This manuscript were published in Developmental & Comparative Immunology

Volume 90, January 2019, Pages 41-46

https://doi.org/10.1016/j.dci.2018.09.001

IDENTIFICATION OF NOVEL LUMBRICIN HOMOLOGUES IN *EISENIA ANDREI* EARTHWORMS

Kornélia Bodó[1], Ákos Boros[2], Éva Rumpler[3], László Molnár[3], Katalin Böröcz[1], Péter Németh[1], Péter Engelmann[1*]

[1]Department of Immunology and Biotechnology, Clinical Center, Medical School, University of Pécs, Hungary

[2]Regional Laboratory of Virology, ANTSZ, Baranya County Institute of State Public Health Service, Pécs, Hungary

[3]Department of Comparative Anatomy and Developmental Biology, Faculty of Sciences, University of Pécs, Hungary

***Corresponding author:** Department of Immunology and Biotechnology, Clinical Center, Medical School, University of Pécs, Pécs, H-7643, Szigeti u. 12, Hungary. Tel: + 36-72-536-288, Fax: + 36-72-536-289, email: engelmann.peter@pte.hu

ABSTRACT

Lumbricin and its orthologue antimicrobial peptides were typically isolated from annelids. In this report, mRNA for lumbricin and -serendipitously- a novel lumbricin-related mRNA sequence were identified in *Eisenia andrei* earthworms. The determined mRNA sequences of *E. andrei* lumbricin and lumbricin-related peptide consist of 477 and 575 nucleotides. The precursors of proline-rich *E. andrei* lumbricin and the related peptide contain 63 and 59 amino acids, respectively. Phylogenetic analysis indicated close relationship with other annelid lumbricins. Highest expression of both mRNAs appeared in the proximal part of the intestine (pharynx, gizzard), while other tested organs had moderate (body wall, midgut, ovary, metanephridium, seminal vesicles, ventral nerve cord) or low (coelomocytes) levels. During ontogenesis their expression revealed continuous increase in embryos. Following 48 hours of *in vivo* Gram-positive bacteria challenge both mRNAs were significantly elevated in coelomocytes, while Gram-negative bacteria or zymosan stimulation had no detectable effects.

Keywords: innate immunity, antimicrobial peptides, earthworms, lumbricin, gene expression

1. INTRODUCTION

Antimicrobial peptides (AMPs) are structurally conserved bioactive molecules during phylogenesis (Boto et al., 2018; Bulet et al., 2004; Nguyen et al., 2011; Zasloff, 2002). Until now, several thousands of AMPs have been isolated from prokaryotes to mammals (Boman, 1995; Zasloff, 2002). They possess a broad range of antimicrobial activity with no or little cytotoxicity (Kumar et al., 2018, Nguyen et al., 2011).

Earthworms operate with complex cellular and humoral immune constituents to maintain their self-integrity (Gupta and Yadaw, 2016). Until now a handful of immune components have been identified in earthworms (Cooper et al., 2002; Mácsik et al., 2015), but only a limited number of antimicrobial molecules (e.g. F1/F2, lysenin/fetidin, lysozyme, lumbricusin, OEP3121) have been characterized (Josková et al., 2009; Lassegues et al., 1997; Kim et al., 2015; Liu et al., 2004; Opper et al., 2013, Zhang et al., 2002).

In addition to lysozyme, just one restricted AMP denoted as lumbricin I, has been isolated and characterized from the earthworm, *Lumbricus rubellus*. This 62 amino acid long peptide exhibits *in vitro* broad antimicrobial spectra against fungi, Gram-positive and Gram-negative bacteria without hemolytic activity (Cho et al., 1998). By now, several lumbricin homologues have been identified and described from other earthworm (Li et al., 2011; Wang et al., 2003) and leech species (Schikorski et al., 2008).

These aforementioned studies revealed the parallel existence of this peptide among annelid species, however it was not detected yet from *Eisenia andrei* earthworms. In this report we describe the characterization, tissue and ontogenetic distributions, and antimicrobial induction of a new lumbricin homologue and a novel lumbricin-related peptide from *E. andrei*.

2. MATERIALS AND METHODS

2.1. Earthworm husbandry

Adult (clitellated) *Eisenia andrei* (Lumbricidae, Annelida) were collected from the breeding stock, maintained at standard conditions (Molnár et al., 2012). Prior to organ and tissue isolations earthworms were placed onto moist tissue paper for overnight depuration to avoid soil contaminations.

2.2. RNA isolation, cDNA synthesis, rapid amplification of cDNA ends (RACE)

Coelomocytes were harvested from the coelomic cavity, followed by the surgical removal of cerebral ganglion and the ventral nerve cord. Total RNA was extracted from the samples according to the manufacturer's protocol using NucleoSpin® RNA isolation kit (Macherey-Nagel GmbH, Düren, Germany). For the 3' RACE PCR reverse transcription (RT) was conducted from total RNA using High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) and Adapter-oligo-dT-anchor primer (Table S1).

After RNAse-H digestion 3' RACE PCR reaction was made using adapter primer and a generic forward primer (Ea-Lumbr-F, Table S1) designed to the conserved sequence regions of the known lumbricin sequences of *L. rubellus* (AF060552) and *Hirudo medicinalis* (EU156756) and the same reagents described previously (Boros et al., 2011). For the 5' RACE RT-PCR reactions sequence specific R1 primers were used for RT (Table S1). Following the RT and RNase-H digestion 3' poly-A-tailing of the cDNA was made using terminal deoxynucleotidyl transferase enzyme and dATP (Boros et al., 2011). The polyA-tailed cDNA was purified using GeneJET PCR purification kit (Thermo-Fisher, Waltham, MA, USA). Semi-nested PCR reactions were conducted using sequence-specific R2 (PCR1) and R3 (PCR2) reverse primers and Adapter-oligo dT-anchor primer (PCR1) the Adapter (PCR2) as a forward primers (Table S1) and the same reagents described previously (Boros et

al., 2011). The thermal program for PCR reactions of the 3' and 5' RACE experiments started with 1 cycle at 94°C for 30 sec, followed by 35 cycles of 94°C for 35 sec, 50°C for 1 min, 72°C for 2 min, and terminated with a final elongation step of 72°C for 5 min. The visible PCR amplicons were purified using either GeneJET PCR purification kit or GeneJET Gel extraction kit (Thermo-Fisher, Waltham, MA, USA) and sequenced directly on an automated sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Stafford, USA) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). The obtained sequences were submitted into the NCBI Genbank (lumbricin accession number: KX816866; LuRP accession number: KX816867).

2.3. Sequence and phylogenetic analysis

Amino acid sequences of annelid lumbricins and its novel homologues from *E. andrei* were aligned by Clustal Omega (Sievers and Higgins, 2014). Phylogenetic analysis was conducted using the maximum likelihood method and Poisson model by MEGA 7.0 (Kumar et al., 2016). The numbers closed to the branch nodes represent the percentage of 1000 bootstrap replications.

2.4. Relative quantification of target genes from adult tissues and embryos

Various organ and tissue samples (pharynx, gizzard, midgut, ovarium, metanephridium, body wall, seminal vesicle, ventral nerve cord and coelomocytes) were collected from at least ten adult earthworms. Earthworm embryos were gathered from their cocoons. Their distinct developmental stages (from E1 to E4) were identified by their specific morphological features (Boros et al., 2010). Total RNA was extracted from ten pooled tissue samples of adult earthworms as well as a pool of ten embryos from all developmental stages

according to the manufacturer's protocol using NucleoSpin® RNA isolation kit (Macherey-Nagel GmbH, Düren, Germany). The amount of total RNA was determined by NanoDrop at 260 nm. RNA samples were stored at – 80 ^{o}C. Reverse transcription was accomplished in 20 µl reactions using High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) and cDNA samples were stored at – 20 ^{o}C. Subsequently, cDNA used as a template in qPCR reactions. Gene specific primers were designed with Primer Express software (Thermo Fisher Scientific) to estimate the expression levels of target genes in the aforementioned tissues (Table S1)**.** Gene expression was measured by an ABI Prism 7500 instrument (Applied Biosystems, Warrington, UK) applying Maxima SYBR Green/Low Rox Master Mix (Thermo-Fisher, Waltham, MA, USA). The amplification profile started at 95 ºC for 10 min, that followed 40 cycles of 35 sec at 95 ºC, 35 sec at 58 ºC, and 1 min at 72 ºC. Quantitative measurements were normalized to *RPL17* mRNA level (Table S1). Three independent experiments were implemented in duplicates.

2.5. *In vivo* microbial challenge

Adult earthworms (three animals/condition) were exposed to heat-inactivated *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (OKI 112001) (each 10^{8}/ml) and zymosan (membrane from *Saccharomyces cerevisiae* in 1 mg/ml final concentration, Sigma-Aldrich, Budapest, Hungary) on filter paper for different time points at room temperature (Cang et al., 2017; Homa et al., 2005; Schikorski et al., 2008). The suspension of microorganisms and zymosan were diluted in *Lumbricus* balanced Salt Solution (LBSS). Control earthworms were exposed on LBSS-immersed filter paper (for composition, please see Engelmann et al., 2005). After the treatments coelomocytes were harvested from the coelomic cavity as we published earlier (Engelmann et al., 2005). Coelomocyte numbers were evaluated by trypan-blue exclusion method and subsequently were centrifuged twice in LBSS

(200g, 5 min, at room temperature). Total RNA extraction, reverse transcription, and qPCR experiments were executed as we described earlier. *RPL17* mRNA level was employed for the normalization process. Normalized expressions of both genes are exhibited in pathogen stimulated earthworms comparison with untreated ones.

2.6. Statistical analysis

Statistical analyses were carried out with Prism v5.0 (GraphPad software, La Jolla, CA USA). Data were calculated from three independent experiments. Data were checked for normality prior to further analysis (Shapiro-Wilk normality test). All data were expressed as mean±SEM. Results were analyzed by one or two way ANOVA followed by Bonferonni post hoc tests. $p<0.05$ was denoted as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Sequence analysis of *E. andrei* lumbricin and LuRP

A large variety of AMPs were isolated from several organisms from plants to humans. Annelid earthworms provided relatively limited information in this field (Tasiemski, 2008). Cho et al. (1998) isolated the first antimicrobial peptide (lumbricin I) from the earthworm, *L. rubellus*. Until recently, several lumbricin homologues were described from other earthworms (*Metaphire tschiliensis,* and *M. guillelmi*) and the leech, *H. medicinalis* (Li et al., 2011; Schikorski et al., 2008; Wang et al., 2003). Based on the available annelid lumbricin sequences a novel generic forward primer was designed for the detection of lumbricin homologues in *E. andrei* by 3' RACE PCR (Table S1). Surprisingly, the 3' RACE PCR showed the presence of not one but two discrete bands (Fig. S1). The sequences determined from the two PCR amplicons showed only 43% pairwise nucleotide (nt) identity. Using sequence specific primers and 5' RACE PCR technique 466-nt and 549-nt-long sequences (without the polyA-tail) of the two mRNAs were determined. The 466-nt-long mRNA called as lumbricin (*Lumbr*) contains a single 192-nt-long ORF encoding a 63-aa-long peptide (average calculated molecular mass: 7413.35 Da), while the 575-nt-long mRNA called as lumbricin-related peptide (*LuRP*) contains a single 180-nt-long ORF encoding a 59-aa-long peptide (average calculated molecular mass: 7066.84 Da). The precursor peptides show 98% (Lumbr) and 66% (LuRP) identity to the antimicrobial peptide lumbricin I from *L. rubellus* (AF06552) as the closest match identified by BLASTp search. The *E. andrei* Lumbr and *E. andrei* LuRP precursor peptides show only 66% pairwise aa identity (Fig 1c). The 3' untranslated regions of both mRNAs contain the AUUAAA and AAUAAA polyadenylation signal sequences (Tian and Graber, 2012) (Fig. 1a, b). Phylogenetically Lumbr and LuRP precursor peptides are also separated from each other; Lumbr is clustered together with the

lumbricin I of *L. rubellus* while LuRP shows closer relationship to the lumbricin homologue of *H. medicinalis* (Fig. 1d).

Interestingly, the N-terminal end of Lumbr of *E. andrei* is 13 aa shorter than the lumbricin I of *L. rubellus* otherwise the sequence of the two peptides are identical (Fig 1c). Typical lengths of lumbricin homologues are ranged between 57 and 76 amino acids (Fig. 1c).

E. andrei lumbricin and its related peptide harbor numerous proline residues (14.3% and 6.8% in molar ratio) similar to lumbricin I and other lumbricin homologues (Cho et al., 1998). Typically proline-rich AMPs were isolated from the arthropods including insects and crustaceans (Graf et al., 2017; Otvos, 2002). Proline amino acids uniquely alter the protein conformation (e.g. folding and cyclisation); thereby it exerts an influence on the secondary structure of proteins (Graf et al., 2017; Vanhoof et al., 1995). Furthermore, aromatic amino acid (His, Trp, Tyr) content of these *Eisenia* lumbricin homologues is relatively high (15-16% in molar ratio), which could further suggest the antimicrobial activity of these peptides (Muñoz et al., 2007).

3.2. Tissue and embryonic expression patterns of *Lumbr* and *LuRP* in *E. andrei*

Since the recent studies (Li et al., 2011; Wang et al., 2003) did not survey extensively the tissue localization of lumbricins in annelids (Tasiemski, 2008), hence we aimed to examine the *Lumbr* and *LuRP* expression patterns in the different organs of *E. andrei*. According to Wang et al. (2003) lumbricin was restricted to the body wall in *M. tschiliensis*, and not present in the intestine or coelomocytes. A lumbricin homologue was isolated from the skin secretions of *M. guillelmi* (Li et al. 2011). Schikorski et al. (2008) investigated *Hm-lumbricin* expression of the microglial cells in the course of leech CNS regeneration. In contrast we demonstrated the presence of *Lumbr* and *LuRP* in a wide variety of *E. andrei* tissues (Fig. 2a). Highest mRNA expressions of both AMPs were detected in the proximal

part of the intestine (including pharynx and gizzard), while other tested tissues had a moderate (body wall, midgut, ovary, seminal vesicle, metanephridium, ventral nerve cord) or low (coelomocytes) level of expression. Higher *LuRP* mRNA expression was demonstrated in all tested tissues and coelomocytes compared to *Lumbr*. The highest expressions of both AMPs were detected in the intestine, because this organ is the most exposed for frequent microbial invasions. According to Fiołka et al. (2012) lysozyme expression is also mainly detectable in the intestine of the earthworm, *Dendrobaena veneta*. Both *lumbricin* isoforms show ubiquitous tissue expression in *E. andrei* (Fig. 2a), in contrast to lysenin that is mainly attributed to large coelomocytes (amoebocytes), eleocytes (Opper et al., 2013) or sessile chloragocytes (Ohta et al., 2000).

First ontogenetic distribution pattern of lumbricin is reported from *L. rubellus*. Cho et al. (1998) detected *lumbricin I* expression in adult *L. rubellus*, but not in the cocoons or developing earthworms. In contrast, both *lumbricin* homologues from *E. andrei* were expressed in the course of embryonic development (Fig. 2b). Their expression displayed continuous increase up to the fourth developmental stage (E4) when the body is entirely segmented and the organ differentiation is completed (Boros et al., 2010). *LuRP* exhibited significantly higher expression compared to *Lumbr* in the different stages of developing *E. andrei* earthworms. One explanation of the gradient increase of *Lumbr*/*LuRP* expression could be the larger body size of the more developed embryonic stages. On the other hand, it is known that numerous symbiotic bacteria colonize the earthworm embryos and their frequencies boost during early embryogenesis (Zachmann and Molina, 1993; Davidson et al., 2010). It is probable that Lumbr and LuRP might control the growth of commensal bacteria in earthworm embryos that is known already about other invertebrate antimicrobial peptides (Roiff and Schmid-Hempel, 2016).

3.3. Induction of *Lumbr* and *LuRP* mRNA expression upon *in vivo* microbial challenge

Proline-rich AMPs possess a wide range of antimicrobial activity against microorganisms (Otvos 2002). Indeed, *L. rubellus* lumbricin I is efficient against Gram-negative, Gram-positive bacteria and fungi without any haemolytic activity (Cho et al., 1998). Follow-up experiments on lumbricin homologues have verified these observations in other annelid species (Li et al., 2011; Schikorski et al., 2008). *L. rubellus* lumbricin I had similar minimal inhibitory concentrations comparing the activity against *E. coli* and *S. aureus* (Cho et al., 1998).

Interestingly, bacterial challenge did not induce the *lumbricin I* expression compared to non-bacteria challenged earthworms revealed by Northern blot analysis in *L. rubellus*. Thus, *lumbricin I* is evidenced constitutive expression in this species (Cho et al. 1998). In contrast, *Hm-lumbricin* expression is modulated overtime by microbial challenge (Schikorski et al., 2008). In particular Gram positive bacteria (*Micrococcus)* and zymosan treatments were more effective on *lumbricin* mRNA expression compared to Gram negative (*Aeromonas*) bacteria exposure in *H. medicinalis* (Schikorski et al., 2008). Li et al. (2011) described that among the tested strains the most sensitive were *Pseudomonas aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive) to lumbricin–PG, however *E. coli* (Gram-negative) was less sensitive to this peptide. Similarly to the aforementioned studies we found significantly elevated mRNA level of *Lumbr* and *LuRP* upon 48 hrs of *S. aureus* bacteria challenge, but there was no any increase of expression upon *E. coli* or zymosan treatments (Fig. 2c and d). Regarding to the kinetics of AMP induction Schikorski et al., (2008) observed induction of *Hm-lumbricin* after 6 hours that is peaked after 24 hours in isolated leech CNS. In comparison, our results evidenced a rather slow induction of *Lumbr* and *LuRP* at 48 hours, but we exposed intact earthworms to microbes.

These discrepancies in the bacterial induction of lumbricin could be explained with the following considerations. First, there can be major differences in the expression levels among species. Second, the applied methods (Northern blot vs. qPCR) have different sensitivity. Third, these effects against the different microbial strains are based on the various structural features: mainly the amino acid compositions of lumbricins and differences between the microbial cell-wall constituents (Tassanakajon et al., 2015, Cunha et al., 2017).

CONCLUSIONS

Our study has revealed the presence of two novel members of the proline-rich lumbricin AMP family in the earthworm *E. andrei*. Hereby, our novel data support the high conservation of lumbricin AMPs in annelid worms and their possible role in the maintenance of earthworm immune homeostasis during ontogeny and pathogenic infections.

ACKNOWLEDGEMENTS

We acknowledge the financial support of Medical School Research Foundation (PTE-ÁOK-KA 2017/04), University of Pécs, the GINOP-232-15-2016-00050 and EFOP-361-16-2016-00004 grants. Á.B. and P.E. were supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. Á.B. was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/ 2-11/1-2012-0001 'National Excellence Program'. The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

REFERENCES

Boman, H.G., 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. 13, 61-92.

Boros, A., Somogyi, I., Engelmann, P., Lubics, A., Reglodi, D., Pollák, E., Molnár, L., 2010. Pituitary adenylate cyclase-activating polypeptide type 1 (PAC1) receptor is expressed during embryonic development of the earthworm. Cell Tissue Res. 339, 649-53.

Boros, A., Pankovics, P., Simmonds, P., Reuter, G., 2011. Novel positive-sense, single-stranded RNA (+ssRNA) virus with di-cistronic genome from intestinal content of freshwater carp (*Cyprinus carpio*). PLoS One. 6, e29145.

Boto, A., Pérez de la Lastra, J.M., González, C.C., 2018. The road from host-defense peptides to new generation of antimicrobial drugs. Molecules 23, E311.

Bulet, P., Stöcklin, R., Menin, L., 2004. Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. 198, 169-84.

Cang, T., Dai, D., Yang, G., Yu, Y., Lv, L., Cai, L., Wang, Q., Wang, Y., 2017. Combined toxicity of imidacloprid and three insecticides to the earthworm, *Eisenia fetida* (Annelida,Oligochaeta). Environ. Sci. Pollut. Res. Int. 24, 8722-30.

Cho, J.H., Park, C.B., Yoon, Y.G., Kim, S.C., 1998. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. Biochim. Biophys. Acta. 1408, 67-76.

Cooper, E.L., Kauschke, E., Cossarizza, A., 2002. Digging for innate immunity since Darwin and Metchnikoff. BioEssays 24, 319-33.

Cunha, N.B., Cobacho, N.B., Viana, J.F., Lima, L.A., Sampaio, K.B., Dohms, S.S., Ferreira, A.C.,de la Fuente-Núñez, C., Costa, F.F., Franco, O.L., Dias, S.C., 2017. The next generation of antimicrobial peptides (AMPs) as molecular therapeutic tools for the treatment of diseases with social and economic impacts. Drug Discov.Today. 22, 234-48.

Davidson, S.K., Powell, R.J., Stahl, D.A., 2010. Transmission of a bacterial consortium in *Eisenia fetida* egg capsules. Environ. Microbiol. 12, 2277-88.

Engelmann, P., Pálinkás, L., Cooper, E.L., Németh, P., 2005. Monoclonal antibodies identify four distinct annelid leukocyte markers. Dev. Comp. Immunol. 29, 599-614.

Fiołka, M., Zagaja, M.P., Hulas-Stasiak, M., Wielbo, J., 2012. Activity and immunodetection of lysozyme in earthworm *Dendrobaena veneta* (Annelida). J. Invert. Pathol. 109, 83-90.

Graf, M., Mardirossian, M., Nguyen, F., Seefeldt, A.C., Guichard, G., Scocchi, M., Innis, C.A., Wilson, D.N., 2017. Proline-rich antimicrobial peptides targeting protein synthesis. Nat. Prod. Rep. 34, 702-11.

Gupta, S., Yadaw, S., 2016. Immuno-defense strategy in earthworms: a review. Int. J. Curr. Microbiol. App. Sci. 5, 1022-35.

Homa, J., Olchawa, E., Stürzenbaum, S.R., Morgan, A.J., Plytycz, B., 2005. Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. Environ. Pollut. 135, 275-80.

Josková, R., Silerová, M., Procházková, P., Bilej, M., 2009. Identification and cloning of an invertebrate-type lysozyme from *Eisenia andrei*. Dev. Comp. Immunol. 33, 932-8.

Kim, D.H., Lee, I.H., Nam, S.T., Hong, J., Zhang, P., Lu, L.F., Hwang, J.S., Park, K.C., Kim, H., 2015. Antimicrobial peptide, lumbricusin, ameliorates motor dysfunction and dopaminergic neurodegeneration in a mouse model of Parkinson's disease. J. Microbiol. Biotechnol. 25: 1640-47.

Kumar, P., Kizhakkedathu, J.N., Straus, S.K., 2018. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. Biomolecules 8, E4.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870-4.

Lassegues, M., Milochau, A., Doignon, F., Du Pasquier, L., Valembois, P., 1997. Sequence and expression of an *Eisenia-fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein. Eur. J. Biochem. 246, 756-62.

Li, W., Li, S., Zhong, J., Zhu, Z., Liu, J., Wang, W., 2011. A novel antimicrobial peptide from skin secretions of the earthworm, *Pheretima guillelmi* (Michaelsen). Peptides. 32, 1146-50.

Liu, Y.Q., Sun, Z.J., Wang, C., Li, S.J., Liu, Y.Z., 2004. Purification of a novel antibacterial short peptide in earthworm *Eisenia foetida*. Acta Biochim. Biophys. Sin. 36, 297-302.

Mácsik, L.L., Somogyi, I., Opper, B., Bovári-Biri, J., Pollák, E., Molnár, L., Németh, P., Engelmann, P., 2015. Induction of apoptosis-like cell death by coelomocyte extracts from *Eisenia andrei* earthworms. Mol. Immunol. 67, 213-22.

Molnár, L., Engelmann, P., Somogyi, I., Mácsik, L.L., Pollák, E., 2012. Cold-stress induced formation of calcium and phosphorous rich chloragocytes granules (chloragosomes) in the earthworm *Eisenia fetida*. Comp. Biochem. Physiol. 163A, 199-209.

Muñoz, A., López-García, B., Pérez-Payá, E., Marcos, J.F., 2007. Antimicrobial properties of derivatives of the cationic tryptophan-rich hexapeptide PAF26. Biochem. Biophys. Res. Commun. 354, 172-7.

Nguyen, L.T., Haney, E.F., Vogel, H.J., 2011. The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. 29, 464-72.

Ohta, N., Shioda, S., Sekizawa, Y., Nakai, Y., Kobayashi, H., 2000. Sites of the expression of mRNA for lysenin a protein isolated from the coelomic fluid of the earthworm *Eisenia foetida*. Cell Tissue Res. 302, 263-70.

Opper, B., Bognar, A., Heidt, D., Németh, P., Engelmann, P., 2013. Revising lysenin expression of earthworm coelomocytes. Dev. Comp. Immunol. 39, 214-18.

Otvos, L. Jr., 2002. The short proline-rich antibacterial peptide family. Cell. Mol. Life Sci. 59, 1138-50.

Roiff, J., Schmid-Hempel, P., 2016. Perspectives on the evolutionary ecology of arthropod antimicrobial peptides. Philos, Trans. R. Soc. B 371, 20150297.

Schikorski, D., Cuvillier-Hot, V., Leippe, M., Boidin-Wichlacz, C., Slomianny, C., Macagno, E., Salzet, M., Tasiemski, A., 2008. Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. J. Immunol.181, 1083-95.

Sievers, F., Higgins, D.G., 2014. Clustal omega. Curr Protoc Bioinformatics 48:1-16.

Tasiemski, A., 2008. Antimicrobial peptides in annelids. Inv. Surv. J. 5, 75-82.

Tassanakajon, A., Somboonwiwat, K., Amparyup, P., 2015. Sequence diversity and evolution of antimicrobial peptides in invertebrates. Dev. Comp. Immunol. 48, 324-41.

Tian, B., Graber, J.H., 2012. Signals for pre-mRNA cleavage and polyadenylation. Wiley Interdiscip. Rev. RNA 3, 385-96.

Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., Scharpé, S., 1995. Proline motifs in peptides and their biological processing. FASEB J. 9, 736-44.

Wang, X., Wang, X., Zhang, Y., Qu, X., Yang, S., 2003. An antimicrobial peptide of the earthworm *Pheretima tschiliensis*: cDNA cloning, expression and immunolocalization. Biotechnol. Lett. 25, 1317-23.

Zachmann, J.E., Molina, J.A., 1993. Presence of culturable bacteria in cocoons of the earthworm *Eisenia fetida*. Appl. Environ. Microbiol. 59, 1904-10.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature. 415, 389-95.

Zhang, X.C., Sun, Z.J., Zhuo, R.P., Hou, Q.M., Lin, G.Q., 2002. Purification and characterization of two antibacterial peptides from *Eisenia fetida*. Prog. Biochem. Biophys. 29, 955-60.

FIGURE CAPTIONS

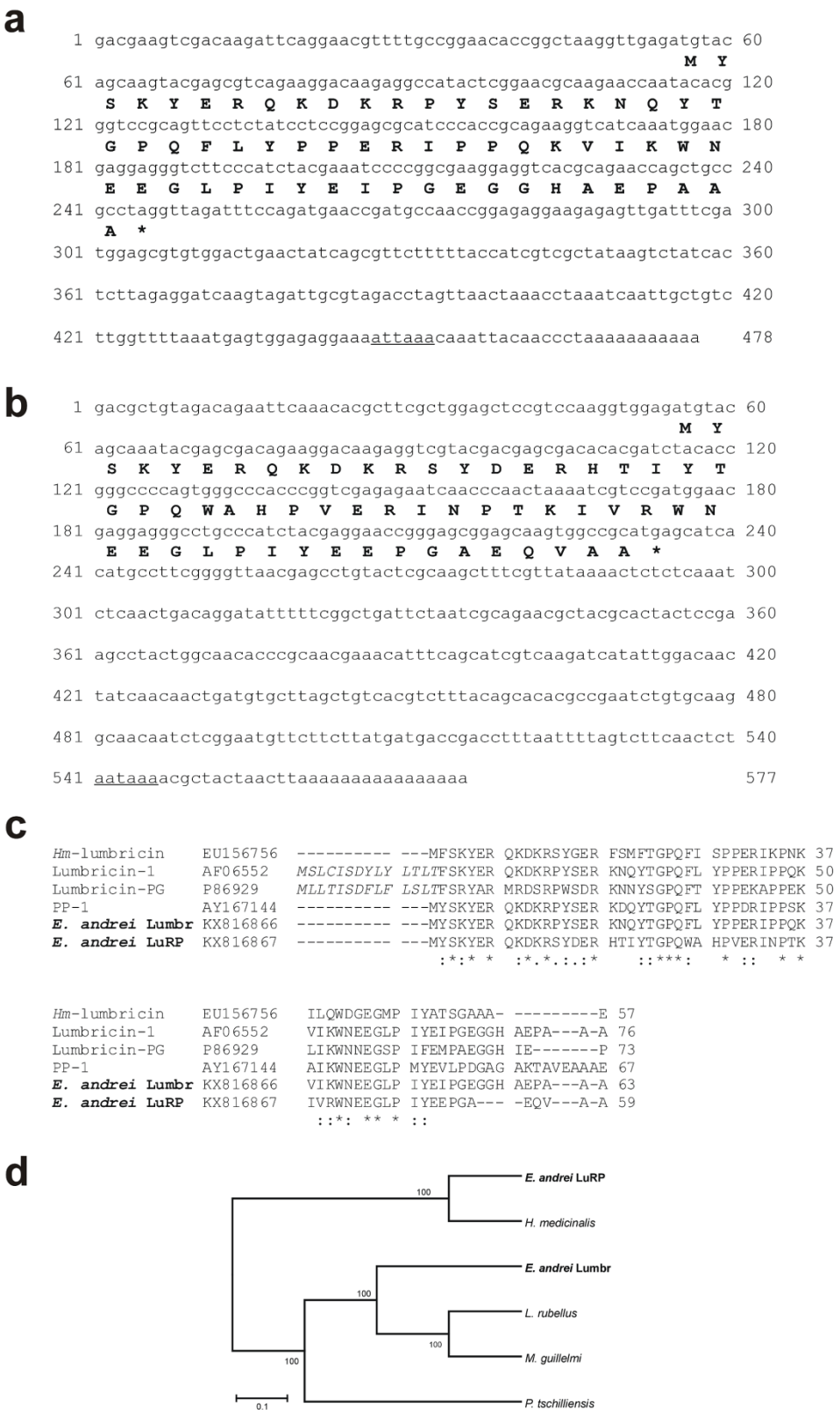

Figure 1. Nucleotide and deduced amino acid sequences of *E. andrei* lumbricin (**a**) and its related protein (*E. andrei* LuRP) (**b**). Amino acid sequences of the open reading frame are

presented under the nucleotide sequences. Stop codons are denoted with asterisks. Polyadenylation signal sequences are underlined. *E. andrei* lumbricin (**a**) is a novel antimicrobial peptide consisted of 63 amino acids and *E. andrei* LuRP (**b**) is made up of 59 amino acids. (**c**). Amino acid sequence alignment of *E. andrei* lumbricin (KX816866) and *E. andrei* LuRP (KX816867) were compared to *L. rubellus* lumbricin I (AF06552), *H. medicinalis* lumbricin (EU156756), *M. guillelmi* lumbricin-PG (P86929) and *M. tschiliensis* antimicrobial–like peptide PP-1 (AY167144). The asterisks (*) signify identical amino acid residues and dots indicate highly conserved (:) or semi-conserved (.) substitutions. Phylogenetic relationship analysis based on the deduced amino acid sequences of *E. andrei* lumbricin and LuRP with the closest annelid molecular relatives by the maximum likelihood method. The numbers closed to the branch nodes represent the percentage of 1000 bootstrap replications (**d**).

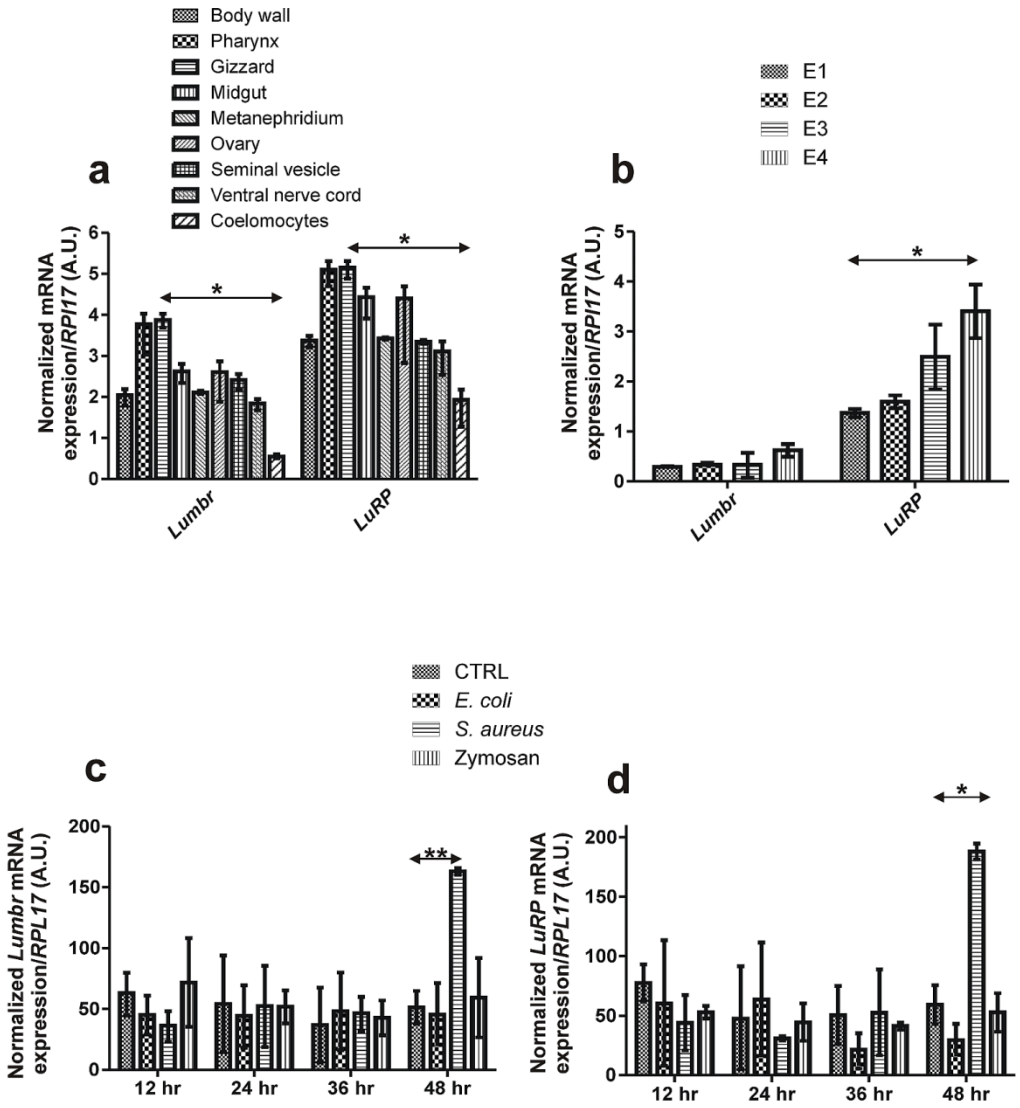

Figure 2. Comparison of *E. andrei* lumbricin (*Lumbr*) and *LuRP* mRNA expression levels from various tissues of *E. andrei* earthworms (**a**). Differential expression levels of *E. andrei Lumbr* and *LuRP* mRNA during the earthworm ontogenesis (b). Induced gene expression levels of *Lumbr* (**c**) and *LuRP* (**d**) were observed upon *in vivo* bacterial stimulation at different time points. Quantitative measurements were normalized to *E. andrei RPL17* mRNA levels. Three independent experiments were performed in duplicates. Results are demonstrated as mean and error bars represent standard error of the mean. Asterisks represent significant *p* (*< 0.05, **<0.01) values. A.U.: arbitrary units.