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1 **IDENTIFICATION OF NOVEL LUMBRICIN HOMOLOGUES IN *EISENIA ANDREI***
2 **EARTHWORMS**

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1 **ABSTRACT**

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

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

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1 1. INTRODUCTION

2

3 Antimicrobial peptides (AMPs) are structurally conserved bioactive molecules during
4 phylogenesis (Boto et al., 2018; Bulet et al., 2004; Nguyen et al., 2011; Zasloff, 2002). Until
5 now, several thousands of AMPs have been isolated from prokaryotes to mammals (Boman,
6 1995; Zasloff, 2002). They possess a broad range of antimicrobial activity with no or little
7 cytotoxicity (Kumar et al., 2018, Nguyen et al., 2011).

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

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

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

25

1 2. MATERIALS AND METHODS

2 2.1. Earthworm husbandry

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

7

8 2.2. RNA isolation, cDNA synthesis, rapid amplification of cDNA ends (RACE)

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

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

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

10

11 **2.3. Sequence and phylogenetic analysis**

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

17

18 **2.4. Relative quantification of target genes from adult tissues and embryos**

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

1 according to the manufacturer's protocol using NucleoSpin® RNA isolation kit (Macherey-
2 Nagel GmbH, Düren, Germany). The amount of total RNA was determined by NanoDrop at
3 260 nm. RNA samples were stored at – 80 °C. Reverse transcription was accomplished in 20
4 µl reactions using High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific)
5 and cDNA samples were stored at – 20 °C. Subsequently, cDNA used as a template in qPCR
6 reactions. Gene specific primers were designed with Primer Express software (Thermo Fisher
7 Scientific) to estimate the expression levels of target genes in the aforementioned tissues
8 (Table S1). Gene expression was measured by an ABI Prism 7500 instrument (Applied
9 Biosystems, Warrington, UK) applying Maxima SYBR Green/Low Rox Master Mix
10 (Thermo-Fisher, Waltham, MA, USA). The amplification profile started at 95 °C for 10 min,
11 that followed 40 cycles of 35 sec at 95 °C, 35 sec at 58 °C, and 1 min at 72 °C. Quantitative
12 measurements were normalized to *RPL17* mRNA level (Table S1). Three independent
13 experiments were implemented in duplicates.

14

15 **2.5. *In vivo* microbial challenge**

16 Adult earthworms (three animals/condition) were exposed to heat-inactivated
17 *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (OKI 112001) (each 10⁸/ml) and
18 zymosan (membrane from *Saccharomyces cerevisiae* in 1 mg/ml final concentration, Sigma-
19 Aldrich, Budapest, Hungary) on filter paper for different time points at room temperature
20 (Cang et al., 2017; Homa et al., 2005; Schikorski et al., 2008). The suspension of
21 microorganisms and zymosan were diluted in *Lumbricus* balanced Salt Solution (LBSS).
22 Control earthworms were exposed on LBSS-immersed filter paper (for composition, please
23 see Engelmann et al., 2005). After the treatments coelomocytes were harvested from the
24 coelomic cavity as we published earlier (Engelmann et al., 2005). Coelomocyte numbers were
25 evaluated by trypan-blue exclusion method and subsequently were centrifuged twice in LBSS

1 (200g, 5 min, at room temperature). Total RNA extraction, reverse transcription, and qPCR
2 experiments were executed as we described earlier. *RPL17* mRNA level was employed for the
3 normalization process. Normalized expressions of both genes are exhibited in pathogen
4 stimulated earthworms comparison with untreated ones.

5

6 **2.6. Statistical analysis**

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

12

1 **3. RESULTS AND DISCUSSION**

2 **3.1. Sequence analysis of *E. andrei* lumbricin and LuRP**

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

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

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

6 *E. andrei* lumbricin and its related peptide harbor numerous proline residues (14.3%
7 and 6.8% in molar ratio) similar to lumbricin I and other lumbricin homologues (Cho et al.,
8 1998). Typically proline-rich AMPs were isolated from the arthropods including insects and
9 crustaceans (Graf et al., 2017; Otvos, 2002). Proline amino acids uniquely alter the protein
10 conformation (e.g. folding and cyclisation); thereby it exerts an influence on the secondary
11 structure of proteins (Graf et al., 2017; Vanhoof et al., 1995). Furthermore, aromatic amino
12 acid (His, Trp, Tyr) content of these *Eisenia* lumbricin homologues is relatively high (15-16%
13 in molar ratio), which could further suggest the antimicrobial activity of these peptides
14 (Muñoz et al., 2007).

15

16 **3.2. Tissue and embryonic expression patterns of *Lumbr* and *LuRP* in *E. andrei***

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

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

11 First ontogenetic distribution pattern of lumbricin is reported from *L. rubellus*. Cho et
12 al. (1998) detected *lumbricin I* expression in adult *L. rubellus*, but not in the cocoons or
13 developing earthworms. In contrast, both *lumbricin* homologues from *E. andrei* were
14 expressed in the course of embryonic development (Fig. 2b). Their expression displayed
15 continuous increase up to the fourth developmental stage (E4) when the body is entirely
16 segmented and the organ differentiation is completed (Boros et al., 2010). *LuRP* exhibited
17 significantly higher expression compared to *Lumbr* in the different stages of developing *E.*
18 *andrei* earthworms. One explanation of the gradient increase of *Lumbr/LuRP* expression
19 could be the larger body size of the more developed embryonic stages. On the other hand, it
20 is known that numerous symbiotic bacteria colonize the earthworm embryos and their
21 frequencies boost during early embryogenesis (Zachmann and Molina, 1993; Davidson et al.,
22 2010). It is probable that *Lumbr* and *LuRP* might control the growth of commensal bacteria in
23 earthworm embryos that is known already about other invertebrate antimicrobial peptides
24 (Roiff and Schmid-Hempel, 2016).

25

1 **3.3. Induction of *Lumbr* and *LuRP* mRNA expression upon *in vivo* microbial challenge**

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

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

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

7

8 **CONCLUSIONS**

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

13

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24

1 FIGURE CAPTIONS

a

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                                                                M Y
61 agcaagtacgagcgtcagaaggacaagaggccatactcggaacgcaagaaccaatacacg 120
  S K Y E R Q K D K R P Y S E R K N Q Y T
121 ggtccgcagttcctctatcctccggagcgcacccaccgagaaggatcaaatggaac 180
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b

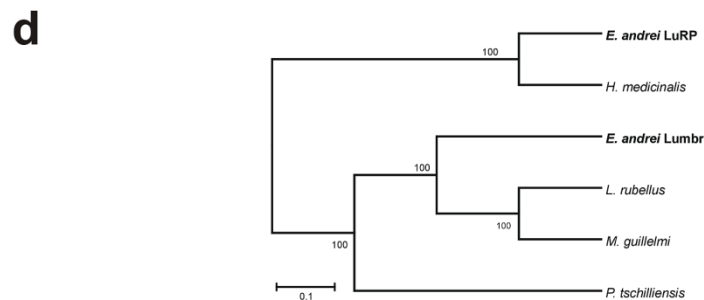
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421 tatcaacaactgatgtgcttagctgtcagctctttacagcacacgccgaatctgtgcaag 480
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541 aataaaacgctactaacttaaaaaaaaaaaaaaaaaa 577
  
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c

<i>Hm-lumbricin</i>	EU156756	-----	---MFSKYER	QKDKRSYGER	FSMFTGPQFI	SPPERIKPNK	37
<i>Lumbricin-1</i>	AF06552	<i>MSLCISDLYL</i>	<i>LTLTFSKYER</i>	QKDKRPYSER	KNQYTGPFQL	YPPERIPPQK	50
<i>Lumbricin-PG</i>	P86929	<i>MLLTISDFLF</i>	<i>LSLTFSRYAR</i>	MRDSRPWSDR	KNNYSGPQFT	YPEEKAPPEK	50
PP-1	AY167144	-----	---MYSKYER	QKDKRPYSER	KDQYTGPFQL	YPPDRIPPSK	37
<i>E. andrei Lumbr</i>	KX816866	-----	---MYSKYER	QKDKRPYSER	KNQYTGPFQL	YPPERIPPQK	37
<i>E. andrei LuRP</i>	KX816867	-----	---MYSKYER	QKDKRSYDER	HTIYTGQWA	HPVERINPTK	37
			::*: * *	:*. * . : : *	::****:	* : : * *	

<i>Hm-lumbricin</i>	EU156756	ILQWDGEGMP	IYATSGAAA-	-----	E	57
<i>Lumbricin-1</i>	AF06552	VIKWNEEGLP	IYEIPGEGGH	AEPA---	A-A	76
<i>Lumbricin-PG</i>	P86929	LIKWNNEGSP	IFEMPAEGGH	IE-----	P	73
PP-1	AY167144	AIKWNEEGLP	MYEVLDPDAG	AKTAVEAAAE		67
<i>E. andrei Lumbr</i>	KX816866	VIKWNEEGLP	IYEIPGEGGH	AEPA---	A-A	63
<i>E. andrei LuRP</i>	KX816867	IVRWNEEGLP	IYEEPGA---	-EQV---	A-A	59
			::*: * * * :			



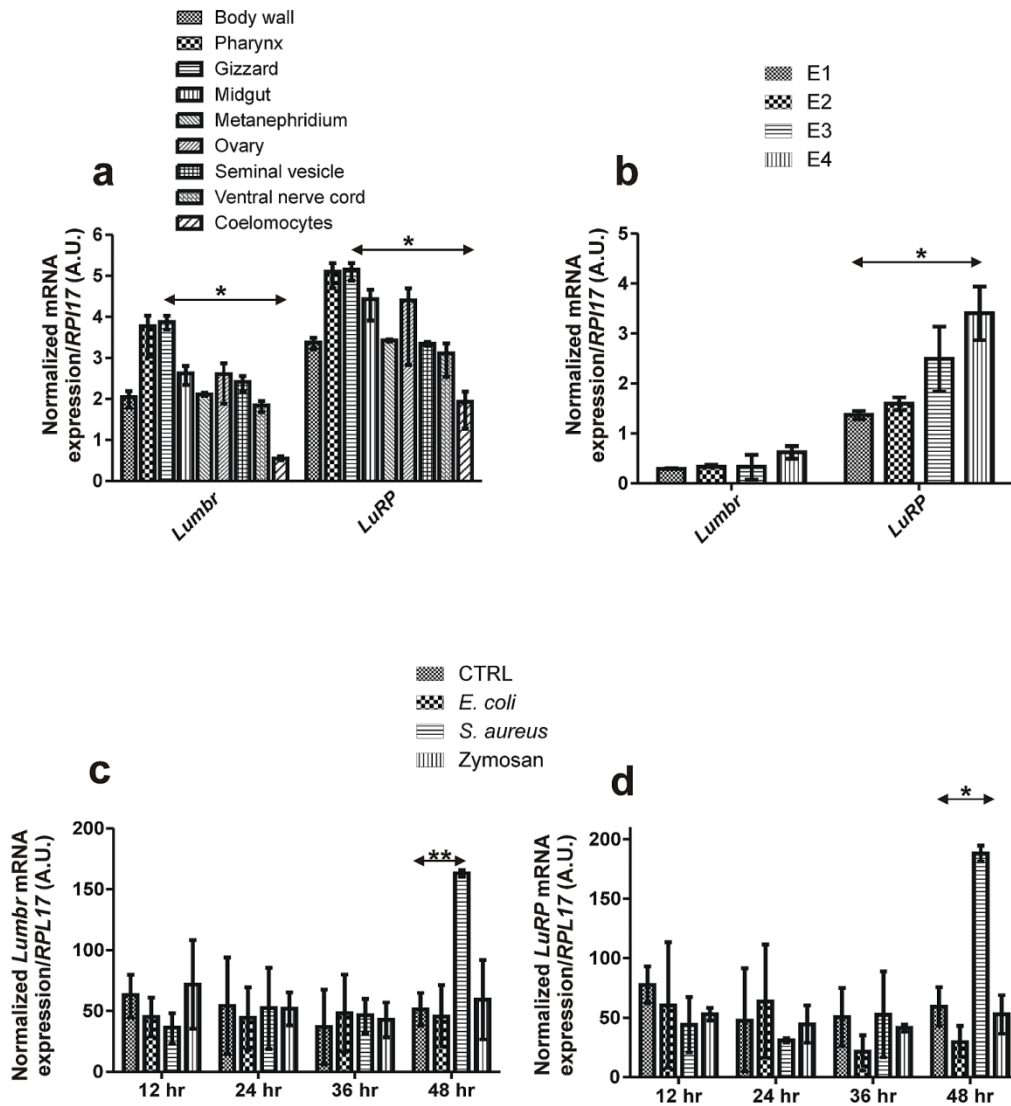
2
3 **Figure 1.** Nucleotide and deduced amino acid sequences of *E. andrei* lumbricin (a) and its
4 related protein (*E. andrei* LuRP) (b). Amino acid sequences of the open reading frame are

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

13

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15



1
2 **Figure 2.** Comparison of *E. andrei* lumbricin (*Lumbr*) and *LuRP* mRNA expression levels
3 from various tissues of *E. andrei* earthworms (a). Differential expression levels of *E. andrei*
4 *Lumbr* and *LuRP* mRNA during the earthworm ontogenesis (b). Induced gene expression
5 levels of *Lumbr* (c) and *LuRP* (d) were observed upon *in vivo* bacterial stimulation at different
6 time points. Quantitative measurements were normalized to *E. andrei* *RPL17* mRNA levels.
7 Three independent experiments were performed in duplicates. Results are demonstrated as
8 mean and error bars represent standard error of the mean. Asterisks represent significant p ($* <$
9 0.05, $** < 0.01$) values. A.U.: arbitrary units.