG immunoglobulin and C3b complement fragment, were described to enhance coelomocyte phagocytic activity, in contrast to IgM and C3d fragment, which did not affect phagocytosis (Laulan *et al.* 1988).

Engulfed material can be eliminated by several mechanisms – either by proteolytic and lysozyme-like enzymes or by an oxidative burst, which involves the production of highly reactive oxygen radicals. Oxygen radicals were detected both in the coelomic fluid and in chloragosomes of chloragogen cells (Valembois *et al.* 1991). However, they do not occur during the phagocytosis of inert synthetic particles (Bilej *et al.* 1990b; Bilej *et al.* 1991) suggesting the possible discrimination between antigenic and non-antigenic structures (Tuckova and Bilej 1996).

2.1.3. Transplantation immunity

The ability to recognize and respond to allografts as well as xenografts and, on the other hand, the ability to accept or not to destroy autografts was observed in many annelid species. This process begins like the reaction to injury. The first major change which occurs after the healing of wounds is, regardless of the graft origin, the accumulation of coelomocytes near the graft sites and their infiltration into the matrix. The response to the xenografts results in complete walling off the graft and its destruction by encapsulation reaction (Parry 1978). The number of invading coelomocytes during the autograft transplantation is markedly lower (Cooper 1970; Hostetter and Cooper 1973) but the reaction seems to be more rapid. The maximum

number of coelomocytes surrounding the graft was detected within 24 hours, returning to the normal level by 72 hours. In contrast, the peak response to xenografts is on day 3 or 4, and normal levels are not reached before day 7. The destruction of xenografts is completed approximately by day 17 after the transplantation. If a second graft is transplanted at this time, an accelerated rejection within 6 and 7 days occurs. Moreover, the number of the invading coelomocytes is 20 - 30 % higher. The increased number of coelomocytes during the re-transplantation is probably caused by an increased proliferating activity of mesenchymal lining of the coelomic cavity and the septa. These data suggest the existence of short-term and very limited memory which is based solely on cells as the transfer of either the coelomic fluid or other substances does not induce any accelerated reaction (Bailey *et al.* 1971; Hostetter and Cooper 1973).

2.2. Humoral defense mechanisms

The coelomic fluid of annelids exerts numerous biological activities that are involved in effective defense mechanisms against invaders. It was documented that it contains various antimicrobial factors like lysozyme (Cotuk and Dales 1984) and antimicrobial peptides (Cho *et al.* 1998; Wang *et al.* 2003; Liu *et al.* 2004; Tasiemski *et al.* 2007). Moreover, coelomic fluid was found to cause the lysis of vertebrate erythrocytes and, subsequently, several hemolytic factors were isolated and described. The majority of proteins with hemolytic properties have hemagglutination activity as well and, more interestingly, a spectrum of antibacterial and bacteriostatic activities against pathogenic soil bacteria (Roch 1979; Valembois *et al.* 1982; Roch *et al.* 1991). Furthermore, it was observed that coelomic fluid lyses eukaryotic cells other than erythrocytes, namely fibroblasts and insect hemocytes (Kauschke and Mohrig 1987) and various tumor cell lines (Bilej *et al.* 1995; Cooper *et al.* 1995). Hereinafter, each activity of the coelomic fluid is thoroughly described.

2.2.1. *Lysozyme*

Lysozyme is a bacteriolytic enzyme which catalyzes the hydrolysis of 1,4-β-D-links between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan of bacterial cell walls and thus efficiently protects against infections caused by Gram-positive bacteria. Lysozyme activity was observed in coelomocyte extracts as well as in the coelomic fluid (Cotuk and Dales 1984). Later, the active protein was isolated and partially sequenced (Ito *et al.* 1999). Based on the N-terminal sequence, a novel class of lysozymes including those of molluscs, echinoderms, nematodes and earthworms was proposed. Recently, cDNA coding for lysozyme-like molecule of *E. fetida* earthworms was characterized and cloned (Radka Josková, personal communication).

2.2.2. *Antimicrobial peptides*

Antimicrobial peptides are an abundant and diverse group of molecules that are produced by many cell types in invertebrates, vertebrates and plants. Depending on their tissue distribution, they ensure either a systemic or a local protection of the organism against pathogens. These peptides exert their antimicrobial activity through either the lipid bilayer of the cell membrane by the formation of multimeric pores (Hancock 1997) or the interaction with DNA or RNA after penetrating into the cell membranes (Boman *et al.* 1993; Cabiaux *et al.* 1994; Park *et al.* 1998; Zasloff 2002).

Most of antimicrobial peptides of invertebrates were found in arthropods. To date, only a limited number of bioactive peptides have been described in annelids. An antimicrobial peptide named Lumbricin I was identified in *Lumbricus rubellus* (Cho *et al.* 1998). Lumbricin I is a proline-rich antimicrobial peptide which is constitutively expressed in adult animals and is not induced by bacterial infection. A Lumbricin I analog named PP-1 was found in the Asian earthworm *Pheretima tschiliensis* (Wang *et al.* 2003). PP-1 is synthesized in the body wall only and its localization in the mucus

of the epidermis suggests its role in the mucosal defense. Furthermore, an antimicrobial short peptide OEP3121 of only 5 amino acids was found in *Eisenia fetida* earthworms (Liu *et al.* 2004).

Recently, an antimicrobial peptide from coelomocytes of a polycheate *Nereis diversicolor* named hedistin was thoroughly described (Tasiemski *et al.* 2007). Hedistin possesses an activity against a large spectrum of bacteria and seems to be constitutively and exclusively expressed in circulating NK-like cells. In addition to C-terminal amidation, which increases the net cationic charge and consequently the electrostatic attraction to target bacterial membrane (Tossi *et al.* 2000; Shai 2002), the hestidin primary structure contains bromotryptophan residues typical for marine organisms (Taylor *et al.* 2000; Shinnar *et al.* 2003). The presence of bromotrypthophan in *N. diversicolor* could be the result of an adaptation involving the recruitment of an enzymatic system already used by numerous marine organisms, in a specialized defense way against microorganisms.

2.2.3. Hemolytic molecules

The coelomic fluid of *E. fetida* earthworms was described to exhibit strong hemolytic activity. A heterogeneous group of proteins having hemolytic properties has so far been described but their relationship is still not clear.

The first hemolytic proteins were described by Du Pasquier and Duprat (Du Pasquier and Duprat 1968) and later on they were named **EFAF** (*Eisenia fetida andrei* factors) and characterized as two glycoproteins secreted by chloragocytes and eleocytes (Roch 1979; Roch *et al.* 1981; Roch 1984). The 45-kDa protein is encoded by a single nonpolymorphic gene and has a pI of 6.0, while the 40-kDa protein is encoded by a gene having four alleles, each representing one of isoforms with pI of 6.3, 6.2, 5.95 and 5.9. Each individual earthworm possesses the 45-kDa protein and 1 or 2 isoforms of the 40-kDa protein (Roch 1979; Roch *et al.* 1987). In addition to EFAFs hemolytic activity, these proteins were found to agglutinate red blood cells

(Valembois *et al.* 1984) and to participate in the cytotoxic activity of the coelomic fluid (Kauschke and Mohrig 1987). Moreover, they exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria (Valembois *et al.* 1982; Lassegues *et al.* 1989; Hirigoyenberry *et al.* 1992), particularly against strains that are pathogenic for earthworms (Valembois *et al.* 1986; Roch *et al.* 1987; Roch *et al.* 1991). In addition to their bacteriolytic activity, they may also mediate opsonization (Sinkora *et al.* 1993) and participate in the clotting of the coelomic fluid (Valembois *et al.* 1988).

It was documented, that 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.* 1981; Roch *et al.* 1989).

Later, EFAFs were characterized at the molecular level and were named **fetidins**. A gene for the 40-kDa protein was cloned (Lassegues *et al.* 1997; Milochau *et al.* 1997) and it was found that its putative amino acid sequence comprises an N-glycosylation site and a peroxidase motif. This is in accordance with the finding that both fetidins have peroxidase activity.

Independently, a 41-kDa hemolytic protein, which is produced by coelomocytes and causes contraction of rat vascular smooth muscles, was characterized and named **lysenin** (Sekizawa *et al.* 1996). Simultaneously, two 42-kDa lysenin-related proteins with weak contractive activity were identified (Sekizawa *et al.* 1997). More recently, a new member of this lysenin-like multi-gene family was cloned and was provisionally called lysenin-related protein 3 (Bruhn *et al.* 2006). Lysenin has a high amino acid sequence homology with fetidin (89 % identity, 95 % positivity), with lysenin-related protein 1 (76 % identity, 89 % positivity) and lysenin-related protein 3 (81 % identity, 90 % positivity). Amino acid sequence of lysenin-related protein 2 corresponds to that of fetidin. All these data suggest a close relationship between these lytic molecules.

The hemolytic activity of lysenin is dependent on the presence of sphingolipids in the membrane (Yamaji *et al.* 1998). Moreover, the presence of cholesterol in the membrane facilitates hemolysis. Upon binding to the spingomyelin, lysenin forms

oligomers and subsequently pores 3 nm in diameter in the target membranes (Yamaji *et al.* 1998; Yamaji-Hasegawa *et al.* 2003).

As sphingomyelin is crucial for the lytic activity of lysenin, it has been proposed to use lysenin as a valuable probe for sphingomyelin detection in sphingomyelin storage diseases, particularly in the cells of Niemann-Pick A patients (Yamaji *et al.* 1998), although the multiplicity of hemolysins in the natural source and the cytolytic activity appeared to be a major obstacle.

At the same time, a 38-kDa hemolytic protein named **eiseniapore** was identified in the coelomic fluid of *E. fetida* earthworms (Lange *et al.* 1997). Eiseniapore requires either sphingomyelin or galactosylceramide to bind to the membrane of erythrocytes and to induce lysis. Similarly to fetidin and lysenin, the presence of cholesterol in the membrane enhances lytic activity of eiseniapore. Eiseniapore was documented to form hexamers, which induce the formation of pores with an outer diameter of 10 nm and an inner diameter of 3 nm (Lange *et al.* 1997; Lange *et al.* 1999).

Eue *et al.* described 3 hemolytic proteins in the coelomic fluid of *E. fetida,* **H_1** (46 kDa), **H_2** (43 kDa) and **H_3** (40 kDa) (Eue *et al.* 1998). Each hemolysin consists of several isoforms with pI between 5.1 and 6.2 and they share structural similarities. Despite the cross-reactivity of the monospecific antisera, hemolysins functionally differ. H_3 protein is bifunctional – besides hemolytic properties which are common to all hemolysins it has also agglutinating activity. Later on, Koenig *et al.* (Koenig *et al.* 2003) isolated and analyzed two hemolytic proteins from the cell lysate (**CL_{39}** and **CL_{41}**) and three hemolysins from the coelomic fluid (H_1, H_2 and H_3) of *E. fetida* earthworms. Using mass spectrometry and bioinformatics tools they demonstrated the identity of CL_{39} with fetidin and CL_{41} with lysenin. Moreover, they showed that $H_{1\text{-}3}$ proteins share sequence components with fetidin but they seem to be glycosylated.

2.2.4. Coelomic cytolytic factor

The coelomic fluid does not cause either the lysis of the coelomocytes of other earthworm species or of the hemocytes of mullusks, nematodes and protozoans. However, it was documented that coelomic fluid of *E. fetida* lyses a broad spectrum of various cell types including chicken fibroblasts, guinea-pig polymorphonuclear leukocytes and insect hemocytes (Kauschke and Mohrig 1987). A proteinase-independent cytolytic effect of the coelomic fluid was observed in experiments with TNF-sensitive tumor L929 cell line. Subsequent isolation of lytic proteins led to the identification of 42-kDa protein, which was named coelomic cytolytic factor – CCF (Bilej *et al.* 1995).

CCF acts in earthworm defense as a pattern-recognition molecule. Upon binding microbial pathogen-associated molecular patterns, namely O-antigen of LPS of Gram-negative bacteria, muramyl dipeptide and muramic acid of peptidoglycan from the cell walls of Gram-positive bacteria and β-1,3-glucans and *N*, *N'*-diacetylchitobiose of yeast, CCF triggers the activation of the prophenoloxidase cascade, which results in the formation of cytotoxic and antimicrobial compounds and thus represents an important invertebrate defense mechanism (chapter 2.2.5). The broad specificity of CCF for pathogen-associated molecular patterns results from the presence of two spatially distinct pattern recognition lectin-like domains. One domain, which shows homology with the polysaccharide and glucanase motifs of β-1,3-glucanases and invertebrate defense molecules, is located in the central part of CCF molecule and interacts with LPS and β-1,3-glucans. The C-terminal tryptophan-rich domain mediates interactions of CCF with *N*, *N'*-diacetylchitobiose, muramyl dipeptide and muramic acid (Fig. 2) (Beschin *et al.* 1998; Bilej *et al.* 2001).

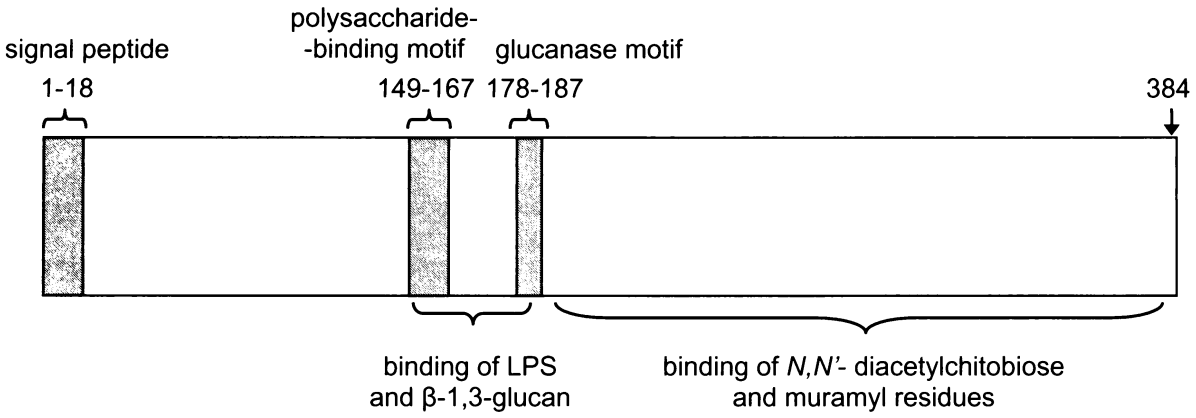

Fig. 2. Structure of *E. fetida* CCF. The broad specificity of CCF for pathogen-associated molecular patterns results from the presence of two distinct domains. One domain, which shows homology with the polysaccharide-binding and glucanase motifs of β-1,3-glucanases and invertebrate defense molecules is located in the central part of the CCF polypeptide chain and interacts with LPS and β-1,3-glucans. The C-terminal domain mediates the interaction of CCF with *N,N'*-diacetylchitobiose and muramyl constituents.

As indicated above, CCF displays amino acid sequence homology with bacterial and animal β-1,3-glucanases (Yamamoto *et al.* 1993; Bachman and McClay 1996; Kozhemyako *et al.* 2004) but it does not exhibit their enzymatic activity. More interestingly, CCF shows homology with the α subunit of the β-1,3-glucan sensitive factor G from the horseshoe crab *Tachyplesus tridentatus* (Seki *et al.* 1994), with the Gram-negative bacteria-binding proteins of various insects (Lee *et al.* 1996; Dimopoulos *et al.* 1997; Shin *et al.* 1998; Kim *et al.* 2000) and β-1,3-glucan recognition protein of arthropods (Ma and Kanost 2000; Ochiai and Ashida 2000). All these invertebrate homologs have been suggested to play a role in invertebrate innate immunity by acting as pattern recognition molecules.

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, thereby providing an efficient mechanism for phagocytosis in earthworm defense reactions (Bilej *et al.* 1995). CCF is also involved in the cell-

mediated cytotoxic reactions (Bilej *et al.* 1998) and potentiates the lytic activity of coelomic fluid against red blood cells from various species (Bilej *et al.* 2000).

More interestingly, CCF shares functional analogies with mammalian tumor necrosis factor. The ability of the coelomic fluid to lyse TNF-sensitive tumor cell line L929 is caused by CCF. This activity is not inhibited by anti-TNF neutralizing monoclonal antibodies, suggesting that the structure of TNF and CCF as well as the mechanism of TNF and CCF mediated lysis differ. In addition to this TNF-like lytic activity, CCF exhibits other similarities with this cytokine. CCF is secreted by macrophage-like coelomocytes upon LPS stimulation, while TNF is produced by LPS-activated macrophages (Aggarwal *et al.* 1985; Bilej *et al.* 1998). Both TNF and CCF have opsonizing properties (Luo *et al.* 1993; Bilej *et al.* 1995) and bind β-1,3-glucans and *N*, *N'*-diacetylchitobiose via lectin-like interactions (Olson *et al.* 1996; Beschin *et al.* 1998). In addition, monoclonal antibodies elicited against the lectin-like TIP domain of TNF cross-react with CCF and, conversely, monoclonal antibody against CCF reacts with TNF without impairing the interaction of TNF with its specific receptor (Lucas *et al.* 1994; Magez *et al.* 1997). However, the activity of CCF is not inhibited by anti-TNF antibody suggesting different mechanisms of TNF- and CCF-mediated lysis.

The lectin-like domain of TNF was shown to be involved in the killing of African and American trypanosomes (Lucas *et al.* 1994; Magez *et al.* 1997; Olivares Fontt *et al.* 1998). Hence, in view of the similar lectin-like activity of CCF and TNF, the possible trypanolytic activity of CCF was investigated (Beschin *et al.* 1999). The coelomic fluid of *E. fetida* as well as purified CCF were described to have a potent trypanolytic activity that can be inhibited not only by anti-CCF monoclonal antibodies but also by *N*, *N'*-diacetylchitobiose and anti-TNF antibodies. The possible target for both CCF and TNF on the trypanosome surface is the N-linked *N*, *N'*-diacetylchitobiose core of the variant-specific glycoprotein (VSG) that acts as a protective coat. This idea is strongly supported by the fact that CCF and TNF are able to lyse only bloodstream forms of parasites expressing VSG but not insect-stage procyclic forms expressing procyclin as a surface protein (Beschin *et al.* 1999; Bloc *et al.* 2002).

It was documented that TNF increases the membrane conductance in mammalian cells, interacting with ion-channels or ion-channel-coupled molecules through a lectin-like domain (Hribar *et al.* 1999; van der Goot *et al.* 1999). Similarly, when endothelial cells or macrophages were activated with CCF, an increase in membrane conductance occurred (Bloc *et al.* 2002). As observed with TNF, the ion-gating effect of CCF appeared when cells from TNF-receptor I and TNF-receptor II knockout mice were used. Moreover, this effect is blocked by *N*, *N'*-diacetylchitobiose and amiloride – an epithelial sodium channel inhibitor – suggesting that the effect is mediated by the lectin-like domain of CCF (Bloc *et al.* 2002).

Surprisingly, despite the functional analogies of CCF and TNF and cross-reactivity of anti-CCF and anti-TNF antibodies, these molecules do not show any gene or amino acid sequence homology, indicating a lack of common evolutionary origin (Beschin *et al.* 1999).

2.2.5. Prophenoloxidase cascade

In invertebrates, mechanical injuries or the presence of foreign objects such as parasites and microorganisms result in melanin deposition around the damaged tissue or intruding object. The melanin will physically shield an intruder and prevent its growth, but perhaps more importantly during melanin production, highly reactive and toxic quinone intermediates are produced. This process named prophenoloxidase activating cascade is controlled by the enzyme phenoloxidase (PO; monophenol monooxygenase). The cascade (for rev. Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004) is triggered by the presence of minute amounts of microbial compounds known as pathogen-associated molecular patterns (PAMPs), such as β-1,3-glucans, lipopolysaccharides or peptidoglycans. Their recognition leads to the activation of serine proteases that subsequently convert the inactive form called prophenoloxidase (proPO) to its active state – phenoloxidase. Subsequently, the active enzyme catalyzes the oxygenation of monophenols to *o*-diphenols and further oxidation of *o*-diphenols

to *o*-quinones. Quinones are non-enzymatically polymerized to melanin, which exhibits antibacterial and antifungal properties (Fig. 3) (Ashida and Yamazaki 1990; Söderhäll *et al.* 1994).

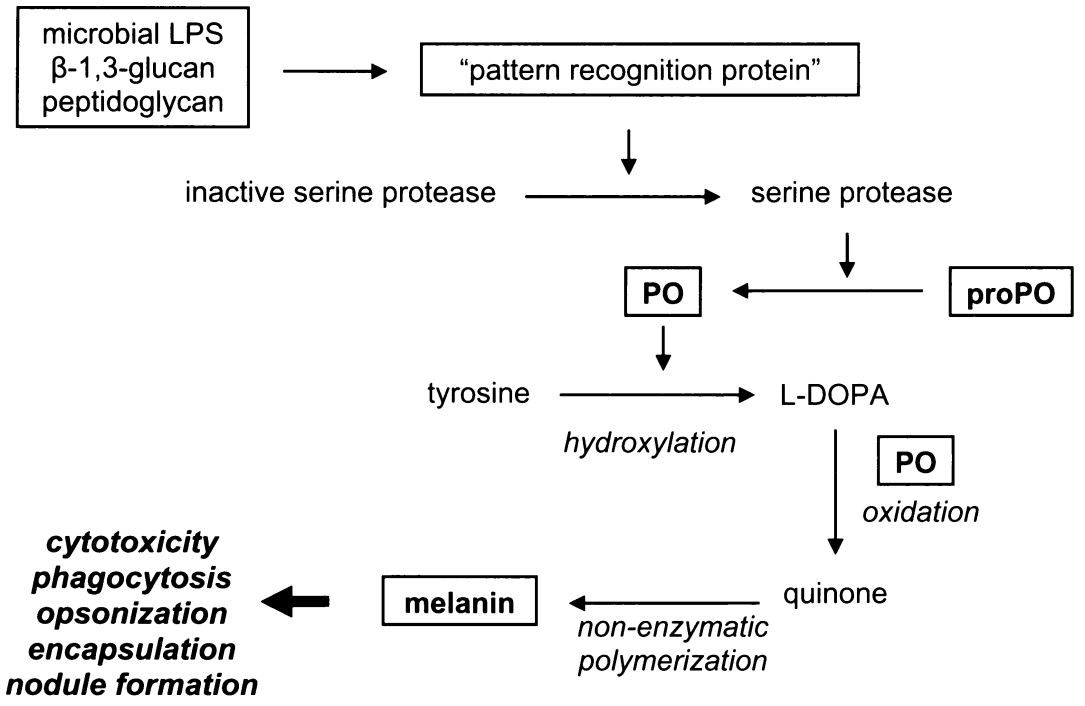

Fig. 3. Prophenoloxidase activating cascade. The recognition of pathogen associated molecular patterns leads to the cleavage of inactive prophenoloxidase to its active state phenoloxidase. Active enzyme catalyzes both hydroxylation and oxidation of phenols to quinones, which are subsequently polymerized to melanin. Melanin exerts various biological activities important in defense reactions.

Prophenoloxidase as well as phenoloxidase have been isolated and characterized in many invertebrate species. Their molecular weight varies between 70 and 90 kDa and all of them contain two functional copper-binding sites. The primary structure of the copper-binding sites is highly conserved among invertebrate proPOs as well as the peptide motif GCGEQNM which is present in α-macroglobulins of both invertebrates and vertebrates (Spycher *et al.* 1987; Hall *et al.* 1989) and in the complement proteins C3 and C4 (Dodds and Day 1993).

Taking into account the deleterious consequences of producing highly reactive quinone intermediates at inappropriate places and times, there is a need to carefully control both the activation and the activity of PO. This control is partially achieved by synthesizing the enzyme as an inactive zymogen that requires proteolytic cleavage in order to become active. The zymogen is stored in certain types of blood cells (crustaceans and some insects; Söderhäll and Smith 1983; Johansson and Söderhäll 1985; Leonard *et al.* 1985; Sritunyalucksana *et al.* 1999) or is mainly present in the plasma (some insects; Ashida 1971; Saul and Sugumaran 1986). Moreover, several inhibitors of PO or proPO activation were identified. Among others, a DOPA-containing peptide (4,2 kDa), which acts as a competitive inhibitor of the PO was found in the housefly *Musca domestica* (Daquinag *et al.* 1995; Daquinag *et al.* 1999).

The finding of the PO activity in the coelomic fluid of *E. fetida* proved the presence of proPO activating cascade in annelids (Seymour *et al.* 1992; Beschin *et al.* 1998). It was documented that proPO cascade of *E. fetida* is directly activated by Gram-negative bacteria and yeasts, while Gram-positive bacteria have to be treated with lysozyme to activate the cascade (Bilej *et al.* 2001). As mentioned above, the activation of the proPO cascade is triggered by a pattern recognition molecule of the coelomic fluid, by CCF, upon the recognition of microbial pathogen-associated molecular patterns (Beschin *et al.* 1998; Bilej *et al.* 2001).

2.2.6. Perforin and calreticulin

Perforin is known to play an important role in the defense of vertebrates. This protein, which is homologous to the C9 complement protein, is present as a monomer in the granules of cytotoxic cells. Once released from the granules of activated cells, it forms a complex with granzymes and proteoglycans that binds to the target cell plasma membrane and promotes entry of the granzymes into the target cell (Podack *et al.* 1985; Young *et al.* 1986a; Young *et al.* 1986b; Young *et al.* 1986c; Li *et*

al. 1994). Subsequently, granzymes trigger the endogenous apoptotic pathway (Smyth and Trapani 1995).

Kauschke *et al.* (Kauschke *et al.* 2001) found that anti-mouse-perforin monoclonal antibody specifically bound to the coelomocytes with small cytoplasmic granules suggesting that cytotoxic perforin-like activity is associated with one cell type only. Moreover, while analyzing the perforin-like fraction of coelomocyte lysate of *E. fetida* earthworms, they found peptides homologous to calreticulin. This finding is not surprising since perforin and calreticulin are often co-purified (Andrin *et al.* 1998). Among others, calreticulin plays two important functions in eukaryotic cells – chaperoning and regulation of Ca^{2+} homeostasis. Moreover, calreticulin was shown to be important for defense mechanisms not only of invertebrates. It is required for stress response (Park *et al.* 2001; Goo *et al.* 2005) and is involved in the phagocytosis (Asgari and Schmidt 2003; Kuraishi *et al.* 2007). The data of Kauschke and her co-workers suggest the presence of both perforin and calreticulin in *E. fetida* earthworms.

3. Aims

The general aim of this work was to study molecules involved in the defense of *E. fetida* earthworms and to elucidate the mechanisms of antimicrobial and cytolytic activity of the coelomic fluid. Although all the defense molecules we have chosen for our studies have already been described earlier; we wanted to further describe them in detail on a gene level, to examine thoroughly their function or to determine their modulation during immune response.

It was previously documented that the coelomic fluid of *E. fetida* earthworms has a potent hemolytic activity. Later on, several proteins with hemolytic activity were independently described. We have focused on two of them, fetidin and lysenin (Sekizawa *et al.* 1996; Lassegues *et al.* 1997; Milochau *et al.* 1997) which display a high amino acid sequence homology and have a similar molecular weight (40 and 42 kDa). Moreover, both proteins bind to sphingomyelin in plasma membranes of target cells, polymerize and subsequently form channels through the lipid bilayer. Our aim was **to clarify the relationship of these two hemolytic molecules, specifically, to prove whether they are either protein isoforms encoded by alleles of one gene or products of two related genes and to determine the level of their expression in coelomocytes of individual earthworms**.

Further, we focused on another coelomic molecule - coelomic cytolytic factor (CCF) which acts in earthworm defense as a pattern recognition molecule. Upon binding microbial pathogen-associated molecular patterns, CCF triggers the activation of a prophenoloxidase cascade which results in the formation of cytotoxic and antimicrobial compounds. Moreover, CCF was shown to have functional analogies with mammalian cytokine TNF. CCF is present not only in the coelomic fluid of *E. fetida*, but also in the coelomic fluid of other earthworm species, namely in *Lumbricus terrestris*. Therefore we decided **to investigate whether some other earthworm species of *Lumbricidae* family possess CCF-like molecules and, potentially,**

to characterize a primary sequence of their CCF-like molecules. Furthermore, we aimed to compare CCF-based biological properties of the coelomic fluids of these different earthworm species.

Furthermore, CCF was shown to interact with ion channels on the surface of vertebrate cells and thus cause membrane depolarization. As this artificial non-physiological experimental observation is based on the interaction of an invertebrate defense lectin with vertebrate cells, it may reflect an evolutionary ancient mechanism of macrophage activation, and therefore we wanted **to elucidate whether the interaction of CCF lectin-like domain with mammalian macrophages and subsequent membrane depolarization affects the activation of these cells**.

Next, we focused on another molecule - calreticulin - which was shown to play an important role in immune response. It was described that perforin-like fraction of *E. fetida* coelomocyte lysate contains peptides having homology with calreticulin (Kauschke *et al.* 2001). As calreticulin was shown to be involved in defense mechanisms of invertebrates, specifically during stress response (Park *et al.* 2001; Goo *et al.* 2005) and phagocytosis (Asgari and Schmidt 2003; Kuraishi *et al.* 2007), we wanted **to prove its presence in *E. fetida* earthworms, characterize a calreticulin-coding cDNA, show the phylogenetic relationships of this molecule and determine its expression in different organs**.

It is obvious that the coelomic fluid of *E. fetida* earthworms exerts various biological activities. In addition to CCF and hemolytic proteins, lysozyme-like activity of the coelomic fluid of *E. fetida* earthworms was described. However, the modulations of these defense molecules during *in vivo* immune response have not yet been addressed. Therefore, **we investigated the effect of experimental challenge with live Gram-positive, Gram-negative bacteria and β-1,3-glucan on the expression CCF and fetidin and, moreover, to determine the levels of hemolytic activity and lysozyme-like activity in the coleomic fluid**.

As was briefly mentioned above, the prophenoloxidase cascade represents one of the most important defense mechanisms in many invertebrates. The final product

of this cascade, melanin, is involved in wound healing as well as in immune responses. The finding of the prophenoloxidase activity in the coelomic fluid of *E. fetida* suggested the presence of prophenoloxidase activating cascade in annelids (Seymour *et al.* 1992; Beschin *et al.* 1998). However, its presence has not yet been directly assigned and, hence, we wanted **to prove the presence of the prophenoloxidase activating cascade as a defense mechanism in earthworms**.

4. Results

4.1. Relationship between hemolytic molecules

The coelomic fluid of *E. fetida* earthworms contains various proteins causing the lysis of red blood cells. These proteins, often described independently by different research groups, comprise *Eisenia fetida andrei* factor (EFAF) (Roch *et al.* 1981; Roch 1984), fetidin (Lassegues *et al.* 1997; Milochau *et al.* 1997), lysenin (Sekizawa *et al.* 1996; Sekizawa *et al.* 1997), eiseniapore (Lange *et al.* 1997) and hemolysins isolated either from the coelomic fluid (H_1, H_2 and H_3) or from the cell lysate (CL_{39} and CL_{41}) (Eue *et al.* 1998; Koenig *et al.* 2003). Even though their nomenclature and terminology differ, their further characterization indicates that they are the same or similar molecules. All hemolytic proteins described so far share biochemical analogies, having a similar molecular mass around 40 kDa, similar pI and ability to bind sphingomyelin and create pores in target lipid membranes.

The aim of our study was to clarify whether fetidin and lysenin are either protein isoforms encoded by different alleles of one gene, or products of two related genes.

Both cDNA and amino acid sequences of fetidin and lysenin display high homology. We took advantage of differences between their sequences in 5'UTR regions to design primers specific for each molecule. However, using primers specific for lysenin in PCR we amplified also fetidin. Subsequent sequencing revealed errors in the published 5'UTR sequence of fetidin. A new set of primers designed based on the presence of different deletions in the sequence of fetidin and lysenin in combination with stringent annealing temperature in PCR reaction provided a reliable tool for amplifying the desired sequence only. Using these specific primers we proved that the coelomocytes of each individual *E. fetida* contain mRNA for both fetidin and lysenin. Moreover, PCR reactions with *E. fetida* genomic DNA as a template

resulted in amplification of both molecules, fetidin and lysenin, suggesting that they are encoded by two distinct genes.

Real-time PCR experiments were performed to see the differences in the expression of genes encoding fetidin and lysenin in individual earthworms. We found out that, while the expression of fetidin is similar in all individuals, the expression of lysenin strongly varies. The expression of lysenin was up to 26 times higher in some individuals than in others.

To address the relationships between hemolytic activity and expression of fetidin and lysenin, coelomic fluids of tested animals were separated in native PAGE and then the gels were applied on an erythrocyte suspension embedded in agarose. After several hours of incubation, four different patterns of hemolytic proteins were observed. In parallel, the hemolytic activity of coelomic fluids of tested animals was quantified. The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids. All animals exhibiting higher hemolytic activity share the same hemolytic pattern consisting of several bands on agarose-embedded erythrocytes, reflecting the presence of more hemolytic proteins in the coelomic fluids of these earthworms.

Surprisingly, we did not prove the presence of either fetidin of lysenin in other earthworm species (*Aporrectodea caliginosa*, *Apporectodea icterica*, *Apporectodea longa*, *Apporectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus*, *Lumbricus terrestris*) as tested by PCR using fetidin and lysenin specific primers. This finding suggests a unique occurrence of these hemolytic molecules in *Eisenia fetida* earthworms.

In conclusion, we document that fetidin and lysenin are encoded by different genes with high homology and their expression differs in individual *E. fetida* earthworms.

Procházková P., Šilerová M., Felsberg J., Josková R., Beschin A., De Baetselier P., Bilej M.: Relationship between hemolytic molecules in *Eisenia fetida* earthworms. *Dev. Comp. Immunol.* 30: 381-392 (2006).

Procházková P., Šilerová M., Felsberg J., Josková R., Beschin A., De Baetselier P., Bilej M.: Relationship between hemolytic molecules in *Eisenia fetida* earthworms. *Dev. Comp. Immunol.* 30: 381-392 (2006).

5. Conclusions

Earthworms, as well as other invertebrates, have developed a variety of defense mechanisms efficiently recognizing and responding to non-self substances. They possess physical and chemical barriers as well as efficient cellular mechanisms (clotting and coagulation responses, phagocytosis of invading microorganisms and encapsulation reactions) and a broad range of antimicrobial factors (lysozyme-like proteins, proteases, cytolytic proteins, antimicrobial peptides and components of the prophenoloxidase activation cascade). The recognition of non-self in invertebrates is based on pattern recognition and lectin-like molecules that are designed to recognize few highly conserved structures present in many different microorganisms. In this study we focused on detailed description of defense molecules of earthworms, on examination of their function and regulation during immune response. From our data we may conclude:

- Two *E. fetida* hemolytic proteins, fetidin and lysenin, are encoded by two separate genes that share high homology and their expression in coelomocytes differs in individual earthworms. Moreover, these two proteins seem to be unique for *E. fetida* earthworms.

- Coelomic fluids of earthworm species *Aporrectodea caliginosa*, *Apporectodea icterica*, *Apporectodea longa*, *Apporectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestris* contain CCF-like molecules having 80-90 % homology with *E. fetida* CCF. Furthermore, CCF of *E. fetida* is particular – it is the only one recognizing *N, N'*-diacetylchitobiose and having cytolytic and trypanolytic activities. The broad recognition repertoire of *E. fetida* CCF probably reflects a particular microbial environment this species lives in.

- *E. fetida* CCF interacts with ion channels on the surface of vertebrate cells via *N, N'*-diacetylchitobiose lectin-like activity, thus inducing membrane depolarization resulting in the release of TNF, IL-6 and NO via NF-κB activation.

- We proved the presence of calreticulin in *E. fetida* earthworms and specified the sequence of calreticulin-coding cDNA. Neighbor-joining phylogeny tree constructed based on the deduced amino acid sequences suggests the common origin of *E. fetida* calreticulin and calreticulins of mollusks. A high calreticulin expression was detected in various tissues, namely epidermis, body wall muscles, ventral nerve cord, sperm cells and mesenchymal lining.

- Parenteral administration of Gram-positive and Gram-negative bacteria or microbial polysaccharides results in augmented CCF levels in the coelomic fluid of *E. fetida* and in increased lysozyme-like activity whereas the hemolytic activity decreases. Moreover, the biosynthesis of CCF, but not fetidin, is up-regulated upon microbial stimulation.

- Phenoloxidase and its inhibitors exist in *E. fetida* earthworms but the level of phenoloxidase activity in the coelomic fluid is lower as compared to other invertebrate species. Moreover, phenoloxidase activation is slower.

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