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SHORT COMMUNICATION

Gene expression during postembryonic segmentation in the centipede *Lithobius peregrinus* (Chilopoda, Lithobiomorpha)

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Abstract Postembryonic segmentation (anamorphosis) is widespread among arthropods, but only partially known as for its developmental mechanics and control. Studies on developmental genetics of segmentation in anamorphic arthropods are mostly limited to the germ band stage, during early phases of embryonic development. This work presents the first data on the postembryonic expression of a segmentation gene in a myriapod. Using real-time PCR, we analyzed *engrailed* expression patterns during the anamorphic stages of the centipede *Lithobius peregrinus*. A variation pattern in *en* RNA level during anamorphosis suggests that gene expression is precisely modulated during this period of development and that *engrailed* is mainly expressed in the posterior part of the body, in the newly differentiating segments of each stage. As anamorphosis is possibly the primitive segmentation mode in arthropods, the postembryonic *en* expression pattern documented here provides evidence for a conservation of *en* role in ontogeny, across the embryonic/postembryonic boundary, as well as in phylogeny, across the same boundary, but in the opposite direction, from primitive postembryonic expression to the more derived expression in clades with exclusively embryonic segmentation.

Keywords *Engrailed* · Segment polarity genes · Real-time PCR · Anamorphosis

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Introduction

In most insects and chelicerates, and in some myriapods, segmentation is completed before hatching, but in many other arthropod taxa, among which millipedes and lithobiomorph centipedes, only a fraction of the trunk segments is completed during embryogenesis. In these species, the final adult number of segments (when fixed) is reached later in ontogeny, as segments emerge sequentially from anterior to posterior, through a series of molts (Fusco 2005). This postembryonic developmental mode is called *anamorphosis*.

Several lines of evidence suggest that anamorphosis is the primitive segmentation mode in arthropods (Hughes et al. 2006); however, very little is known about this important developmental process beyond the level of descriptive morphology. Postembryonic segmentation is traditionally considered to be the product of the proliferative activity of an ill-defined "subterminal generative zone," but basic information about morphogenesis and gene expression associated with anamorphosis is lacking. Studies on the developmental genetics of segmentation in anamorphic arthropods are mostly limited to the germ band stage, during early phases of embryonic development. This is in part due to difficulties in applying in situ and immunohistochemical protocols to postembryonic stages, owing to the mechanical and chemical interference of the thick cuticle.

The embryonic expression of several *Drosophila* segmentation genes homologues has been studied in other insects, as well as in some chelicerates, myriapods, and crustaceans (see Damen 2007 and references therein). Available data suggest that the more downstream levels of the segmentation gene network are more conserved than the upstream levels (Damen 2007). This reflects a marked disparity in earlier segmentation processes, also visible in

morphogenesis, as exemplified by the difference between short, intermediate, and long germ band developmental modes.

Among the late expressed genes in the segmentation cascade, the segment polarity gene *engrailed* (*en*) is one whose role is among the most conserved. We hypothesize that this gene may have a role in segmentation also during the postembryonic development of the lithobiomorph centipedes. As for all other myriapods investigated so far, lithobiomorph data on segmentation gene expression are limited to the embryonic phase of development. These include two segment polarity genes (*en* and *wingless*; Hughes and Kaufman 2002), a pair-rule gene (*even-skipped*; Hughes and Kaufman 2002), and a few later expressed genes involved in segmental neurogenesis (*ASH, Delta* and *Notch*; Kadner and Stollewerk 2004).

This work presents the first data on the postembryonic expression of a segmentation gene in a myriapod. Using real-time PCR (qPCR), we analyzed the *engrailed* expression pattern during the anamorphic stages of *Lithobius peregrinus*. Like all lithobiomorphs, the post-embryonic development of *L. peregrinus* is hemianamorphic: the juvenile hatches with an incomplete number of trunk segments, to reach the final segmental arrangement, with 15 leg-bearing segments, following a precise schedule of segment addition across five stages (anamorphic phase of postembryonic development). Subsequently, the animal continues to grow and molt without any further variation in its segmental organization (epimorphic phase of postembryonic development).

Materials and methods

Experimental animals

Adult centipedes were collected during the years 2008–2009 in San Stino di Livenza (northeastern Italy) and housed in plastic boxes (35×23×20 cm) with a hardened poured plaster of Paris floor to maintain an adequate level of humidity and pieces of wood barks to let the animals hide underneath. The boxes were sprayed with water every few days, and living crickets were provided weekly as food.

Eggs were collected periodically by rinsing out the boxes and woods with water and catching the eggs in a sieve. The eggs were kept in Petri dishes with a humid plaster floor, to hatch in 15–20 days since deposition. Since hatching, the individual specimens were bred separately in Petri dishes with the same humid medium on the floor. They were checked daily for molting and were fed with living fruit flies. All stages were kept at 21±1°C under natural photoperiod.

For qPCR experiments, specimens of seven postembryonic stages were sacrificed the first day after the molt to the selected stage: juveniles from the first (L0) to the fifth (L4) stage (often referred to as "larval"), juveniles of the first postanamorphic stage (often referred to as "postlarval," PL1) and adults of different ages.

RNA extraction and cDNA synthesis

For each stage, a different number of specimens were processed together to obtain 1 μg of total RNA (from 36 L0, to two adults). All stages were treated the same, and 60 embryos were used to validate the protocols. Individuals were killed at the selected stage by freezing in N_2 and stored at −80°C, before being transferred to a ceramic mortar and ground to powder in liquid nitrogen with other individuals of the same stage. Total RNA was isolated using the SV Total RNA Isolation kit (Promega, Madison, WI), according to the manufacturer's instructions. To remove contaminating DNA, the isolated RNA was treated with RNase-free DNase I (Promega) at 25°C for 15 min, and a reaction was then stopped by adding DNase Stop Solution. Both reagents were included in the SV Total RNA Isolation kit (Promega). The concentration and purity of the RNA were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, USA). For all RNA samples, A260/A280 and A260/A230 ratios were in the range of 2.0–2.1 and 1.9–2.0, respectively.

To synthesize the first-strand cDNA, a mixture of total RNA (1 μg) and random primers (0.5 μg/reaction) in a volume of 10 μl were heated at 70°C for 5 min and then chilled on ice for 5 min. To this mixture, ImProm-II™ 1× reaction buffer, $MgCl_2$ solution (2.25 mM), dNTP mix (0.5 mM each dNTP), RNasin Ribonuclease Inhibitor solution (10 U/μl) and ImProm-II Reverse Transcriptase (1 μl/reaction) were added, to give a total volume of 10 μl. This mixture was equilibrated at 25°C for 5 min, extended at 42°C for 60 min, and the enzyme was then inactivated at 70°C for 15 min. To minimize the sample treatment disparity, all RNA samples were reverse-transcribed simultaneously.

Primer design, gene isolation and sequence analysis

Specific primers for reference and target genes were designed to conserved regions on the basis of comparison of sequences from several arthropods, obtained from the GenBank database (see Electronic Supplementary Material for the sequences of all primers used).

Since no information is available about the temporal expression for the genes of interest, differently aged individuals were pooled for cDNA amplification. PCR cycling was performed using a standard protocol. The

products were cloned into the pGEM®-T Easy Vector (Promega) and sequenced (BMR Genomics). In order to avoid cloning or PCR artifacts, several clones were screened for each fragment obtained.

Sequence similarity search was performed using the program "Blast" (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was carried out with the program "ClustalW" (http://www.ebi.ac.uk/Tools/clustalw/index.html) and was edited in the GeneDoc software version 2.7.000 (www.psc.edu/biomed/genedoc).

Real-time PCR assays

qPCR was performed on a Rotor Gene 3000 (Corbett Research, Sidney, Australia) using the fluorescent marker SYBR Green (Applied Biosystems, Foster City, USA) to generate semiquantitative data. PCR premixes containing all reagents except for target cDNAs were prepared and aliquoted by a Robotic Liquid Handling System (CAS-1200, Corbett Robotics) into PCR tubes (Corbett Research).

The PCR reaction was carried out following the program: 1 cycle at 95°C for 10 min and 40 cycles consisting of 10 s at 95°C, 15 s at 59°C, and 30 s at 72°C. This was followed by the measurement of fluorescence during a melting curve in which the temperature raised from 72 to 95°C in sequential steps of 1°C for 45 s. This ensured the detection of one gene-specific peak and the absence of primer dimer peaks (see Electronic Supplementary Material for a melting curve example).

A fluorescence threshold was manually set to 0.05 on the fluorescence log scale, to determine the cycle threshold number (*Ct*) at which the fluorescence passed the detection threshold. For each cDNA sample, relative expression levels of target gene were normalized by two reference genes: *actin* (*ACT*) and *elongation factor 1-α* (*EF1-α*). The amplification efficiency ($E = 10^{-1/\text{slope}_{\text{CPvs[cDNA]}}}$, Pfaffl 2001) for each sample was calculated on the basis of the results of three amplification reactions, each with a different quantity of the template (6, 18, and 54 ng of total reverse-transcribed RNA). Each reaction was performed in duplicate, and only amplifications with $1.9<E<2.1$ were considered acceptable. The amplification efficiency, obtained for each sample, was used to estimate the relative level of expression (R) between stage L0, that we chose as the "calibrator," and the stage of interest ("sample"):

$$R = \frac{E_{en}^{\Delta C_{en}}}{E_{\text{ref}}^{\Delta C_{\text{ref}}}}$$

where E_{en} is the amplification efficiency of *engrailed* mRNA, E_{ref} is the amplification efficiency of reference mRNA, $\Delta C_{en}=Ct_c-Ct_s$ is the difference between calibrator and sample cycle thresholds for *en* mRNA, and $\Delta C_{\text{ref}}=Ct_c-Ct_s$ is the difference between calibrator and sample cycle thresholds for reference mRNA. qPCR assays, using the same reference gene, were replicated three to four times for each postembryonic stage.

Models

Two alternative simple models for *engrailed* expression pattern during anamorphosis, which differ in the number of *en*-expressing segments at each stage, have been comparatively evaluated. Model A assumes that, at each stage, *en* is expressed in the new forming segments at the rear of the trunk as well as in the already formed trunk segments. Model B assumes that *en* expression is localized in the new forming segments differentiating at each anamorphic stage.

Each model provides for three variants that differ in the growth pattern of the tissues that express *engrailed* within each *en*-expressing segment. The gene could be expressed in domains (e.g., a transversal row of cells) that grow across stages along a single direction (i.e., with linear size, variant 1), in domains (e.g., an epithelium) that grow as a surface (i.e., with the square of linear size, variant 2), or in domains that grow as a volume (i.e., with the cube of linear size, variant 3).

The two models, with their three variants, are structurally identical, do not have adjustment parameters and are based on the same approximations of gene expression and body growth patterns. These assume a uniform level of gene expression among the *en*-expressing tissues and constant segment growth rates across anamorphosis. However, violations of these assumptions do not substantially alter the relative fit of the two models to observed data, which in essence depends on the number of *en*-expressing segments (see below). Fixed parameters of the two models are based on standard descriptions of *Lithobius* anamorphosis (e.g., Andersson 1976) and per-molt growth rates previously estimated on samples of *L. peregrinus* bred in our laboratory under the same conditions of the animals used in this study. These estimates are very close to the corresponding values recorded in closely related species (e.g., *L. forficatus*, Andersson 1976).

For each stage (x), the expected relative level of gene expression (R) is calculated as the ratio between the volume of the *en*-expressing tissues (V_E) and the volume of the whole body (V_B) with respect to the same ratio at the reference stage L0 ($x=0$, $R(0)=1$), that is

$$R(x) = \frac{V_E(x)}{V_B(x)}$$

Expressing volumes as relative values with respect to volumes at stage L0, V_B is directly computed from

observed per-molt average volume growth rate for the whole body (r_B=1.46)

$$V_B(x) = r_B^x$$

while, V_E is computed as

$$V_E(x) = \frac{N_E(x)}{N_E(0)} \cdot \left(r_S^{\frac{D}{3}}\right)^x$$

where the first term of the right-hand side product is the number of trunk segments expressing the gene at stage x (numerator) with respect to the same number at stage L0 (denominator), and the second term is the relative portion of tissues expressing the gene for each *en*-expressing segment with respect the same measure in L0. The fixed parameter r_S (=1.31) is the observed per-molt volume growth rate for an average segment (this accounts for trunk growth to the exclusion of trunk elongation due to anamorphic segment addition). D is the number of spatial dimensions of growth for the tissues expressing the gene in each segment, thus the term D/3 corrects for the allometric growth of tissues developing along a limited number of spatial dimensions (1 or 2).

The two models differ exclusively for the N_E values. In model A, $N_E(x)=N_T(x)$, where $N_T(x)$ is total number of leg-bearing segments at stage x, while in model B, $N_E(x)=N_F(x)$, where $N_F(x)$ is the number of new trunk segments in formation during stage x (Table 1). The three variants of each model differ in the D value (1, 2, or 3).

Results and discussion

Engrailed, actin, and elongation factor 1-α sequences

The *L. peregrinus* orthologue of *en* was cloned from a juvenile cDNA using standard methods. We recovered two different fragments with a high sequence similarity to the *en* gene of other arthropods: *Lp_enS* (180 bp; GenBank accession number FR750353) and *Lp_enL* (228 bp; GenBank accession number FR750354). The two sequences differ in a short intervening sequence lacking stop codons, between the EH2 and EH3 domains. This linker, also identified in the *Lithobius atkinsoni en* homologue (Hughes and Kaufman 2002), bears a similarity to the sequence of one of the *engrailed* paralogues of the spider *Cupiennius salei* (Damen 2002; Fig. 1). Further analyses are required to establish whether the two *Lp_en* sequences represented splicing variants or paralogue genes, and to investigate their possible different function. Different *en* paralogues, with distinct functions, have been reported for chelicerates (Damen 2002) and crustaceans (Gibert et al. 2000).

In addition, cDNA partial sequences for *actin* (*LpACT*; GenBank accession number FR750355) and *elongation factor 1-α* (*LpEF1-α*; GenBank accession number FR714838) were isolated as well.

Table 1 Synoptic table of *Lithobius* anamorphosis outlined in terms of descriptive morphology (Andersson 1976). N_C, number of completed leg-bearing segments (number of functional limb pairs); N_F, number of newly forming leg-bearing segments (number of limb bud pairs); N_T, total number of leg-bearing segments (N_C+N_F)

stage	N_C	N_F	N_T
L0	7	1	8
L1	7	3	10
L2	8	2	10
L3	10	2	12
L4	12	3	15
PL1	15	0	15

Engrailed expression pattern

Using qPCR, it was impossible to discriminate between the different isoforms of *Lp_en*, so the analyzed expression data represent the sum of the expression level for all isoforms of the gene.

Embryos express high levels of *en*, in conformity with what is reported by Hughes and Kaufman (2002) for *L. atkinsoni*. While useful for validating our qPCR protocol, this group of specimens was nevertheless excluded from subsequent analyses because the procedure does not allow to refer expression data to a precise phase of the embryonic segmentation.

The effects of the reference gene and the developmental stage on *en* relative expression values were tested with a two-way ANOVA on all the experimental groups to the exclusion of L0 (chosen as "calibrator" of *en* expression, value set to 1). Expression levels in the six stages differ significantly (n=40, p<0.0001), whereas there is no significant difference between *EF1-α* and *ACT* data normalization (n=40, p>0.26). This suggests that both genes are appropriate as reference genes for quantitative expression studies in this species. Accordingly, the data obtained using either reference gene were lumped together in subsequent analyses.

The *engrailed* expression pattern through postembryonic development shows a peak at stage L1 to decrease toward a minimum value in the adult stages (Fig. 2). The significant variation in *en* RNA level during anamorphosis suggests that gene expression is precisely modulated during this period of development. However, adult *en* expression, although significantly lower than all other stages to the exclusion of L4 (Fischer's test LSD, α=0.05), differs significantly from 0 (t test, n=6, p<0.0011). As the segmental pattern is completed before adulthood, adult

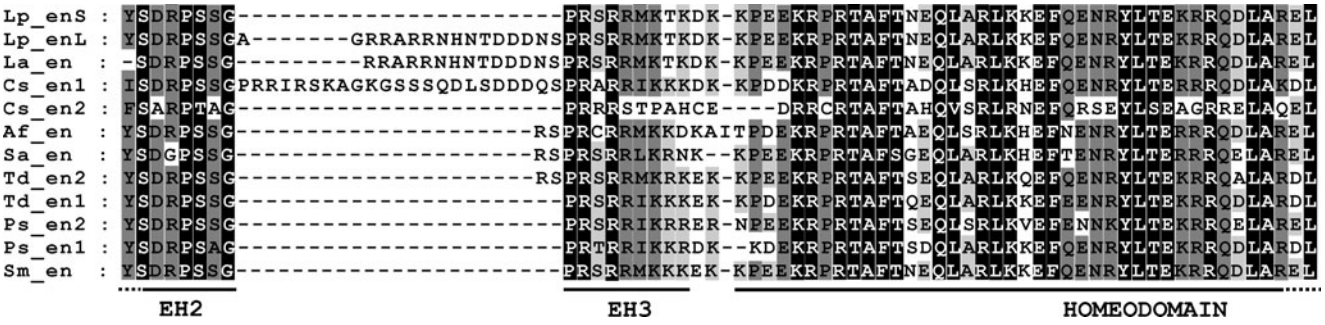

Fig. 1 Sequence alignments of arthropod orthologues of *engrailed*. All sequences except those of *L. peregrinus* (Lp_en) were acquired from Genbank with the following accession number: *Lithobius atkinsoni* La_en (AF434998), *Strigamia maritima* Sm_en (AAL13136), *Artemia franciscana* Af_en (CAA50279), *Schistocerca americana* Sa_en (AAA29807), *Thermobia domestica* Td_en1 (AF104006), *Thermobia domestica* Td_en2 (AF104007), *Porcellio scaber* Ps_en1 (AF254262), *Porcellio scaber* Ps_en2 (AF254262), *Cupiennius salei* Cs_en1 (CAA07503), *Cupiennius salei* Cs_en2 (CAC87039). Localization of domains EH2, EH3, and the homeodomain is shown. Orthologous sequences from arthropod species were aligned with fragments from *L. peregrinus* using the ClustalW program and amino acids are shaded according to the degree of conservation using GeneDoc: *black* (100% similarity), *gray* (80–90% similarity), *light gray* (60–70% similarity)

engrailed expression indicates that the gene products are likely involved in other functions, besides those strictly related to the process of segmentation. Thus, in order to get better estimates of the *engrailed* expression specifically associated to segmentation, we rescaled the observed expression values in each pre-adult stage by subtracting a quantity equal to the average transcript level in adults, assuming a uniform level of *en* expression not related to segmentation across stages. A one-way ANOVA on this transformed dataset confirms the significance of the stage as a factor affecting *en* expression levels in L1-PL1 ($n=34$, $p<0.0001$).

The transformed dataset was used for comparing the two models (A, B) of *en* expression pattern (Fig. 3). Models were matched to the observed data by calculating the residuals sum of squares (RSS) between observed and expected values ($n=34$).

Model B (*en* expression restricted to the terminal trunk region) shows RSS values consistently lower than those of the alternative model, with the variant that assumes the *en*-expressing tissues growing with the square of linear dimensions performing slightly better than the other two. This result is robust with respect to substantial variations in growth parameters, the difference in fitting between the two models depending essentially on the number of *en*-expressing segments.

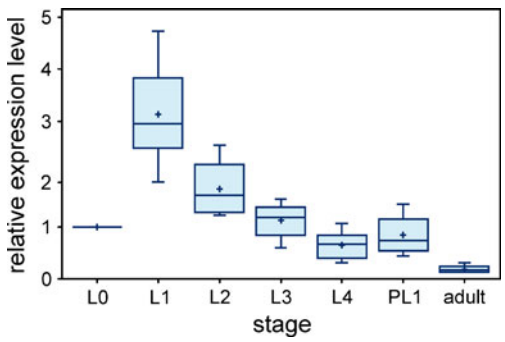

Fig. 2 Relative levels of *engrailed* expression during postembryonic development in *L. peregrinus*. Data are normalized with respect to the expression level of two reference genes using L0 as calibrator stage (value set to 1). *Boxes* represent the interval between lower and upper quartiles, with median (transverse line) and mean (small cross); *vertical lines* are ranges of variation

In model A, the number of *en*-expressing segments is estimated as the number of leg-bearing segments (Table 1). However, adding a constant value to these figures, to include, for instance, legless trunk segments (+1 for the massilliped segment and possibly +2 for the segments of the genital region, see Fusco 2005) and/or the head segments (+6, see Hughes and Kaufman 2002), has the sole effect of making the pattern smoother across the stages (results not shown). Different implementations of model A cannot significantly improve its fit, as, according to this model, the mass of *en*-expressing tissues is expected to increase roughly in proportion to body size.

Model B has a better fit to the observed data, although with evident limits. A partial fit was not unexpected, as the chosen estimator for the number of *en*-expressing domains, the number of limb bud pairs (Table 1), cannot be an accurate indicator alone of the state of the ongoing process of segmentation. As a matter of fact, different segmentation processes (in different structures) have different ontogenetic schedules, and some of these are not even in pace with the molt cycle (Minelli et al. 2006). For instance, the central nervous system segmentation is completed at stage L3 (Scheffel 1969), while the respiratory system segmentation is not completed before stage PL2 (Kaufman 1961). Late-terminating segmentation processes can explain the residual observed expression of *en* after the traditionally recognized conclusion of anamorphosis (PL1), which is simply based on the count of leg pairs. More complex implementations of model B can significantly improve its fit, for instance

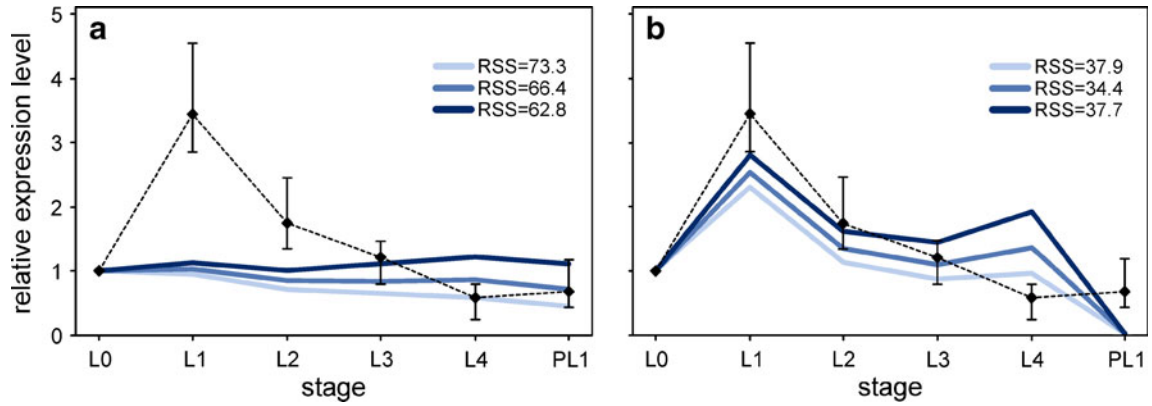

Fig. 3 Observed median levels of expression for *engrailed* during anamorphosis in *L. peregrinus* (*diamonds* and *dotted line*), rescaled by subtracting the average adult level of *en* expression and expected expression levels under two simple models (with three variants each, *thick lines*). **a** *En* expressed in all trunk segments. **b** *En* expressed only in the most posterior segment in formation at each stage. *Vertical lines* are interquartile ranges; *light blue line*, *en*-expressing tissues growing with liner size (variant 1); *medium blue line*, *en*-expressing tissues growing with the square of liner size (variant 2); *dark blue line*: *en*-expressing tissues growing with the cube of liner size (variant 3). *RSS*, residual sum of squares

adopting a less approximate body growth function (there is little growth between the first two stages), or using a combination of different morphological markers to quantify the *en*-expressing domains (results not shown). However, we refrained from an arbitrary work of ad hoc parameter adjustment, preferring to compare the two hypotheses in their most simple form.

Comparative remarks

In *L. atkinsoni* embryo, a three-to-four cell-wide stripe of tissue expresses *en* at the posterior boundary of each segment. These stripes appear sequentially in the embryo, from anterior to posterior, reflecting the short germ band mode of development of this arthropod. The expression stripe width does not vary across germ band stages, and at the time of the appearance of the seventh leg-bearing segment (the most posterior fully formed leg-bearing segment of the future hatching L0 juvenile), all the *en* stripes of the head and trunk are still visible in in situ embryo preparations (Hughes and Kaufman 2002).

qPCR data on postembryonic segmentation in *L. peregrinus* suggest a different expression pattern during anamorphosis. *Engrailed* appears to be mainly expressed in the posterior part of the body, in the newly differentiating segments of each stage, in tissue domains within each segment which grow most probably as expanding epithelia, rather than as stripes of cells of invariant width. This dissimilarity with respect to embryonic expression was somehow anticipated, in consideration of the full three-dimensional architecture of the body of the juvenile, compared to the almost bidimensional constitution of the embryo at the germ band stage, and the fact that, with respect to germ band embryo, the already individualized segments of anamorphic stages are morphogenetically far more advanced than the segments in formation.

However, besides the different modes of *en* expression in the two ontogenetic phases, our data are perfectly compatible with the retention of a segment polarity role for *en* in postembryonic segmentation.

The high level of conservation of the *en* expression domain across arthropods, usually marking the posterior margin of prospective sclerites, likely reflects the gene's fundamental role in segmental patterning (Damen 2007). In fact, a different domain of expression has been recorded on the dorsal embryonic tissues of the pill millipede *Glomeris marginata* (Janssen et al. 2004), possibly in relation to the dorsoventral mismatch in segmental patterning typical of the Diplopoda. However, these differences in the precise localization of *en* expression within the segmental boundaries marked by sclerite anteroposterior extension have not cast doubts about the conservation of its segment-polarity role (Janssen et al. 2008).

The postembryonic *en* expression pattern documented here provides complementary evidence on the key role of *en* in segmentation. This shows a conservation of *en* role in ontogeny, across the embryonic/postembryonic boundary, but also in phylogeny, across the same boundary, but in the opposite direction, from a primitive postembryonic expression to the derived embryonic expression in epimorphic arthropods. In fact, the scattered phylogenetic distribution of epimorphosis (the developmental mode that sees the final segmental arrangement attained during embryogenesis) among arthropods (Fusco 2005) and paleontological data on ontogenetic fossil series (e.g., trilobites) both suggest that an ontogeny with most of the segments formed

during postembryonic development could be the ancestral mode of development for the Arthropoda (Hughes et al. 2006).

As in the transition from sequential to simultaneous segmentation in insect embryonic development, recorded in several insect lineages (Davis and Patel 2002), in the transition from postembryonic to embryonic (germ band) segmentation, the conservation of the role of relatively downstream segmentation genes, like *engrailed*, seems to play an important part.

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Gene expression during postembryonic segmentation in the centipede *Lithobius peregrinus* (Chilopoda, Lithobiomorpha)

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Development Genes and Evolution

Electronic supplementary material

- **primer sequences**
- **melting curve of real-time PCR**

primer sequences

The *engrailed* (***Lp_en***) primer pair was designed on the sequence of *Lithobius atkinsoni* (GenBank accession number AF434998).

Actin (***LpACT***) was isolated using a pair of primers based on the sequences of other arthropod species: *Plutella xylostella* (AB282645), *Manduca sexta* (L13764), *Ornithodoros moubata* (AY547732), *Homarus americanus* (AF399872), *Ceratitis capitata* (M76614). Specific primers (ActLp, used in qPCR assays) were designed on the amplicon sequence obtained.

For *elongation factor 1-α* (***LpEF1-α***) a primer pair designed on the sequence alignment of some Litobiomorpha species was used (*Lithobius forficatus*, AF240799; *Pokabius bilabiatus*, AF240804; *Bothropolys multidentatus* AF240789; *Australobius scabrior*, AY310166).

Gene	**Forward primer sequence 5'-3'**		**Reverse primer sequence 5'-3'**		**Length (bp)**
Lp_en	**EngFor4**	TATTCCGACCGGCCATCGT	**EngRev4**	CAATTCCCGAGCCAGATCCT	222
LpEF1-α	**ElFacFor**	GCTGGAATCTAGCCCCAAC	**ElFacRev**	CAATGTGAGCAGTGTGGCA	504
LpACT	**ActFor**	AACTGGGATGACATGGAGAAG	**ActRev**	GGGTACATGGTGGTACC	689
	ActLpFor	TACAATGAGCTGCGTGTCG	**ActLpRev**	ATGGAGTTGAAGGTGGTCT	575

melting curve of real-time PCR

Standard Melting Curve as generated by Rotor Gene 3000 (Corbett Research, Sidney, Australia). The software plots the negative first derivative of fluorescence over temperature (-dF/dT) versus the temperature.

Each peak is formed by the dissociation of a single PCR product: blue, *elongation factor 1-α*; red, *actin*; green, *engrailed*. There is no evidence of non-specific amplification or primer-dimerisation. The graph, from a single assay, is representative of all the experiments.