

The expression of *wingless* and *Engrailed* in developing embryos of the mayfly *Ephoron leukon* (Ephemeroptera: Polymitarcyidae)

Brigid C. O'Donnell · Elizabeth L. Jockusch

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Abstract The expression of the segment polarity genes *wingless* (*wg*) and *engrailed* (*en*) is highly conserved across arthropods, and these genes play a crucial role in patterning of the segmental body plan. Investigations of the expression and function of *wg* and *en* have focused primarily upon holometabolous insects, with the notable exception of recent detailed work in *Oncopeltus* (Hemiptera), *Schistocerca*, and *Gryllus* (Orthoptera). An increase in the phylogenetic breadth of our understanding of molecular patterning is crucial to ascertain the extent of conservation and divergence in molecular patterning mechanisms during insect embryogenesis. We examined the expression of *wg* mRNA transcripts and localization of En protein during embryogenesis in the mayfly *Ephoron leukon* (Ephemeroptera: Polymitarcyidae). These data represent one of the first embryonic gene expression pattern data for a mayfly, a lineage that may be the sister group to all other winged insects. Many aspects of *wg* and En expression are highly conserved, notably their expression in juxtaposed stripes in each parasegment, as well as expression domains in the

procephalon, mouthparts, thoracic limbs, and nervous system. Future work in mayflies can be used to determine if conservation extends to other components of the segmentation hierarchy.

Keywords *Ephoron leukon* · *Wingless* · *Engrailed* · Mayfly · Embryogenesis · Segment polarity genes

Introduction

The role of the segment polarity genes *wingless* (*wg*) and *engrailed* (*en*) in insect development has been studied intensively, and accumulating evidence suggests that parasegments demarcated by *wg* and *en* expression were present in the most recent common ancestor of all arthropods (Damen 2002; reviewed recently by Damen 2007; Hughes and Kaufman 2002a) as well as in the sister group to arthropods, the onychophorans (Eriksson et al. 2009). In *Drosophila*, *wg* and *en* are expressed in adjacent stripes that delineate the posterior and anterior regions of each parasegment along the main body axis (Baker 1987, 1988; Fjose et al. 1985; Kornberg et al. 1985). *wg* (or *wnt-1*) encodes a secreted glycoprotein that functions as a signaling factor and is part of the large family of *Wnt* genes (Nusse and Varmus 1992), with nine paralogues recently isolated from *Tribolium* (Bolognesi et al. 2008b). *en* encodes a homeobox-containing transcription factor (Desplan et al. 1985) that activates the diffusible signaling protein encoded by *hedgehog* (Zecca et al. 1995) which maintains the expression of *wg* in neighboring posterior cells (Ingham and Hidalgo 1993). Similarly, *wg* maintains expression of *en* in the adjacent anterior cells of most parasegments (Klingensmith and Nusse 1994; Nusse and Varmus 1992). *Drosophila* mutant for *wg* or *en* produce

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B. C. O'Donnell · E. L. Jockusch
Department of Ecology and Evolutionary Biology,
University of Connecticut,
U-3043, 75 North Eagleville Road,
Storrs, CT 06269, USA

E. L. Jockusch
e-mail: elizabeth.jockusch@uconn.edu

Present Address:

B. C. O'Donnell (✉)
Department of Biological Sciences, Plymouth State University,
Boyd Science Center, Room 208, 17 High Street,
Plymouth, NH 03264, USA
e-mail: bcodonnell@plymouth.edu

embryonic phenotypes with deleted regions of segments (Nüsslein-Volhard and Wieschaus 1980).

Our understanding of body plan formation, segmentation, and appendage patterning in insects is based primarily on data from *Drosophila melanogaster* and *Tribolium castaneum*, which are holometabolous insects (those developing with a pupal stage). For insects outside of Holometabola, *wg* and/or *en* expression has been documented in *Thermobia* (Thysanura), *Periplaneta* (Blattodea), *Tenodera* (Mantodea), *Gryllus*, *Acheta*, *Schistocerca* (Orthoptera), and *Oncopeltus* (Hemiptera; Angelini and Kaufman 2005a; Dearden and Akam 2001; Giorgianni and Patel 2004; Jockusch and Ober 2004; Mahfooz et al. 2004; Miyawaki et al. 2004; Patel et al. 1989; Peterson et al. 1998; Rogers and Kaufman 1996). In *Tribolium*, ectopic expression of *wg* leads to increased *en* expression (Oppenheimer et al. 1999), and knockdown of *wg* leads to loss of most *En* expression (Bolognesi et al. 2008a; Ober and Jockusch 2006). *Tribolium* and *Oncopeltus* embryos in which *wg* expression has been knocked down have malformed segment boundaries (Angelini and Kaufman 2005a; Bolognesi et al. 2008a; Ober and Jockusch 2006). Although segment defects were not observed in *Gryllus* embryos in which *wg* was knocked down, gene knockdowns targeting components of the Wnt signaling cascade or multiple Wnt paralogues led to severely truncated embryos lacking posterior segments in *Gryllus* (Miyawaki et al. 2004), *Tribolium* (Bolognesi et al. 2008b), and *Oncopeltus* (Angelini and Kaufman 2005a). Posterior segments were also deleted in *Oncopeltus* with reduced expression of *en* (Angelini and Kaufman 2005a).

Currently available data suggest that the expression and function of segment polarity genes are relatively conserved across the pool of insect species studied to date (see above references), yet this conclusion is based upon data drawn from a limited sample of the total number of lineages circumscribed by Insecta (only about a third of the thirty orders of insects have been sampled). An expanded and inclusive set of focal organisms used in studies of evolutionary developmental biology is necessary to make well-supported inferences about ancestral states and the sequence of evolutionary transformations (see similar themes explored in Jenner and Wills 2007 and Travis 2006). Mayflies (Ephemeroptera) are an evolutionarily important lineage for comparative studies of molecular development as they constitute the presumed sister group (perhaps along with Odonata, the dragonflies and damselflies) to all or most other winged insects (Gullan and Cranston 2005; Kjer 2004; Regier et al. 2010; Simon et al. 2009; Willman 2004; Zhang et al. 2008). Thus, information on developmental mechanisms in mayflies will help clarify the ancestral state for processes of segmentation and appendage development in winged insects. In this study,

we document the expression of *wg* mRNA transcripts and *En* protein localization during embryogenesis in a mayfly species, *Ephoron leukon* (Polymitarcyidae). Our data show that the expression of *wg* and *En* in the procephalon, body segments, and appendage primordia is relatively conserved in this species. Additionally, this work establishes that field collection of gravid females of *E. leukon* leads to a tractable system for developmental studies of mayfly embryogenesis.

Materials and methods

Embryo collection and fixation

Eggs of *E. leukon* were field collected from females on the Housatonic River (near Cornwall, Connecticut, USA) from 2001 to 2005. Species identification was confirmed by consultation with Steve Burian (Southern Connecticut State University, personal communication). Emergence of *E. leukon* occurs over several days in late July or early August and is typified by extremely dense and synchronized hatches of the aquatic nymphs into winged adults. Mating swarms occur over a period of 1–1.5 h directly after dusk, and females typically mate immediately after hatching to the winged stage. To collect fertilized eggs, a black light was positioned on the bank of the river and females were captured, held by the wings and the abdomen immersed into water to induce oviposition. A single female may deposit more than a thousand eggs, each of which is approximately 300 μm along the long axis, including the polar cap (O'Donnell, unpublished). Thus, it is possible to collect hundreds of thousands of fertilized eggs for laboratory culturing from a single evening's hatch.

Eggs were transported back to the laboratory and placed in small glass bowls filled with filtered river water. Eggs were allowed to develop at room temperature (RT) in ambient light conditions with periodic recharging with fresh filtered river water for 4–6 weeks or until eyespots/ocelli developed in a majority of the eggs (the signal of the onset of embryonic diapause in this species). These collection and maintenance procedures mirror those used for *Ephoron virgo* (Olivier) in the Netherlands (Greve et al. 1999), and we have used them to obtain and raise eggs from several different mayfly species (B. O'Donnell, unpublished data).

From our in-lab cultures, we collected embryos of a variety of developmental stages: from the heart stage/torpedo stage through the onset of diapause. Development is arrested shortly after the late post-elongated germ band (EGB) stage and resumes only after extended exposure to cold temperatures, as in several other species of *Ephoron* which undergo an obligate diapause (Britt 1962; Edmunds et al. 1956; Giberson and Galloway 1985, Greve et al. 1999; Watanabe and Takao 1991). Watanabe (1998) and

Giberson and Galloway (1985) documented the effects of temperature upon embryonic development and egg hatching rate in the congeners *Ephoron shigae* and *Ephoron album*, respectively. Several studies have demonstrated the ultimate impacts of temperature on nymphal development, timing of emergence, and duration of the emergence period in *E. leukon*, *E. album*, and *E. shigae* (Giberson and Galloway 1985; Snyder et al. 1991; Watanabe and Ohkita 2000; Watanabe and Takao 1991). The seasonal diapause is quite useful for providing late-stage embryos and early nymphal stages throughout the year as embryos can be maintained in this state for over a year. Embryos held in diapause can be kept in the lab year round, and clutches of eggs can be brought to room temperature for rearing of late-stage embryos through hatching and early nymphal stages.

Eggs were subjected to a 6-min soak in 50% fresh bleach solution to soften the chorion and then fixed for 30–50 min (older stage embryos were fixed for longer times) in 6% formaldehyde in phosphate-buffered saline + 0.1% Tween (PBTw). Heptanes were added (2:1 ratio of fixative to heptanes) and eggs were agitated on a platform shaker at high speed. Eggs were rinsed in PBTw and stored in absolute methanol at -20°C .

Gene amplification

RNA was extracted from a mixture of embryonic stages of freshly collected embryos of *E. leukon* using a Trizol-based method (Gibco BRL). cDNA was synthesized using MuLV reverse transcriptase and an oligo dT primer with incubations of 5 min at 65°C , 1 h at 37°C , and 10 min at 75°C . *wg* was amplified from *E. leukon* (GenBank accession number EU931674) using the degenerate primers 5'*wg*1: GARTGYAARTGYCAYGGYATGTCTGG and 3'*wg*2: ACTICGRCACCARTGGAATGTRC (Brower and DeSalle 1998). PCR reaction conditions were $94^{\circ}\text{C}/1$ min for denaturation, $56^{\circ}\text{C}/1$ min for annealing, and $72^{\circ}\text{C}/1$ min for extension for 36 cycles with a final extension step of 5 min at 72°C . A band of the appropriate size was excised from the gel, cleaned, and cloned into a dual-promoter vector (TOPO TA Cloning Kit, Invitrogen). Colonies were picked directly into a PCR mixture, and their inserts were then amplified with M13 forward and M13 reverse primers; inserts of the appropriate size were cleaned and cycle-sequenced. *wg* was amplified from several other mayfly species from a total of six different families: *Paraleptophlebia* (Leptophlebiidae), *Baetisca* (Baetiscidae), *Ephemerella* (Ephemerellidae), *Siphonurus* (Siphonuridae), *Caenis* (Caenidae), and *Ephemerella* (Ephemeridae; GenBank accession numbers: EU931673, EU931675–EU931679) using genomic DNA obtained via a CTAB extraction (modified from Murray and Thompson 1980). *wg* bands of the appropriate size from the additional taxa were

excised from 1% agarose gels, cleaned, and cycle-sequenced using standard cycle sequencing recipes and reaction conditions. Cycle sequencing used ABI BigDye, version 1.1, and resulting products were electrophoresed on an ABI3100 automated DNA sequencer.

Riboprobe synthesis and in situ hybridization

DIG-labeled riboprobes for *E. leukon* were synthesized with the DIG RNA Labeling Kit (Roche BioChemicals, Germany). Probes were hydrolyzed using 0.2 M sodium carbonate and 0.2 M sodium bicarbonate solution (2:3 ratio) at 60°C for 10 min, precipitated with 7.5 M ammonium acetate, and resuspended in hybridization buffer (see below). Probe strength was assessed by the dot-blot method (Huang et al. 1998) with reference to known standards.

Fixed eggs of *E. leukon* were placed in a small glass cuvette containing 1 ml of PBTw and dipped 5–30 times (dependent upon developmental stage) in a sonicator (FS30 Ultrasonic Cleaner, Fisher Scientific) to extract embryos from the egg membranes. Embryos were then rinsed several times in PBTw and pre-hybridized in buffer (50% formamide + 5XSSC (sodium chloride + sodium citrate solution) + 1X Denhardt's reagent + 0.1% Tween + 0.1% CHAPS + 200 $\mu\text{g}/\text{ml}$ herring sperm DNA (Nulsen and Nagy 1999) or dextran sulfate-enhanced hybridization buffer: 50% formamide + 4XSSC + 1X Denhardt's reagent + 5% dextran sulfate + 0.1% Tween + 250 $\mu\text{g}/\text{ml}$ tRNA (Broadus and Doe 1995) at 56°C for a minimum of 1 h up to overnight. Both hybridization buffers produced robust staining. Probes were subsequently added at a concentration ranging from 0.1 to 1 $\mu\text{g}/\text{ml}$, dependent upon the developmental stage. Incubation lasted >18 h at 56°C . Embryos were rinsed five times quickly and then for a total of four more washes spaced by at least 30 min between washes and then left overnight at 56°C in plain hybridization buffer (50% formamide + 5XSSC + 0.1% Tween).

The next day, embryos were rinsed in 2XSSC for 1.5 h at 56°C then in 1XSSC for an additional hour at 56°C . Next, embryos were rinsed twice in PBTw at RT and incubated in a 1:1,500 dilution of anti-digoxigenin-labeled Fab fragments (Roche BioChemicals) in 2% bovine serum albumin (Fisher Scientific) in PBTw for 2 h at RT. Embryos were then rinsed in PBTw every 10 min for a total of 1 h and equilibrated in alkaline phosphatase buffer (100 mM Tris-Cl, + 100 mM NaCl + 50 mM MgCl_2 + 0.1% Tween, pH9.5) for 15 min. The spatial distribution of *wg* mRNA transcripts was detected by development with the nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate-4-toluidine color reaction for up to 12 h at room temperature. Rinsing with PBTw stopped the color reaction, and embryos were counterstained with DAPI to label nuclei and stored in 80% glycerol at -20°C .

Immunohistochemistry

Fixed embryos were rinsed in phosphate-buffered saline + 0.1% Triton-X (PBTr) then incubated overnight at 4°C in a 1:50–1:400 dilution of En4F11 (Patel et al. 1989) in 2% bovine serum albumen in PBTr. The next day, embryos were rinsed every 10 min for a total of 1 h at RT then incubated with a peroxidase-conjugated secondary antibody (Jackson Immunochemicals) for 2 h at RT. Embryos were then rinsed in PBTr every 10 min for an hour before equilibration in 1X stable peroxidase substrate buffer (1XHP; Pierce, Rockford, IL, USA) then developed in a 1:10 dilution of metal-enhanced diaminobenzadine substrate (Pierce) in 1XHP buffer for 15 min at RT. Embryos were rinsed, stained with DAPI, and stored in 80% glycerol at –20°C. All embryos were viewed with a Zeiss Axioskop 2 Plus compound microscope and images were collected with an Olympus digital camera and image collection software (Magnafire; Meyer Instruments, Houston, TX, USA).

Results

Characterization of *E. leukon* *wg*

Amplification of a portion of the *wg* (*Wnt-1*) gene from *E. leukon* produced a 477-bp sequence, and its identity was confirmed by BLAST search results and by the presence of a number of conserved residues ($n=42$) between the inferred *E. leukon* Wnt-1 protein sequence and other Wnt-1 protein sequences (Fig. 1a). Partial *wg* sequences from *E. leukon* plus six additional mayfly species ranged from 441 to 480 bp in length, owing to a variable insertion region (Fig. 1b). Protein-specific BLAST searches of the mayfly insertion region did not return any significant similarity to known proteins; thus, the functional significance of this region is unknown.

Segmental expression of *wg* during *E. leukon* embryogenesis

During embryogenesis, *E. leukon* embryos add segments progressively to the posterior as is typical in short germ insects (Anderson 1972), and many of the embryonic stages are highly similar to those seen in other insects (Fig. 2). Embryogenesis in *E. leukon* proceeds in a manner highly similar to that seen in other burrowing mayfly species (e.g., *Ephemera japonica*, Tojo and Machida 1997, 1999), including the related polymitarcyid species, *Tortopus incertus* (Tsui and Peters 1974).

Stripes of *wg* expression appear before segments are morphologically distinguishable (i.e., before lateral furrows

are evident along the anteroposterior axis of the body). Stripes are added progressively from anterior to posterior (Fig. 3a–e), with the exception that the intercalary stripe appears midway through segmentation after the anterior-most abdominal stripes of *wg* expression have already formed (Fig. 3c). During *E. leukon* embryogenesis, this is the sole instance of a *wg* stripe appearing out of anterior to posterior order. *wg* stripes in each segment are positioned several rows anterior to the posterior edge of each segment.

When segmental *wg* expression is initiated, it appears as two clusters separated by a small gap along the midline. Developmentally older and more anterior segments express *wg* in a continuous stripe across the midline (Fig. 3d, e). Just before the completion of segmentation, *wg* expression in the gnathal and thoracic segments begins to fade mid-ventrally (Fig. 3e), eventually resolving to weak or no expression ventrally by the EGB stage (Fig. 3f). At this stage, the abdominal segments retain *wg* across their midlines (Fig. 3f). Subsequently, *wg* is expressed in discrete clusters in the abdominal segments (Fig. 3g).

wg expression in the procephalon

wg expression in the procephalon is visible in the ocular region, as well as in the antennae, labrum, and stomodeum. In the earliest stages of development, *wg* is expressed strongly in the procephalon in two patches recessed slightly from the edges of the embryo (Fig. 3a). These patches of *wg* expression become positioned laterally (Fig. 3b–d) as the embryo elongates and segmentation continues. By about mid-segmentation, the *wg* patches become bisected into a large anterior and smaller posterior domain (Fig. 3d, e, h).

The antennae of *E. leukon* express *wg* very early in embryogenesis. Faint paired expression domains are visible as segmentation commences (Fig. 3b). During segmentation, antennal *wg* stripes extend along the ventral margin of the elongating structures (Fig. 3c–e), but expression never extends across the ventral midline of this region. After the completion of segmentation, *wg* expression becomes faint and somewhat discontinuous along the ventral antennal margin (Fig. 3f, g).

wg is expressed in post-EGB embryos in two patches at the middle and anterior-most region of the procephalon (Fig. 3g), corresponding to the labral buds, which will eventually move medially and fuse to form the mature labrum—this is the first obvious expression of *wg* in the labral region (Fig. 3i, k). The stomodeum (the presumptive mouth and anterior limit of the gut) is positioned directly posterior to the labrum and expresses *wg* early in embryogenesis as a faint patch directly between the nascent

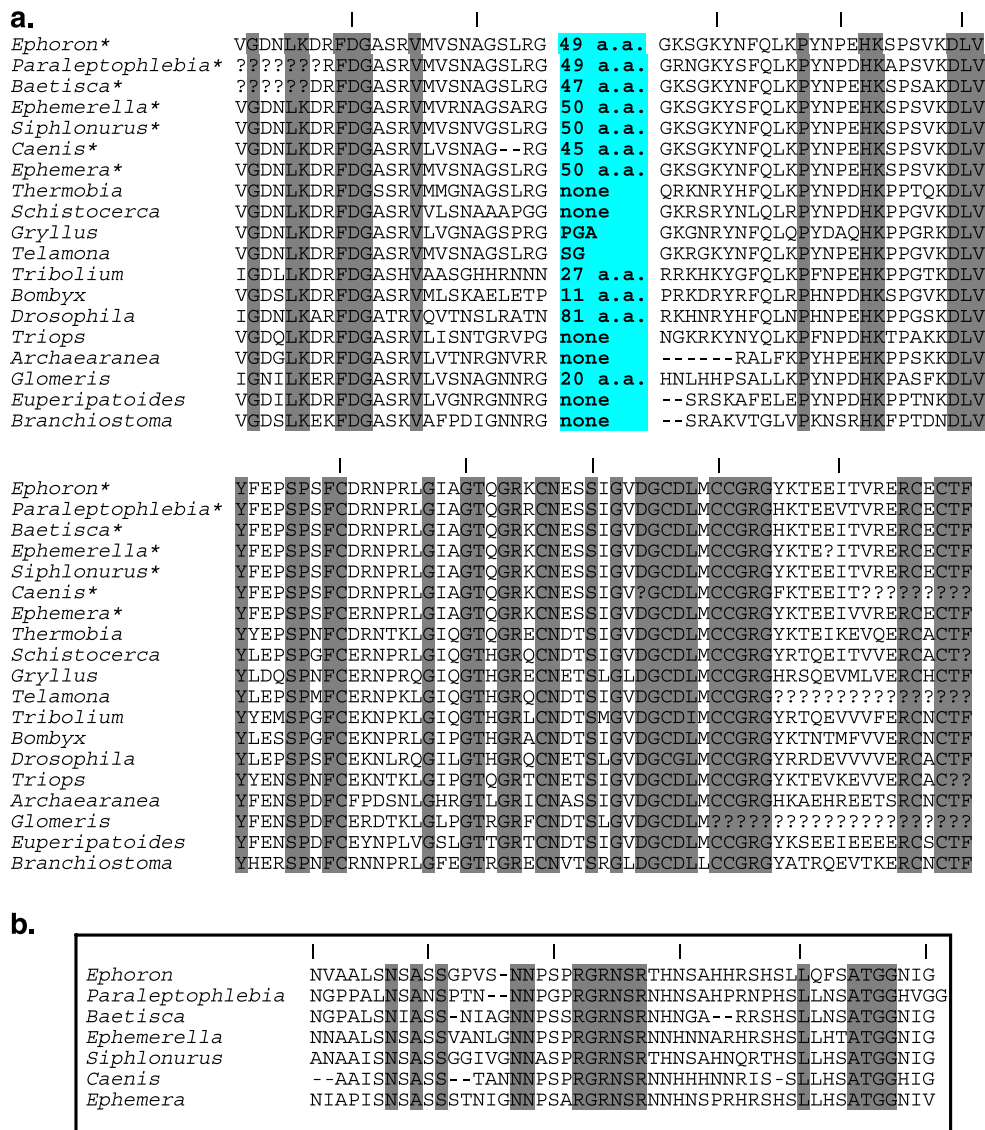


Fig. 1 a Alignment of a portion of the inferred Wg (Wnt-1) protein sequence in *Ephoron leukon*, six additional mayfly species (mayfly species names are followed by asterisks), a firebrat (*Thermobia domestica*, GenBank accession number AF214035.1), grasshopper (*Schistocerca*, AAD37798.1), cricket (*Gryllus*, BAB19660.1), treehopper (*Telamona*, AAS57853.1), beetle (*Tribolium*, NM_001114350.1), moth (*Bombyx*, ABX57129.1), fruitfly (*Drosophila*, AAF52501.1), tadpole shrimp (*Triops*, AAC32377.1), spider (*Achaeareanea*, BAD12586.1), millipede (*Glomeris*, CAE83648.1), onychophoran (*Euperipatoides*, EU347403), and lancelet (*Branchiostoma*, AAC86432). The blue highlighted region indicates the variable length

insertion region (e.g., “49 a.a.” refers to an insertion of 49 amino acid residues). The specific amino acids for insertions of fewer than five amino acids are shown; all others are omitted for clarity. Forty-two amino acids were conserved across all included taxa and are denoted by gray shading. The ruler at the top of the alignment shows tick marks at every ten amino acids and does not count amino acids from the highlighted variable region. **b** Alignment of only the mayfly-specific insertion region of Wnt-1. Conserved residues are shaded in gray ($n=16$). Dash indicates a gap in the alignment and question mark indicates missing data

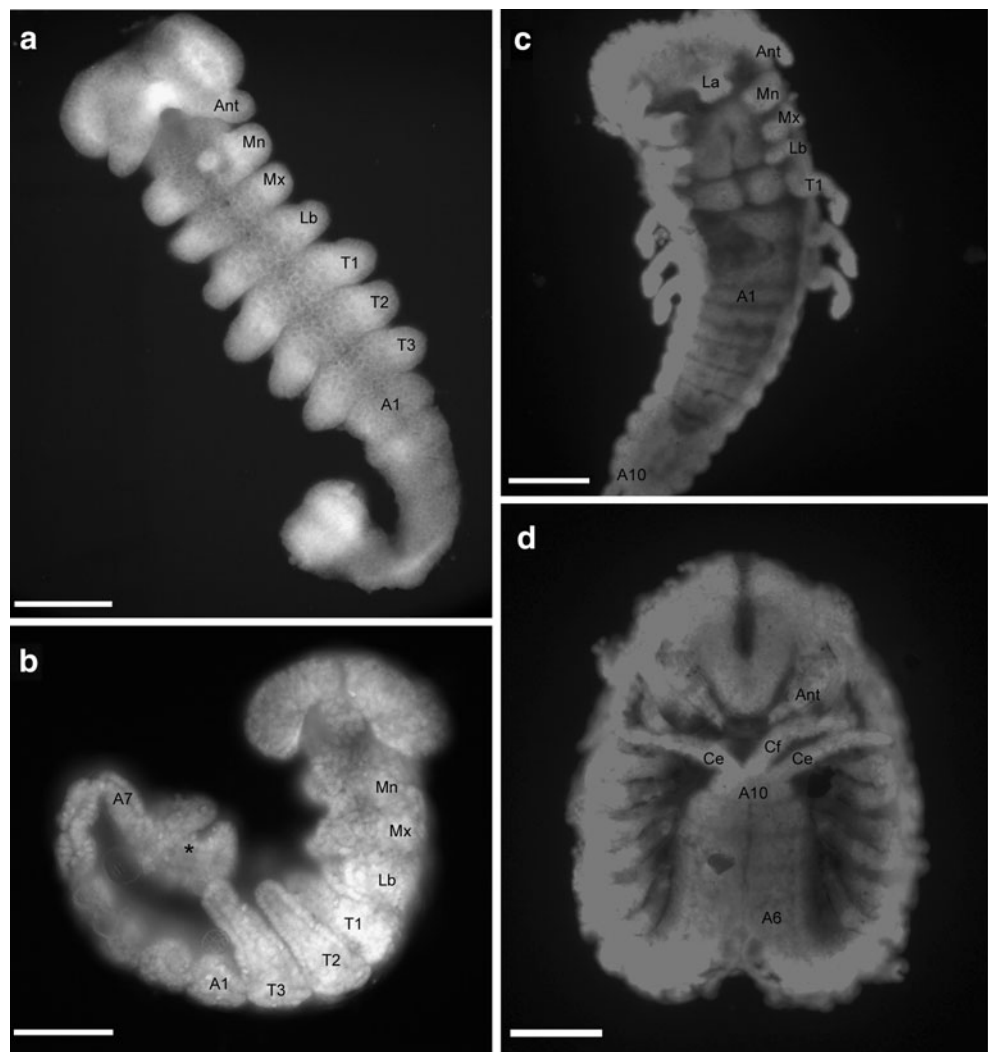
antennae (Fig. 3b). Stomodaeal expression remains apparent in the pre- and post-EGB stages (Fig. 3c–e, g, k).

Expression of *wg* in the mouthparts and thoracic legs

The expression of *wg* in the developing postoral appendages of *E. leukon* (mandibles, maxillae, labium, and thoracic legs) is inherited from the original segmental

stripes. During the early segmenting stages, *wg* initially extends in a stripe along the ventral margin of each of the elongating appendages (Fig. 3d–h, j–l). Later, at the EGB stage, mandibular expression becomes restricted to two patches near the distal tip of the appendages (Fig. 3f). In contrast, maxillary and labial *wg* expression at the EGB stage consists of continuous stripes across these appendages out to the distal-most tips (Fig. 3f). In early post-EGB stage

Fig. 2 Select stages of *E. leukon* embryos stained with DAPI to highlight morphology. Ventral view (**a**, **c**, **d**) or lateral view (**b**); anterior is up for all embryos. **a** Elongating germ band stage. Two abdominal segments are clearly distinguishable. **b** Elongating germ band stage, slightly older than in **a**. Elongated thoracic limbs, seven of the eventual ten abdominal segments and the folding of the posterior abdomen (*asterisk*) are evident. **c** Post-EGB stage. The overall widening of the embryo in the post-EGB stages is shown here. **d** Post-EGB stage, slightly older than in **c**. Embryos at this stage have ventrally folded the posterior of the abdomen. Two lateral cerci (*Ce*) flank a median caudal filament (*Cf*). *Ant* antenna, *La* labrum, *Mn* mandible, *Mx* maxilla, *Lb* labium, *T1* thoracic segment 1, *A1* abdominal segment 1. Scale bars 200 μ m (**a**, **b**, **d**), 250 μ m (**c**)



embryos (Fig. 3k), maxillary expression first becomes relegated to two domains, and older post-EGB stages (Fig. 3g) express *wg* in three distinct patches, including one patch at the tip of the elongating palp. Late-stage *wg* expression in three discrete regions of the maxillae may result from downregulation in a medial region that will give rise to the endites, or branches, as well as from downregulation along the ventral edge of the palp (Fig. 3g). In contrast, labial expression in the post-EGB stages consists of a single stripe that does not extend to the distal most tip of the limb (Fig. 3g, k).

Additional regions of *wg* expression

The earliest expression of *wg* in *E. leukon* embryos occurs in the most posterior region of the embryo, the so-called proliferative zone (Fig. 3a). A posterior patch of *wg* is visible throughout segmentation (Fig. 3b–e), but is absent by the EGB stage (Fig. 3f). At the post-EGB stages, *wg* patches occur in each abdominal segment (Fig. 3g).

Additionally, in post-EGB embryos, *wg* is expressed at the base and along the inner margins of the cerci, along the caudal filament and in the posterior region of the hindgut (Fig. 3m). We did not obtain robust staining in the oldest embryonic stages and in young nymphs. This is likely due to a combination of factors: (1) decreased penetration of riboprobes and antibodies that leads to no visible signal or (2) increased “stickiness” of tissue that creates dark, non-specific staining.

Segmental localization of *En* during *E. leukon* embryogenesis

En expression in the segments occurs progressively over early embryogenesis, paralleling the pattern seen for *wg*. Like *wg* stripes, segmentally repeated stripes of *En* appear prior to the development of morphologically distinguishable segments and are added in an anterior to posterior progression (Fig. 4a–d). Comparison of *wg* and *En* expression patterns following the appearance of

segmental boundaries suggests that En stripes are situated just posterior to the *wg* stripes; confirmation of this pattern requires double stains for *wg* mRNA and En protein. During limb elongation phases, En staining does not extend fully to the distal-most reach of the appendages (Fig. 4c, d). However, post-EGB stage embryos express En in stripes that extend to the lateral edges of each segment (Fig. 4e).

Localization of En in the procephalon, mouthparts, thoracic legs, and ventral region

En localization in the procephalon of *E. leukon* is evident in the ocular and antennal regions. Ocular expression is evident at the EGB stage (Fig. 4e). Later expression, if present, is obscured. Antennal expression occurs in paired stripes in the head during segmentation (Fig. 4b–d), but by the post-EGB stage, the antennae express En only in their distal tips (Fig. 4e). Labral expression of En is not observed at any of the stages of embryogenesis that we examined.

Developing appendages of *E. leukon* inherit En expression from the segmental expression initiated early in embryogenesis, as is also seen for *wg* expression. As appendages elongate, En expression extends along the ventral margins of the limbs (Fig. 4c, d) and out to the tips of the limbs in post-EGB stages (Fig. 4f, g). En expression in the mouthparts and thoracic legs of *E. leukon* remains relatively unchanged in the late stages we have examined, including the post-EGB stage (Fig. 4e–g), indicating that downregulation of *wg* is not attributable to loss of En expression in these regions. Late in embryogenesis, clusters of cells along the mid-ventrum express En in each segment (Fig. 4e–g); these are presumed to belong to the nervous system.

Discussion

Expression of *wg* in mayflies relative to other arthropods

wg expression in the segmenting embryos of *E. leukon* is similar to that of numerous arthropods. Both the sequential appearance of segmentally reiterated stripes and the out-of-sequence appearance of the intercalary expression are highly conserved across insects (Dearden and Akam 2001; Giorgianni and Patel 2004; Jockusch and Ober 2004; Jockusch et al. 2004; Miyawaki et al. 2004; Nagy and Carroll 1994; Niwa et al. 2000; Ober and Jockusch 2006). Striped expression has also been observed in several crustacean, myriapod, and chelicerate taxa (Damen 2002; Duman-Scheel et al. 2002; Hughes and Kaufman 2002a; Janssen et al. 2004; Nulsen and Nagy 1999; Prpic 2004; Williams and Nagy 1996).

Variation in the intensity of *wg* expression along the dorsoventral axis occurs in many taxa. Lower levels of *wg* expression ventrally, as seen in *E. leukon* (Fig. 2g, j, k), are also seen in the myriapods *Glomeris marginata* (in which *wg* expression is also absent dorsally) and *Lithobius atkinsoni* (Hughes and Kaufman 2002a; Janssen et al. 2004) as well as the mysid shrimp *Mysidium columbiae* where lateral expression is consistently more robust than ventral expression (Duman-Scheel et al. 2002). In the spider *Cupiennius salei*, *wg* is only expressed in the appendages and never extends across segments (Damen 2002). Downregulation of expression of *wg* along the dorsoventral axis of each segment occurs in the embryos of *Drosophila* and *Tribolium*, resulting in discrete patches of expression (Baker 1987, 1988; Nagy and Carroll 1994), and in *Drosophila*, the differential regulation of *wg* along the dorsoventral axis has been characterized (Bejsovec and Martinez Arias 1991). Thus, while segmentally reiterated expression of *wg* is a conserved component of arthropod segmentation, this perceived conservation masks considerable evolutionary variation in the dorsoventral extent and dynamics of segmental expression across taxa. The functional significance of this variation is not yet clear. Janssen et al. (2008) have argued that in millipedes, *wg* is required only for the patterning of ventral segment boundaries, while dorsal segment boundary formation is independent of *wg*.

Strong *wg* expression in two patches in the procephalon occurs in regions that likely correspond to the “head blobs” described in *Drosophila* (Liu et al. 2006; Schmidt-Ott and Technau 1992) as well as the dual patches in the cephalon of *Tribolium* and *Schistocerca* embryos (Dong and Friedrich 2005; Liu et al. 2006). Bisection of the *wg* domains in the procephalon of *E. leukon* embryos closely parallels the splits seen in *Tribolium* and *Schistocerca* (Dong and Friedrich 2005; Liu et al. 2006). In these two insects, this subdivision has been implicated in the partitioning of dorsal and ventral protocerebral neuroectoderm into subcomponents of the visual system and the protocerebrum, respectively (Liu et al. 2006). The division of an originally large patch of *wg* expression in the procephalon into subdomains has been suggested to be associated with the development of stemmata in *Tribolium* and compound eyes in *Schistocerca*, as contrasted with the derived condition of *Drosophila* larvae which do not manifest this subdivision (Baker 1988; Liu et al. 2006; Schmidt-Ott and Technau 1992).

In first-instar hatchlings of *E. leukon*, five pigmented spots on the dorsum of the head are visible: two lateral pairs (one anterior and one posterior pair) and a single crescent-shaped structure at the midline which correspond to the ocelli or light-sensing organs. Mayflies including *E. leukon* hatch without compound eyes. As documented in several heptageniid mayflies, the compound eyes of mayfly nymphs originate from the posterior pair of ocelli as a

single ommatidium (Needham et al. 1935), with additional ommatidia added progressively in late nymphal development (Clifford et al. 1979; Needham et al. 1935). Further investigation of the molecular patterning of the compound eyes in *E. leukon* requires chronicling *wg* expression over late embryogenesis and through nymphal development, complemented with expression and functional data for genes involved in retinal determination, e.g., *eyeless*, *sine oculis*, and *eyes absent* (Bonini et al. 1993; Cheyette et al. 1994; Quiring et al. 1994).

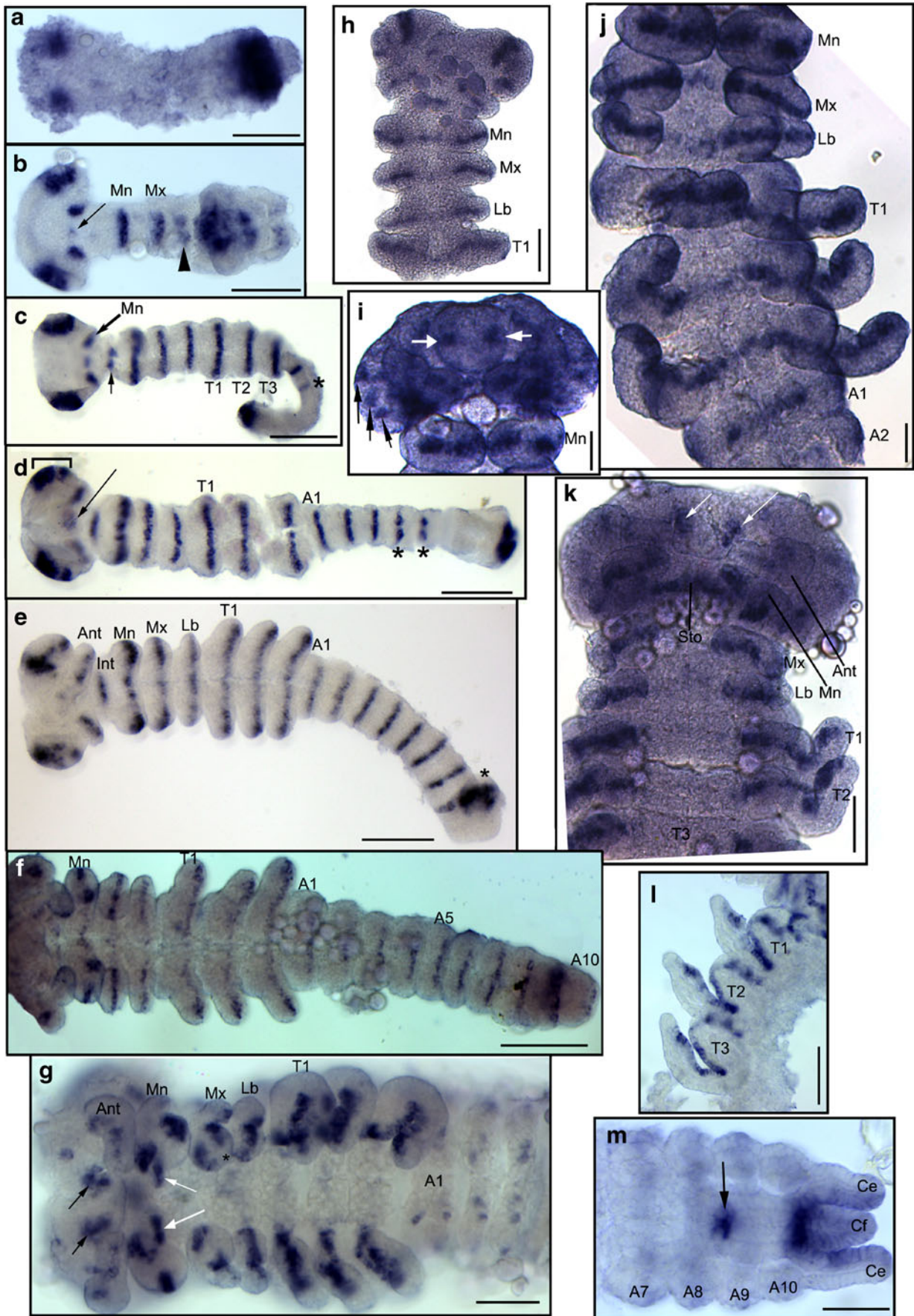
As is true for segmental stripes, *wg* expression along the ventral edges of gnathal appendages is highly conserved across arthropods (Angelini and Kaufman 2005b). At the EGB stage, *E. leukon* expresses *wg* in two patches in the mandibles and in continuous stripes across the maxillary and labial appendage primordia, similar to the expression patterns reported for *Tribolium* and *Schistocerca* (Giorgianni and Patel 2004; Jockusch et al. 2004). The mandibles of young *E. leukon* nymphs are composed of an inner toothed region (the canines and molar surface) plus “tusks” that are oriented at a right angle to the toothed region; these mandibular tusks are unique to mayflies and are found in several burrowing species (McCafferty 1975). In *E. leukon*, the tusks first appear as small nubs in nymphs past the second instar (O'Donnell 2009). In *Drosophila*, *wg* acts cooperatively with *decapentaplegic* (*dpp*) to upregulate the expression of Distal-less and consequently promote the outgrowth of appendages (Lecuit and Cohen 1997). Determining whether *wg* plays a role in regulating the outgrowth of the mandibular tusks in *E. leukon* will require a study of the post-embryonic stages of development. Specifically of interest is whether a novel patch of *wg* is expressed at the frontal margin of the mandible prior to tusk outgrowth, as would be expected in a *Drosophila*-based spatial reiteration model of mouthpart branching (Panganiban et al. 1995). Data drawn from *E. leukon* will add important information to our understanding of the origin and modification of mouthparts with a secondary axis.

Expression of En in mayflies relative to other arthropods

The segmental expression of En in *E. leukon* is highly similar to that of several other arthropods and invertebrates (Abzhanov and Kaufman 2000; Patel et al. 1989; Prud'homme et al. 2003). The absence of labral En expression is typical of all insects examined except *Drosophila* (e.g., Patel et al. 1989; Peterson et al. 1998; Rogers and Kaufman 1996). Similarly, clusters of En-expressing cells in the ventral region have been documented in a host of arthropods including several crustaceans (*Procambarus clarkii*, *Porcellio scaber*; Abzhanov and Kaufman 2000), *Marmokrebs* (Sintoni et al. 2007), and insects (*Thermobia domestica*: Peterson et al. 1998; *Schistocerca*: Boyan and Williams

Fig. 3 Expression of *wg* in *E. leukon* through embryogenesis. Ventral view (a–k, m) or lateral view (l): Anterior is to the left (a–g, m) or up (h–l). **a** Torpedo stage. Two head spots and strong expression in the posterior growth zone are evident. The extra tissue beyond the posterior-most expression is an artifact of the preparation. **b** Early segmentation stage. Posterior-most region of the embryo is folded ventrally. Stomodaeal (presumptive mouth) expression has appeared (arrow) directly between paired patches of *wg* in the antennae; labial expression (arrowhead) is weaker at the ventral midline. **c** Early abdominal segmentation stage. Intercalary expression is evident between the antennal and mandibular segments (arrow). Two abdominal *wg* stripes are present, with fainter expression in the more posterior stripe (asterisk). **d** Mid-abdominal segmentation stage. Stomodaeal expression remains evident (arrow), while ocular expression has been subdivided into anterior and posterior sections (single bracket). The thoracic appendages are just beginning to elongate, five abdominal stripes are present, and the newest expression in abdominal segments four and five is composed of discrete clusters of *wg*-expressing cells (asterisks) separated by a region of lower expression at the ventral midline. **e** Late segmentation stage. *wg* expression is downregulated along the ventral body wall of anterior segments, while segmental expression remains strong in the appendages, the intercalary segment, the distal-most region of the mandibular segment, and the posterior growth zone also maintains strong *wg* expression (asterisk). **f** EGB stage (the phylotypic stage for insects). All body segments are visible: three gnathal, three thoracic, and ten abdominal. Ventral body wall expression has been downregulated in the anterior-most segments of the embryo. **g** Post-EGB stage. *wg* is expressed in the paired labral buds (black arrows) and along the ventral margin of the antennae. Paired crescents of expression are evident in the gnathal region (white arrows). Palp and endite regions of the maxilla and labium are distinct and expression has been downregulated distally in the maxillary endite (asterisk), but not in the labial endite. Additionally, punctate expression is visible in the abdominal segments (only the first three abdominal segments are visible in this specimen). **h** Anterior end of an elongating embryo (stage is between that seen in d and e). **i** Procephalon of an embryo just prior to the EGB stage. The labral lobe expresses *wg* in patches (white arrows). Expression of *wg* in the lateral cephalon is also visible (black arrows). **j** Gnathal and thoracic regions of the embryo in i showing detail of *wg* stripes across the segments and into the gnathal and thoracic appendages. **k** Anterior of a post-EGB stage embryo showing loss of segmental expression outside of the appendages. The labrum has two patches of expression (white arrows) and stomodaeal staining is present. **l** Thoracic limbs of a post-EGB embryo showing that ventral stripes that do not extend to the very distal tips of the limbs. **m** Posterior of a post-EGB embryo showing two expression domains, one in the anterior hindgut (arrow) and another ring at the base of the cerci (Ce) and caudal filament (Cf), corresponding to the posterior hindgut

2002; Siegler et al. 2001; *Tribolium*: Patel et al. 1989; *Drosophila*: DiNardo et al. 1985). Mutational analyses in *D. melanogaster* suggest that En functions in neural specification during embryogenesis (Brower 1986; DiNardo et al. 1985). In addition, studies in *Schistocerca* and *Marmorkrebs* have implicated En in neuronal patterning of the cephalon and segmental neuromeres (Boyan and Williams 2002; Siegler et al. 2001; Sintoni et al. 2007). Definitive evidence as to whether or not the ventral clusters of En expression in late stage *E. leukon* embryos precede neuronal structures of the nymphs awaits functional work



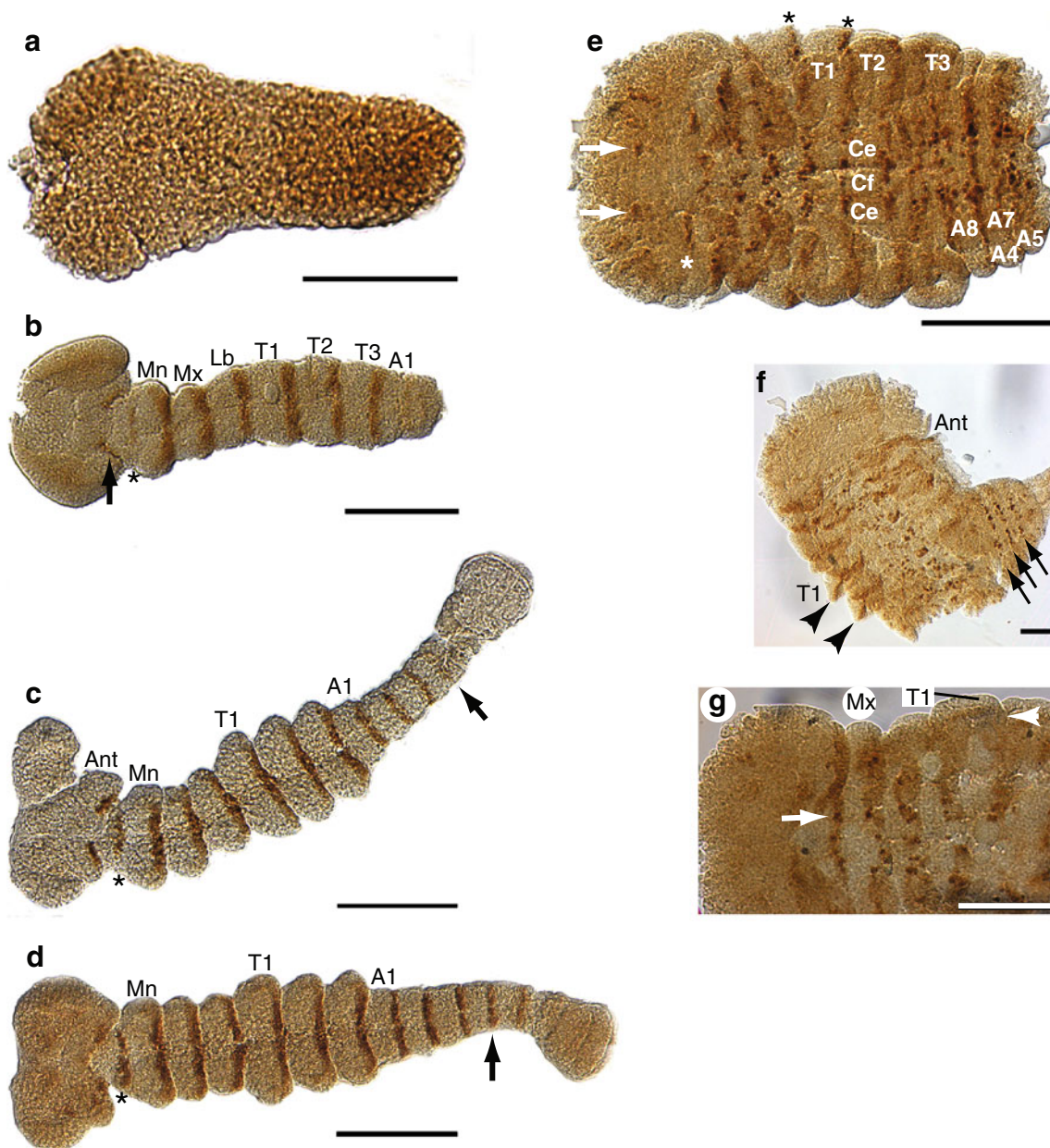


Fig. 4 Localization of the En antibody 4F11 during *E. leukon* embryogenesis. Ventral view, anterior is to the left in **a–g**. **a** Heart stage, slightly younger stage than the torpedo stage seen in Fig. 3a. En expression is strongest in the posterior of the embryo. **b** Early abdominal segmentation stage; note that the posterior abdomen is missing. A stripe of intercalary expression is first evident at this stage (*asterisk*). Antennal expression is visible (*black arrow*). En stripes are situated at the very posterior of each segment and in contrast to *wg* (and En in later stages); expression extends to the lateral edges of each segment. **c**, **d** Mid-abdominal segmentation stages showing four (**c**) and five (**d**) abdominal stripes of En. In **c** and **d**, intercalary expression is indicated (*asterisk*) and expression in abdominal segment 4 is marked with an *arrow*. During limb elongation, En expression terminates before reaching the distal tips of the appendages. **e**

EGB stage. The posterior of the body is folded up against the ventrum (abdominal segments are labeled to illustrate folding); see Fig. 2d for clarity. Two faint patches of En are visible in the cephalon (*white arrows*) and the antennae express En distally (*white asterisk*). Stripes of En extend to the very edges of the segments (*two black asterisks at top of panel*). **f** Post-EGB stage embryo with posterior region unfolded. En expression in the first and second limbs is marked with *arrowheads*. Punctate staining in the most posterior abdominal segments is indicated (*arrows*). **g** Anterior of a late stage embryo. Punctate stain in the gnathal and thoracic segments near the ventral midline is indicated (*arrow*). An *arrowhead* indicates En expression in the first thoracic limb, which is aligned parallel to the main body axis, with the very distal tip curved inward. Scale bar 100 μ m (**a**, **b**), 200 μ m in (**c–g**)

as our data provide purely correlative data in terms of timing and location of En expression.

Engrailed paralogues

En expression in *E. leukon* as revealed with the En4F11 antibody may have provided only a partial picture of expression as two copies of En have been isolated from the mayfly *Ephemera vulgata* (Ephemeroidea; and a host of other insects (see Peel et al. 2006)). This suggests the strong likelihood that *E. leukon* has at least two copies of En in its genome. We do not know whether En4F11 recognizes one or both En paralogues in *E. leukon*. Expression of the two paralogues in *Schistocerca* embryos is highly similar, with differences reported only in the timing of expression of each gene copy in the mandibles and antennae (Peel et al. 2006). Minor differences in expression of *engrailed* paralogues have also been noted in *Thermobia* (Peterson et al. 1998), *Periplaneta* (Marie and Bacon 2000) and *Drosophila* (Coleman et al. 1987). However, in two crustaceans, Abzhanov and Kaufman (2000) documented divergent expression during late embryogenesis of the two En paralogues in tandem with two different antibodies (4D9 and 4F11). Cloning and expression details for each copy of En are essential to determine whether the En4F11-based expression profile reflects a composite or partial picture of En expression in *E. leukon*. The En4D9 antibody did not produce discernible signal in *E. leukon* embryos, while En4F11 worked robustly. A lack of signal may reflect a mismatch between the homeodomain epitope recognized by the 4D9 antibody (ELGLNEA-QIKI; Serrano et al. 1995) and the sequence of the homologous region of mayfly En. In *E. vulgata*, both En paralogues have several amino acid mutations in this region (En-1: DLGLHENQIKI, En-2: ELKLNESQIKI; Peel et al. 2006; underlined residues indicate divergent sites between the epitope region of the 4D9 antibody and the mayfly paralogues), which may account for the lack of signal when *E. leukon* embryos were exposed to En4D9.

Mayflies as a developmental model

Some components of the molecular mechanisms underlying the patterning of the segmental body plan of arthropods and their close relatives are highly conserved, including the widely documented expression of abutting stripes of *wg* and *en* in parasegments (Damen 2002; Eriksson et al. 2009). Expression data from the mayfly *E. leukon* documented here provide additional evidence for this conservation across insects. In addition, this work provides a starting point for elevating a mayfly species onto the stage for expanded study of molecular development, along with recent expression data from the mayfly *Ephoron eophilum* documented by Niwa et

al. (2010). Future work will focus on genes that have been documented to have divergent expression and function across arthropods, including those that lie upstream and downstream of the segment polarity genes as well as genes implicated in branched appendage patterning (e.g., in *Tribolium*, *Oncopeltus*, and *Gryllus*; Angelini and Kaufman 2004, 2005a; Choe and Brown 2007, 2009; Miyawaki et al. 2004). Data from other components of the segmentation cascade (e.g., pair rule and Hox genes) and particular appendage patterning genes (e.g., *decapentaplegic*) in *E. leukon* will be helpful to calibrate the character and timing of evolutionary transitions for the majority of insect lineages (Angelini and Kaufman 2005b; Hughes and Kaufman 2002b). In addition, paralogues of *wg* and *en* have been isolated across insects (Bolognesi et al. 2008b; Peel et al. 2006), and functional studies in *Tribolium* demonstrated that knockdown of *wg/Wnt-1* alone has only a limited ability to disrupt segmental patterning (Bolognesi et al. 2008a). Therefore, screening of the *E. leukon* genome for additional members of the *Wnt* and *en* families followed by careful documentation of expression is critical to more fully understand the roles of different members of these gene families in *E. leukon*. Lastly, gene expression data provide one type of data to understand the developmental basis of morphology. In turn, developing RNA interference (RNAi) methods for mayflies is of interest to probe the functional effects of important patterning genes. The recent demonstration of working RNAi protocols in thysanuran embryos (Ohde et al. 2009) is a promising development for the tractability of the development of functional assays in a greater number of non-model organisms.

Recently, Sommer (2009) argued that evo–devo should proceed by concentrating on a small number of model systems and investing heavily in the development of functional tools for these systems. While we agree that it is important to be able to study some systems in depth, we believe that studying the full phylogenetic breadth contributed by the “evo” component of evo–devo is equally important. One major reason that breadth is required is that accurate inferences of ancestral states are critically dependent on taxon sampling. Failure to sample key lineages can lead to ill-informed assumptions about ancestral conditions (see Jenner 2006). Comparisons across deep phylogenetic distances, such as between major lineages of arthropods or between arthropods and vertebrates, rely implicitly on inferring the morphology and development of the most recent common ancestor of each group. A good example of this comes from the recent debate about whether segmentation is ancestral for bilaterians, and the recent discovery that Notch and Delta, which are required for segmentation in vertebrates, are also used in segmentation in a diversity of arthropods (Damen 2007; Pueyo et al. 2008; Stollewerk et al. 2003). As far as is known, these genes are not required

for segmentation in the major insect models *Drosophila* and *Tribolium*, so prior to the availability of data from other taxa, there was no reason to argue against the conclusion that segmentation evolved independently in different segmented lineages. As this example shows, restriction of studies to only a few taxa will necessarily inhibit our ability to infer ancestral states or the sequence of evolutionary transformations that produced the current diversity of life.

Mayflies add a major new phylogenetic branch to the insect systems in which developmental patterning has been studied at the molecular level. Because of their phylogenetic position, developmental data from mayflies are especially important for accurate inferences about the most recent common ancestor of winged insects. Data from representatives of a small number of orders have identified major developmental shifts that have occurred during the evolution of insects. One well known example is the conversion of *Hox3* from a canonical hox gene to a gene involved in extraembryonic patterning, which occurred after the most recent common ancestor of branchiopod crustaceans and insects and before the divergence of orthopterans from their sister group (Hughes and Kaufman 2002b; Papillon and Telford 2007). Similarly, alterations in the C-terminal end of the Hox gene *Ultrabithorax* which cause transcriptional repression of *Distal-less* and suppression of appendage development occurred after the divergence of collembolans from other hexapods and preceding the diversification of holometabolous insects (Galant and Carroll 2002; Ronshaugen et al. 2002). The large phylogenetic gaps during which these transitions occurred reflect the poor sampling of taxa in this portion of the tree. Developmental data from mayflies and other unsampled (or poorly sampled) lineages that diverged during these time periods can be used to further pinpoint when major developmental transitions such as these occurred. On the other hand, data from mayflies also offer the opportunity to study the origin of specialized mayfly traits, like asymmetric mouthparts (e.g., mandibles in nymphal stages) and the structurally and functionally diverse abdominal gills.

Note added in proof Recently Niwa et al. (2010) described the expression of *wg* and two other genes in the mayfly *Ephoron eophlium*; the *wg* expression pattern is similar to the one described here for *E. leukon*.

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