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Polyembryonic development: insect pattern formation in a cellularized environment

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SUMMARY

The polyembryonic wasp *Copidosoma floridanum* produces up to 2000 individuals from a single egg. During the production of individual embryos the original anteroposterior axis of the egg is lost and axial patterning must subsequently be reestablished within each embryo. The mechanism by which this occurs is unknown. In most insects, egg polarity is established during oogenesis and early development takes place in a syncytium. In *Drosophila melanogaster*, the syncytium is considered essential for establishing the morphogenetic gradients that initiate segmental patterning. However, we found that development of *C. floridanum* occurs almost exclusively in a cellularized environment. To determine whether the *D. melanogaster* patterning cascade is conserved in the absence of a syncytium, we analyzed the expression of Even-skipped, Engrailed and Ultrabithorax/Abdominal-A during polyembryonic development. Here we show that in spite of the absence of a syncytium, the elements of the *D. melanogaster* segmentation hierarchy are conserved. The segment-polarity gene *Engrailed* and the homeotic genes *Ultrabithorax/Abdominal-A* are expressed in a conserved pattern relative to *D. melanogaster*. However, we detect an alteration in the expression of the *Even-skipped* antigen. Even-skipped is initially expressed in segmentally reiterated stripes and not in a pair-rule pattern as it is in *D. melanogaster*. We also observe that the expression of these regulatory proteins does not occur during the early proliferative phases of polyembryony. Our results indicate that a syncytium is not required for segmental patterning in this insect.

Key words: pattern formation, polyembryonic development, Even-skipped, Engrailed, Ultrabithorax, Abdominal-A, *Copidosoma floridanum*

INTRODUCTION

Most metazoan embryos undergo holoblastic cleavage, which compartmentalizes the embryo into separate spatial domains. Developmental asymmetry is created by localizing maternal determinants to separate blastomeres (reviewed by Davidson, 1986) with subsequent pattern formation frequently based on (e.g. in *C. elegans* and *Xenopus*) receptor-ligand mediated cell-cell interactions (Priess, 1994; Kessler and Melton, 1994). In contrast, most insects undergo syncytial cleavage, where nuclear divisions are not followed by cytokinesis. For example, in *D. melanogaster* up to 6000 nuclei reside in a common cytoplasm before cellularization (Turner and Mahowald, 1976). Early pattern formation within the syncytium depends on the diffusion of maternally encoded transcription factors. These transcription factors form gradients that act in a concentration-dependent fashion to trigger a cascade of downstream genes (reviewed by Hülskamp and Tautz, 1991; St. Johnston and Nüsslein-Volhard, 1992).

At the top of the *D. melanogaster* segmentation gene cascade lie maternally provided gene products which are asymetrically distributed in the oocyte during oogenesis. These genes provide the embryo with its axial polarity. They function to transcriptionally regulate the gap genes, resulting in the subdivision of the syncytial blastoderm into broad domains of gene expression. The gap genes in turn initiate transcriptional regulatory interactions that position the expression of the pair-rule genes, providing a double-segment periodicity to the blastoderm. They in turn regulate the segment-polarity genes. The final outcome of this regulatory pathway is the expression of the homeotic genes in discrete regions along the anteroposterior axis, which provides the embryo with region- and segment-specific identities. It is believed that these regulatory interactions depend on diffusion of DNA-binding factors in the syncytial blastoderm. However, such a patterning mechanism is also thought to be incompatible with the cellularized environment in which the majority of other metazoans develop (Wolpert, 1994).

It has also been suggested, but never directly shown, that patterning in primitive insects does not rely on diffusion within a syncytium (reviewed by Patel, 1994; Tautz and Sommer, 1995). In a large number of species, referred to as short germband insects, segments develop through proliferation rather than the sequential subdivision of the blastoderm as in

D. melanogaster. Consequently, segmental patterning of short germband insects is thought to rely at least in part on cell-cell interactions (Akam and Dawes, 1992). These insects like most others, however, still initiate development in a syncytium. Exactly when the transition from a syncytium to a cellularized environment occurs relative to the activity of the segmentation genes remains unknown for any insect other than *D. melanogaster*. Thus, it remains unclear whether a syncytial stage is essential for proper segmental patterning of insects.

One approach to resolving this issue is to study insects that do not exhibit early syncytial cleavages. There are two such groups described in the literature. The first is the primitive insect order Collembola (Anderson, 1972). The second is a small group of parasitic Hymenoptera (Silvestri, 1906; Patterson, 1921) whose closest relatives undergo syncytial cleavage and long germband embryogenesis similar to *D. melanogaster*. These wasps are also unusual in that they are polyembryonic: a single wasp egg laid in the egg of its lepidopteran host divides clonally to produce a brood of genetically identical siblings that feed together inside the host caterpillar (Ivanova-Kasas, 1972). The wasp *Copidosoma floridanum* exhibits the most extreme form of polyembryony known (Silvestri, 1906; Patterson, 1921; Strand, 1989). Following cleavage of the egg, the embryonic cells of *C. floridanum* continue to proliferate for 9 days inside its host, *Trichoplusia ni*, creating as many as 2000 embryos from a single egg. This pattern of development poses at least two mechanistic problems for the operation of the segmenation gene cascade as understood in *D. melanogaster*. First, if cleavage is truly holoblastic, it would prevent diffusion of transcriptional regulatory factors essential for early *D. melanogaster* patterning. Second, the original anterior-posterior axis of the egg is likely lost during 9 days of proliferation and would have to be reestablished in each embryo. To begin characterization of axis formation in this polyembryonic insect, we asked the following questions: (1) does embryogenesis of *C. floridanum* occur within a completely cellularized environment, (2) is the *D. melanogaster* segmentation gene cascade conserved under these conditions, and (3) have elements of the *D. melanogaster* patterning system been co-opted for novel functions in forming multiple embryos per egg?

We found that embryonic development of *C. floridanum* occurs almost exclusively in a cellularized environment. The *D. melanogaster* segmentation proteins are not expressed during the early proliferative phases of polyembryony, but they are utilized in the final stages of development which result in the production of individual wasps. En and Ubx/Abd-A are expressed in a conserved pattern relative to *D. melanogaster*. However, the expression pattern of the pair-rule gene *Eve* is altered from that detected in *D. melanogaster*. We find no evidence of a pair-rule periodicity to Eve expression, rather, it is expressed exclusively in a segment-polarity pattern. We discuss the implications of these findings relative to the cellular and molecular processes underlying segmentation in insects.

MATERIALS AND METHODS

Animal rearing

C. floridanum and it host *Trichoplusia ni* were reared as described by Baehrecke et al. (1993). Host larvae were physiologically staged as described by Strand (1989).

Cell injections

Newly laid *C. floridanum* eggs were dissected from the host egg in physiological saline in a polyethyleneglycol (PEG)-treated Petri dish. They were transferred with a PEG-treated pipette tip into a nine-well glass dish in saline and treated with 100 μg/ml proteinase-K for 4 minutes. After treatment they were transferred to an injection well filled with TC-100 medium (JRH Scientific) and placed on an inverted Nikon Diaphot microscope with Hoffman modulation contrast optics. Iontophoretic injections were performed with an Intra 767 Electrometer (WPI) and Narashige micromanipulator. We used tetramethyl rhodamine or fluorescein dextran (3×10^3 M_r; anionic and lysine fixable) as tracers (Molecular Probes). Injection electrodes made from 1.0 mm o.d. Kwik-Fil Borosilicate glass capillaries (WPI) were pulled on a Sutter electrode puller to an approximate tip diameter of 0.1 μm. Microelectrodes were backfilled with 1 mg/ml of diluted dye and with 200 mM potassium chloride. Eggs were held using microforge-polished holding pipettes filled with corn oil, and observed under a 40× objective. Injections were performed with a few brief current pulses (5-15 nA). Cell injections in proliferative morula and embryonic primordia were performed using the same protocol with no proteinase-K step.

Phalloidin staining

Eggs were treated with 100 μg/ml of proteinase-K for one minute, fixed in 4% formaldehyde in PEM buffer (100 mM Pipes, 2. mM EGTA, 1 mM $MgSO_4$) for 20 minutes, and transferred to a PBS-Triton (0.5% Triton X-100; PBST) solution for 30 minutes. Embryos were then transferred to a 1:3 phalloidin 0.2 μM (Molecular Probes): PBST solution overnight at 4°C. Subsequently, embryos were washed twice (30 minutes each wash) in PBST, washed once in PBS and transferred to 80% glycerol supplemented with 4% isopropylgallate.

SEM

For scanning electron microscopy (SEM), embryos were fixed in 2.5% glutaraldehyde overnight at 4°C, briefly sonicated in PBS and dissected from the enveloping membrane using glass needles. Embryos were dehydrated through a graded series of ethanol, critical point dried, gold coated and examined using a Hitachi scanning electron microscope. Some embryos were fractured after dehydration in order to observe individual cells.

Antibody staining

To characterize antigen expression during *C. floridanum* embryogenesis, embryos were dissected in physiological saline at one day intervals from first to third instar *T. ni* and at 5 hour intervals from fourth and fifth instar *T. ni*. Embryos were transferred to a nine-well glass dish in saline and treated with 100 mg/ml proteinase-K for 1.5 minutes. Embryos were then fixed in 4% formaldehyde in PEM buffer for 18 minutes, sonicated briefly and fixed for another 10 minutes. Antibody reactions were carried out as described by Patel et al. (1994). The following concentrations of primary antibodies, diluted in PBST plus 1% BSA, were used: Eve (mAb2B8; Patel et al., 1992) 1:3, En (mAbEN4F11; Patel et al., 1989) 1:2 and Ubx/AbdA (mAbFP6.87; Kelsh et al., 1994) 1:3. Secondary antibodies (biotinylated horse anti-mouse IgG; Jackson) and rhodamine-conjugated streptavidin (Jackson) were diluted 1:200 in PBST. After completion of the antibody reactions, embryos were counterstained with FITC-conjugated phalloidin. For double labelling, antibody reactions were carried out sequentially. The first antibody was detected with donkey anti-mouse IgG conjugated to Cy5 (Jackson; 1:200 dilution). The second antigen was detected as described above. Embryos were observed and processed using a Biorad 6000 Laser Scanning Confocal Microscope as described by Paddock et al. (1993).

RESULTS

Embryogenesis of *C. floridanum*

To familiarize the reader with the unusual embryogenesis of

C. floridanum, we first provide a generalized description of development (Fig. 1). The life cycle begins when the adult wasp deposits her egg into the host's egg. The wasp egg is enclosed within a thin chorion which forms a small stalk at the anterior end. The first cleavage of the wasp egg occurs 2 hours after oviposition (Figs 1a, 2A) and forms three cells: two equally-sized blastomeres from cleavage of the zygotic nucleus and a large polar cell located anteriorly which contains a polar nucleus. Though polar body nuclei are aborted in most insects, they remain viable and form a polar body cell in *C. floridanum*. The second cleavage is unequal and one blastomere forms one large and one small daughter, while the second blastomere forms two equally sized blastomeres (Figs 1b, 2E). Both large and small cells contribute to future embryos. Subsequent cleavages are asynchronous. Meanwhile, the nucleus of the polar cell divides without cytokinesis, forming a syncytial compartment at the anterior that migrates as an extraembryonic membrane over the dividing blastomeres. The embryo then ruptures out of its chorion and continues development unconstrained by the egg shell. Approximately 24 hours after oviposition, the embryo is enveloped by the polar body-derived extraembryonic membrane. We refer to this structure as the primary morula (Figs 1c, 2G). The inner cells of the primary morula are the source of all embryos that subsequently develop.

The formation of the primary morula resembles early embryogenesis of other metazoans undergoing holoblastic cleavage. However, the next stages of development do not have a counterpart in embryogenesis of other non-polyembryonic insects or metazoan species. Prior to the hatching of the host larva, the cells of the primary morula continue to divide and the morula splits, forming several separate proliferating morulae. When the host larva hatches, these morulae collectively form a 'polymorula', which consists of many proliferating morulae surrounded by the extraembryonic membrane (Figs 1d, 3A). The polymorula increases in size during the host's first to third instars, as new proliferating morulae are formed (Figs 1e,f, 3B,C). At ecdysis to the host's fifth instar, the majority (>1000) of embryos begin to differentiate (Figs 1g, 4A). Each future embryo consists of approximately 20 cells surrounded by a syncytial membrane (Fig. 4B). As cells in each embryo continue to divide, the primordia form solid blastulas comprised of tightly interdigitated cells (Fig. 4D,E). At this stage morphological characters that define the anterior-posterior axis of the embryo appear (Grbić et al., unpublished data). Post-gastrulation development continues in a manner very similar to *D. melanogaster*, including segmentation, germband extension and retraction (Fig. 1h,i). Individual embryos within the polymorula are randomly oriented. The resulting larvae then consume the host, pupate and emerge as adults.

Cell injections

To assess whether *C. floridanum* embryogenesis proceeds entirely within a cellularized environment, we injected selected blastomeres with dextran-conjugated fluorescent dyes during all stages of embryogenesis. The same developmental stages were also stained with phalloidin to visualize the shape of cells and the boundaries between cells. When either blastomere of a 2-cell embryo was injected (n=25), dye diffused between them (Fig. 2A,B). Staining with FITC-conjugated phalloidin showed that on the surface of the embryo cleavage appeared complete (Fig. 2C), but a focal plane 5 μm deeper revealed that cleavage between the blastomeres and the polar cell was incomplete (Fig. 2D, arrows). Complete cellularization was initiated after the second cleavage. In thirty separate injections, we found that the small blastomere retained the dye (Fig. 2E,F). In contrast, the large blastomeres remained dye coupled to one another and the polar cell (Fig. 2F). Connections between the large blastomeres and the polar cell were detected by phalloidin staining (data not shown).

While the primary morula is a solid mass of approximately one hundred cells (Figs 1c, 2G), the number of cells present in individual proliferating morulae varies (Fig. 3A,B). For example, the morulae present in first instar hosts consist of several hundred rounded cells (Fig. 3B), whereas most morulae in third instar hosts consist of 20 to 30 cells. Both the primary morula and proliferating morulae lack all signs of the original anterior-posterior axis of the egg and show no obvious polarity. To assure that we adequately tested whether these stages were completely cellularized, we injected one cell per embryo using the following approach. For the primary morula stage, we successfully injected 62 embryos. In the first sample (n=25), we injected individual cells, selected randomly, around the outer perimeter of the morula, while in the second sample (n=27) we injected cells in the interior of the morula. In each case, dye remained confined to the injected cell (Fig. 2G,H). In the third sample (n=10), we injected the enveloping membrane surrounding the embryonic cells. When this was done, the dye always diffused throughout the membrane but never diffused into an embryonic cell (data not presented).

We then injected individual cells in 36 proliferating morulae. These morulae were in polymorulas dissected from second or third instar hosts (Fig. 3A). Since morula size varies during this period, we selected morulas of varying size located within the exterior and interior of the polymorula. Again, we injected peripherally located cells in approximately half the morulas (n=17) and cells within the interior of the others (n=19). In every case, dye remained confined to the injected cell (Fig. 3C,D). Lastly, we injected individual cells in embryos from fifth instar hosts (see Fig. 1g). At the 20 cell stage (see Fig. 4A,B) we injected cells in 30 embryos. The dye always remained confined to the injected cell (Fig. 4B,C). At the blastula stage, we injected selected cells at different positions along the anterior-posterior and dorsal-ventral axes of individual embryos (n=40). In each case dye remained in the injected cell (Fig. 4F). We conclude that, except for a brief period in early development, embryogenesis of *C. floridanum* proceeds in a cellularized environment. Molecules larger than our tracer (3×10^3 M_r) do not diffuse between embryonic cells, and only the extraembryonic membrane remains syncytial.

Eve expression in *C. floridanum*

We sought to determine whether the *D. melanogaster* segmentation gene cascade remains conserved in the absence of a syncytium and how it is modulated during the proliferative phases of polyembryony. Three monoclonal antibodies to the *D. melanogaster* segmentation and homeotic proteins Eve, En and Ubx/AbdA have been shown to recognize their cognate proteins in divergent arthropod species (Patel et al., 1989; Patel, 1994; Kelsh et al., 1994). We have used these antibodies to analyze segmentation in *C. floridanum*.

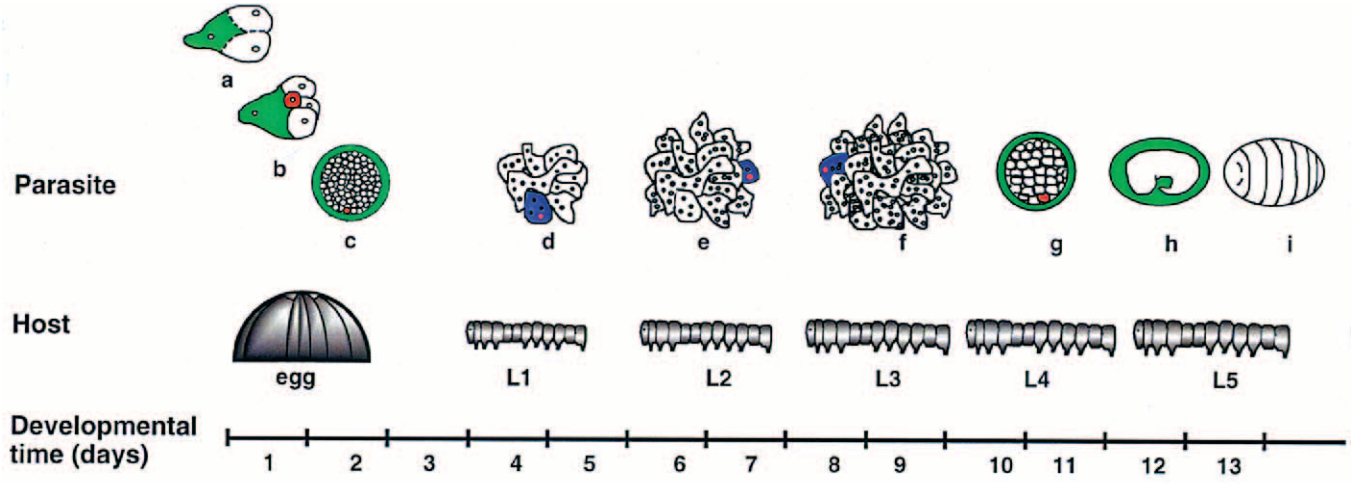

Fig. 1. Life cycle of *C. floridanum*. The top line diagrams the developmental stages of the parasitic wasp, the middle line diagrams the corresponding stages of the lepidopteran host (egg and larval instars; L1-5), and the bottom line indicates developmental time. The stages of wasp development where we injected cells that are dye uncoupled are marked in red (see Figs 2, 3, 4). The life cycle begins when the adult wasp deposits her egg into the host's egg. The first cleavage of the zygotic nucleus (a) results in two equally sized blastomeres. A third large, anterior cell (green) contains the polar body nuclei and gives rise to the extraembryonic membrane. The second cleavage (b) is unequal; one blastomere forms one small (red) and one large blastomere, while the other blastomere forms two equally sized progeny. Further cleavages are asynchronous, and after 24 hours a primary morula (c) is formed. The morula consists of embryonic cells surrounded by the extraembryonic membrane (green) derived from the polar cell. (d-f) After the host egg hatches, the primary morula becomes subdivided, creating progressively more proliferating morulae during the first through early fourth instar of the host. Individual proliferating morulae in a polymorula are marked in blue. In the host fourth instar (L4), proliferating morulae synchronously form individual embryonic primordia (g). During the early fifth instar of the host, the wasp embryos undergo germband extension and retraction (h), and are fully segmented 48 hours later (i). These images are not drawn to scale.

The first indication of segmental periodicity in *D. melanogaster* occurs when pair-rule genes such as *Eve* are activated in a pattern of seven-stripes, in alternating segments. Eve expression starts as a broad domain, missing only from the terminal regions of the blastoderm and resolves into the pair-rule stripes (one stripe for every two future segments) by repression of the inter-stripes. The *D. melanogaster* Eve expression later evolves from a pair-rule pattern to a segmentally reiterated pattern (Frasch et al., 1987; Lawrence et al., 1987).

We surveyed *C. floridanum* embryos over the entire course of embryogenesis for expression of the *Eve* antigen. We did not detect any Eve expression in eggs, primary morulas or proliferating morulas up to the host fourth instar (day 8). However, Eve expression was detected in individual embryonic primordia

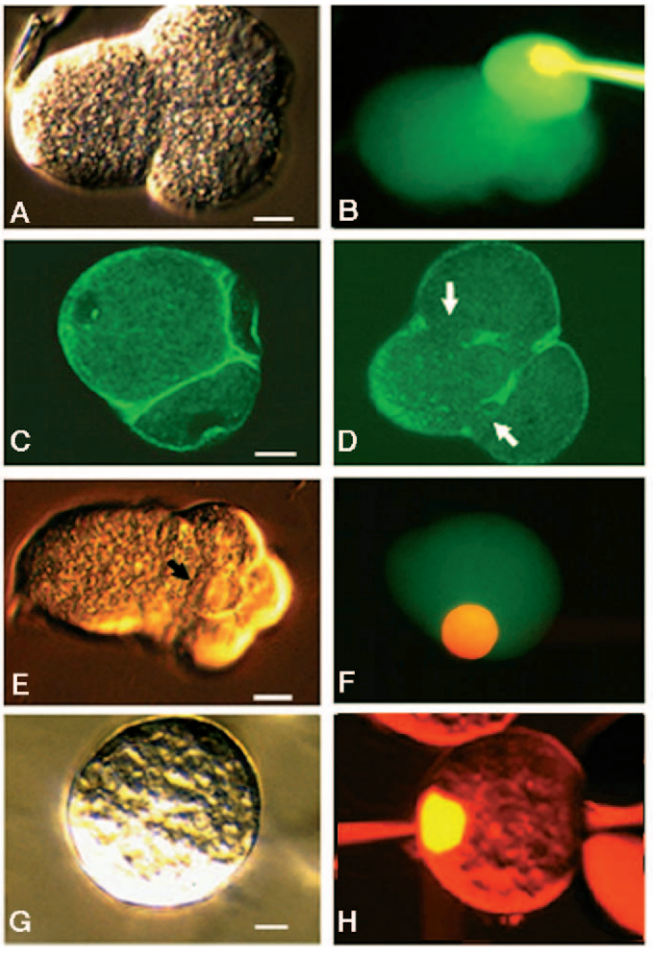

Fig. 2. Confocal, Hoffman and fluorescent images of egg cleavage and the primary morula stage. (A) The first cleavage of the *C. floridanum* egg (scale bar, 10 μm). (B) Injection of dye into a 2-cell stage blastomere (cells containing dye are green, high concentrations of the dye are yellow). Dye spreads first to the polar cell and then to the sister blastomere. (C) Cortical actin staining with FITC-conjugated phalloidin on the surface of the embryo (scale bar, 10 μm). (D) Same as C but with focal plane 5 μm deeper. Incomplete cleavage between the blastomeres is marked by arrows. (E) Hoffman image of the second cleavage with arrow marking the small blastomere (scale bar, 10 μm). (F) An embryo at second cleavage, injected with two dyes. The small blastomere (see Fig. 1b) was injected with rhodamine-conjugated dextran (orange) and the large sister blastomere was injected with fluorescein (green). Dye remains confined to the small blastomere but diffuses between the large blastomeres. (G) Hoffman image of the primary morula (scale bar, 18 μm). (H) Dye (yellow) remains confined to the injected cell (see Fig. 1c).

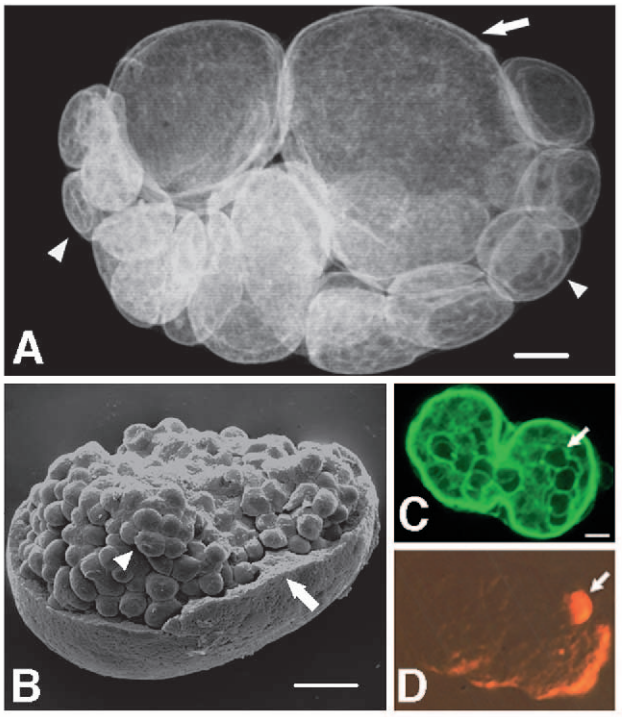

Fig. 3. Confocal, SEM and fluorescence images of the proliferating morula stage. Confocal image of a polymorula dissected from a first instar host. Arrow and arrowheads mark individual morulae within the polymorula. (B) SEM of a single proliferating morula from the polymorula (see Fig. 1d-f). Arrow marks the extraembryonic membrane and arrowhead marks a single embryonic cell (scale bar, 10 μm). (C) Confocal image of an individual proliferating morula stained with FITC-conjugated phalloidin (scale bar, 6 μm). Arrow marks a single cell. (D) Dye injected into this cell (arrow) remains confined to the cell.

at the end of the host fourth instar (day 9). Expression extended from the posterior end to 55% of the length of the newly formed primordium (Fig. 5A), resembling the early *D. melanogaster* Eve syncytial blastoderm pattern. As expected for a nuclear antigen, Eve staining was restricted to the nucleus. Unlike *D. melanogaster*, however, we found no evidence for a two-segment or pair-rule periodicity to Eve expression in *C. floridanum*. At the onset of gastrulation, the initial broad domain resolved directly into segmental stripes of Eve expression. Eve stripes formed sequentially, in a rapid anteroposterior progression, created by the disappearance of Eve from the interstripe regions (Fig. 5B). Individual Eve stripes were one to two cells wide, with three to four cells between the stripes. Stripes encircled the embryo completely. This is the same distance that separates individual Engrailed stripes as they form (see below) and is equivalent to a single segment. As the germband extended, increasing numbers of Eve stripes were detectable, followed by a zone of Eve expression in the tail region, in which the individual stripes had not yet resolved (Fig. 5C, the posterior Eve zone is between the two arrows). Once the germband was fully extended, however, the Eve pattern was detected in 15 stripes (Fig. 5D). Eve expression was not observed in the head. In the condensed germband, Eve was detected in several bilaterally paired neuroblasts (Fig. 5E), in a pattern that is conserved in all insects examined (Patel et al., 1992, 1994).

We were surprised to find that the Eve pair-rule expression pattern was missing while the segmentally reiterated expression was conserved. To ensure that we had not missed a temporally brief pair-rule expression pattern, we examined a large number of *C. floridanum* polymorulae (n=50) from the host fourth and fifth instar. Although each primordium within a polymorula initiates embryonic development synchronously, subsequent development of embyros becomes asynchronous. In each polymorula, therefore, we would see one to two thousand embryos at slightly different stages of development and Eve stripe formation. We were unable to detect a pair-rule pattern in any embryo at any time. We also note that in *D. melanogaster* and the beetles in which Eve expression has been described, the primary pair-rule stripes seem to narrow continuously from when they are first established. The primary stripes are roughly 4-5 cells wide (Frasch et al., 1987; Patel et al., 1992, 1994). These initially thick stripes narrow to 1-2 cells wide when Eve expression becomes segmentally iterated. Two distinct sizes of Eve stripes were not seen in *C. floridanum*. Stripes were one to two cells wide when formed and remained so throughout germband extension.

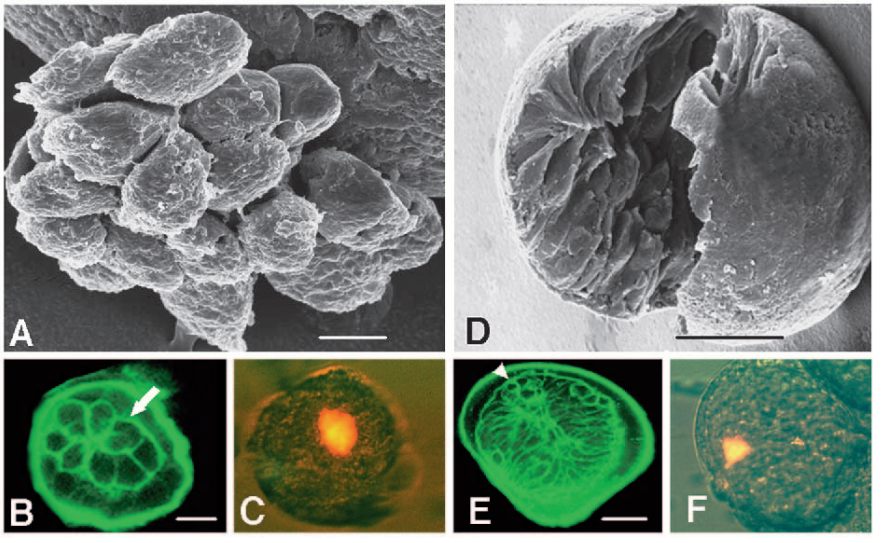

Fig. 4. Confocal, SEM and fluorescence images of embryos undergoing morphogenesis. (A) SEM showing the cells of a morula undergoing compaction to form an embryonic primordium (see Fig. 1g). The extraembryonic membrane has been removed (scale bar, 6 μm). (B) Confocal image of the same stage as A with the embryonic cell mass enveloped by membrane (scale bar, 5 μm). (C) Dye (orange) injected into a single cell of the embryonic primordium remains confined to the cell. (D) SEM of an embryonic primordium. The embryo is fractured to reveal the tightly interdigitated cells present immediately prior to morphogenesis (see Fig. 1g,h). The extraembryonic membrane has been removed (scale bar, 15 μm). (E) Confocal image of the same stage as (D) surrounded by the extraembryonic membrane (scale bar, 15 μm). (E) Arrowhead marks direction of the anterior-posterior axis. (F) Dye (orange) injected into a single cell of this stage remains confined to the cell.

Segment-polarity and homeotic gene patterns

We then examined the spatiotemporal relationship of Eve to expression of Engrailed (En), a *D. melanogaster* segment-polarity gene. In *D. melanogaster*, En is expressed in the posterior 1-2 cells of each segment primordia, and Eve is required for expression of all the En stripes (MacDonald et al., 1986). However, these stripes do not appear sequentially, rather the even-numbered segmental En stripes appear before the odd-numbered stripes (DiNardo et al., 1985) reflecting their regulation by *Eve* and other pair-rule genes (DiNardo and O'Farrell, 1987; Frasch et al., 1988; Manoukian and Krause, 1992). As expected, En staining was restricted to the nucleus in *C. floridanum*. However, the temporal appearance of the *C. floridanum* En pattern, after the appearance of the first two gnathal stripes, does not show a pair-rule influence. The mandibular and labial (first and third) En stripes appeared simultaneously (Fig. 6A). This was followed by the nearly simultaneous formation of the maxillary (second), thoracic and first abdominal stripes (Fig. 6B). Stripes appeared quite rapidly and did not emerge from a posterior growth zone as observed in some short germband insects. The remaining segmental stripes were expressed sequentially, in an anterior-posterior progression, resulting in the mature, 15 stripe En pattern in the fully extended germband (Fig. 6C). These 15 stripes corresponded to three gnathal, three thoracic and nine abdominal segments. In the fully segmented embryo, En marked the posterior of every segment, as it does in all arthropods examined (Fig. 6D). The segmental restriction of Eve expression preceded the onset of En expression. As the En stripes appeared, they coincided precisely with the Eve stripes (Fig. 6E).

To examine whether other, downstream elements of the *D. melanogaster* segmentation cascade were conserved, we used a monoclonal antibody that recognizes the expression pattern of both the Ultrabithorax (Ubx) and Abdominal-A (Abd-A) proteins (referred to as Ubx/Abd-A). The *Ubx* and *abd-A* genes are members of the homeotic selector group in *D. melanogaster*, responsible for the specification of metathoracic and abdominal segment identity (Lewis, 1978; Sanchez-Herrero et al. 1985). The earliest expression of *C. floridanum* Ubx/Abd-A was observed following gastrulation, in a broad abdominal domain (Fig. 6F). The initial expression of Ubx/Abd-A in the embryo was restricted to the prospective abdomen, excluding only the most terminal region of the trunk. This uniform domain resolved into eight stripes, generated by the fading of the antigen from a portion of each segment (Fig. 6G). When embryos were double-labelled with both Ubx/Abd-A and En, we observed that the anterior boundary of the expression domain for Ubx/Abd-A was the posterior compartment of the metathoracic segment, and the posterior boundary was the anterior compartment of the penultimate segment (Fig. 6H).

DISCUSSION

C. floridanum develops in a cellularized environment

We have shown that an injected dextran dye of 3×10^3 M_r does not diffuse between cells subsequent to the early cleavages of *C. floridanum*. This is the first rigorous demonstration that any insect species undergoes holoblastic cleavage and develops in a cellularized environment. The transcription factors that initiate the *D. melanogaster* segmentation cascade are all much larger molecules than our tracer. This suggests that *C. floridanum* develops in the absence of the syncytial environment previously thought to be essential for the operation of the *D. melanogaster* segmentation gene cascade. Despite the absence of a syncytium, En and Ubx/-Abd-A are expressed in a conserved pattern in the post-gastrulation stages of *C. floridanum* development. The expression of Eve however, is not completely conserved and lacks a pair-rule phase to its expression.

How ubiquitous is syncytial patterning in insects?

With the possible exception of the Collembola and certain parasitic Hymenoptera, early development of insects proceeds in a syncytium (Anderson, 1972). However, the precise stage at which the transition from a syncytial to a cellular environment occurs is unknown in most species. In long germband insects, such as *D. melanogaster*, cellularization occurs after the entire anteroposterior body plan has been established in the syncytial stages (Sander, 1976). In short germband insects, like the grasshopper, only the terminal portions of the embryo appear to be fated during the syncytial stage, while the rest of the body is added later, through proliferation in a presumably cellularized environment (Sander, 1976). As with *C. floridanum*, difficulties arise in explaining how the *D. melanogaster* segmentation gene cascade would function in the cellularized environment of a short germband insect. It has been suggested that patterning molecules could diffuse between neighboring cells in a proliferating germband (Patel, 1994). However, cell coupling in a short germband insect has not yet been tested. Alternatively, molecular information, albeit less refined, could be differentially distributed during the early syncytial stages to different parts of the germband. The final stages of segmental patterning could then occur cell autonomously (Nagy et al., 1994). If short germband embryos are indeed developing from a posterior growth zone, a third alternative is possible. Maternal transcriptional regulatory factors could be sequestered in a high concentration at the posterior pole of the embryo during the initial syncytial stages. As cells divide they could exit the growth zone with different concentrations of the maternal factors, thereby creating a concentration gradient. Downstream genes could then be regulated by dilutions of the maternal factors (Patel, 1994; Tautz and Sommer, 1995).

Patterning in the cellularized environment of the *C. floridanum* embryo is clearly different from short germband embryos and none of these proposed alternatives are likely to be operative. First, there is no syncytial stage prior to the formation of the embryonic primordium in which diffusion of transcription factors could occur. Second, we have eliminated cell coupling and passive diffusion between cells as a possible patterning mechanism during the entire course of embryogenesis. Finally, the enormous proliferation of cells that occurs during the proliferative stage of *C. floridanum* development makes activation of downstream genes by dilution of potentially localized maternal factors unlikely.

How is the *C. floridanum* patterning cascade activated?

We have shown that complete cellularization occurs after the

second cleavage in *C. floridanum* eggs, and thereafter high levels of cell proliferation occur before the formation of individual embryonic primordia. The proliferative phase of embryogenesis that occurs in polyembryonic insects does not have a counterpart in monoembryonic species. None of the antigens we examined (Eve, En or Ubx/Abd-A) were expressed during the proliferative period, indicating that these genes have not been co-opted for novel functions during this period. Nonetheless, both the morphology and some of the gene expression patterns of the *D. melanogaster* germband are conserved in the final stages of *Copidosoma* embryogenesis. It is likely that changes in the genetic program that led to polyembryony occurred upstream of the pair-rule genes, perhaps in programs that regulate the fate of polar bodies or the proliferation of embryonic cells.

The activation of the components of the *D. melanogaster* segmentation cascade in the absence of a syncytium returns us to the question of how axis formation is regulated in polyembryonic species. It is unlikely that *C. floridanum* evolved an entirely novel patterning system, since Hymenoptera are relatively closely related to Diptera and polyembryony has evolved independently in four families of parasitic Hymenoptera from monoembryonic ancestors (Ivanova-Kasas, 1972). As mentioned above, our data eliminates the possibility of intercellular diffusion or serial dilution of maternally localized factors that have been proposed as potential modifications to allow the *D. melanogaster* segmentation hierarchy to function in a cellular environment. It is still possible that transcription factors involved in patterning could be *specifically* translocated across the cell membrane, as has been demonstrated for a portion of the Antennapedia protein (Joliot et al., 1991). This would provide a mechanism to form a concentration gradient in the absence of a syncytium. Alternatively, patterning in the cellularized environment of a polyembryonic wasp could utilize cell-cell interactions. Primitive, short germband insects like the grasshopper are likely to be patterned at least in part by cell-cell interactions, as are the holoblastically cleaving ancestors of insects. Vertebrates, nematodes, some molluscs and horseshoe crabs utilize cell-cell interactions during axis formation (Van den Biggelar and Guerier, 1979; Itow et al., 1991; reviewed by Kessler and Melton, 1994 and Priess, 1994). In such systems, signal transduction cascades involving membrane bound receptors might function in the initial steps of differentially activating the conserved downstream elements of the segmentation cascade. Such a system may have become redundant with the evolution of syncytial development in insects.

Modulations of pair-rule genes in insect development

The expression of cognates of the *D. melanogaster* segmentation gene pathway have now been analyzed in several different species of insects which exhibit both short and long germband development (Patel et al., 1989; Fleig, 1990; Tear et al., 1990; Fleig et al., 1992; Nagy et al., 1992; Patel et al., 1992; Sommer and Tautz, 1993; Brown et al., 1994). It has been consistently observed that the final expression of the segment-polarity and homeotic genes is conserved, regardless of the type of embryogenesis. The segmentation gene pathway, however, appears to diverge at the level of the pair-rule genes. In *D. melanogaster*, a long germband insect, Eve is first expressed as a broad band in the syncytial blastoderm, followed by a pair-rule pattern of seven stripes in odd-numbered parasegments (Frasch et al., 1987; Lawrence et al., 1987). Eve then becomes segmentally iterated as seven additional stripes appear de novo in even-numbered parasegments. In *Tribolium*, a holometabolous short germband beetle, Eve also exhibits both a pair-rule and segmental expression pattern, although the stripes appear in a marked anterioposterior progression, with the segmental Eve pattern achieved by splitting the individual pair-rule stripes (Patel et al., 1994). However, in *Schistocerca*, a hemimetabolous, short germband grasshopper, Eve is never expressed in a periodic pattern (Patel et al., 1992). We found a fourth pattern of Eve expression in *C. floridanum*, where there is no double-segment periodicity to the Eve expression pattern and Eve is expressed only in a segmentally reiterated pattern.

En expression in *C. floridanum* is largely consistent with the lack of a pair-rule expression phase to the Eve antigen. In *D. melanogaster*, even-numbered En stripes appear before the odd-numbered stripes, reflecting its underlying pair-rule regulation (DiNardo et al., 1985). In the honeybee, *Apis mellifera*, En expression similarly lags in even numbered segments of the gnathal and thoracic region, but En stripes appear sequentially in the posterior germband (Fleig, 1990). In *C. floridanum*, only gnathal En stripes show alternate expression with all remaining stripes appearing sequentially in a rapid anterior to posterior progression. The lack of alternate expression of En in the posterior germband of *A. mellifera* and *C. floridanum* suggests that patterning in the Hymenoptera may differ from the Diptera. *A. mellifera*, however, also represents a monoembryonic, long germband ancestor of polyembryonic Hymenoptera. The further loss of pair-rule regulation in the thoracic region of *C. floridanum* therefore suggests additional changes in patterning have also occurred in the evolution of polyembryony.

It has been proposed that the function of Eve in primitive insects is as a terminal homeotic gene, consistent with its terminal expression pattern in vertebrates and its terminal position in the mouse homeotic gene cluster (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Patel et al., 1992; Joly et al., 1993). The grasshopper, which does not express Eve in a pair-rule or segment-polarity pattern, likely represents a primitive developmental pattern for insects and may not use the syncytial segmentation patterning seen in *D. melanogaster*. Yet, polyembryonic wasps are not only advanced holometabolous insects, but descendants of monoembryonic species that have a syncytial blastoderm (Ivanova-Kasas, 1961) and likely rely on syncytial patterning. We suggest the pair-rule expression of Eve may have been secondarily lost in polyembryonic insects, as a consequence of establishing segmental pattern in a cellularized environment.

Is *C. floridanum* a short germband insect?

Although Sander (1976) suggested that polyembryonic wasps undergo some form of short germband development, our results indicate that *C. floridanum* undergoes long germband development. First, Eve is expressed early in a domain similar to *D. melanogaster* and the mature stripe pattern is formed in a brief anteroposterior progression. Second, the En pattern also develops rapidly with simultaneous formation of two gnathal stripes followed by formation of the remaining stripes in a quick anterior-posterior progression. Third, germband

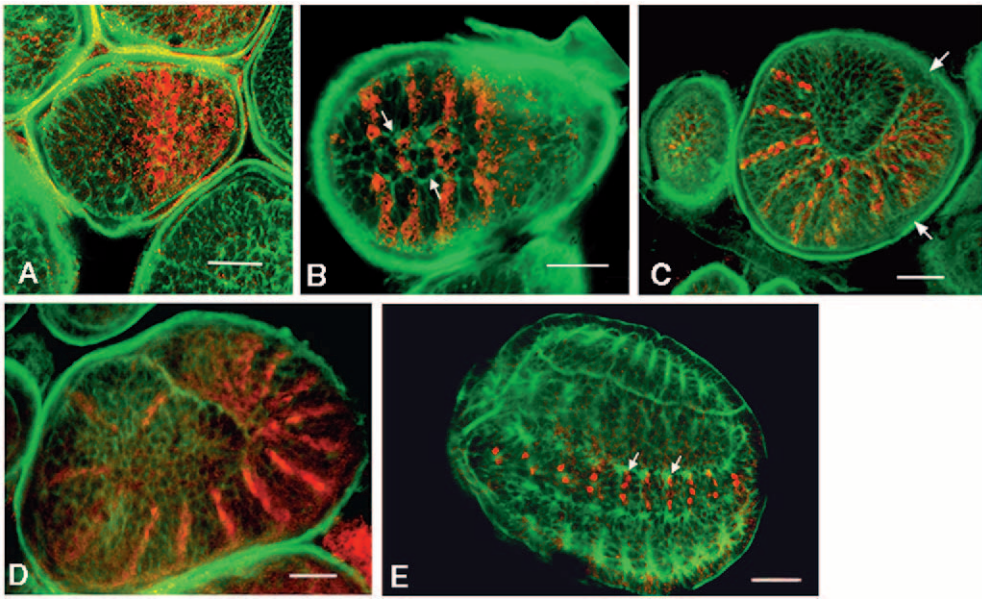

Fig. 5. *C. floridanum* Even-skipped protein expression patterns. Confocal images of embryos stained with the anti-Eve antibody mAb2B813 and counterstained with phalloidin (green). (A) Early Eve expression in the newly formed embryonic primordium. The posterior of the embryo can be recognized at this time by a cluster of cells not visible in this focal plane. (B) Eve expression at the onset of gastrulation. The three most anterior stripes of Eve are visible, followed by a continuous zone of Eve expression in the trunk, not visible in this focal plane. As the gastrulation furrow proceeds, the antigen is absent in gastrulating cells. Arrows mark the rounded, mesodermal cells of the ventral midline. Each Eve stripe is 1-2 cells wide with an interstripe space of 3-4 cells that extends to the dorsal midline of the embryo. (C) Eve expression in the extending germband. The zone of Eve expression in the trunk region from which stripes form in an anterior to posterior progression is marked by arrows. (D) Mature expression pattern of Eve in the fully extended germband. (E) Eve expression in neuroblasts that form bilaterally paired clusters of cells along the ventral midline (arrows). Anterior is to the left in all panels. B is a ventral view, C and D) are lateral views. As an embryo grows, it forms a snail-like coil with the presumptive head on top, visible in the middle of panels C and D. The rest of the embryo coils counter-clockwise with the terminus hidden from view. Scale bars, 25 μm.

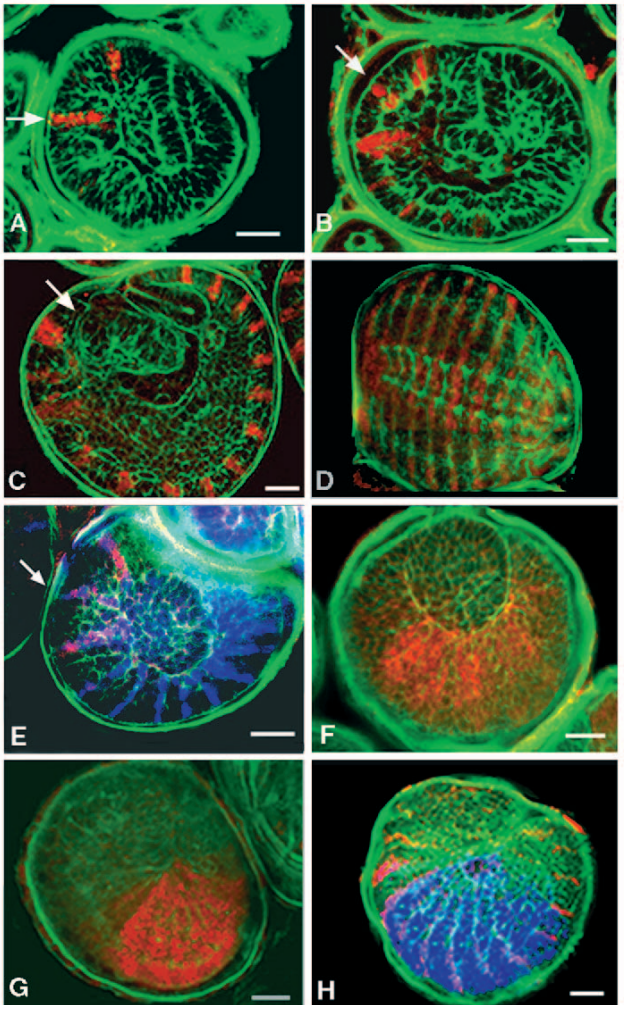

Fig. 6. *C. floridanum* En, Eve and Ubx/Abd-A protein expression patterns. Confocal images of embryos stained with the anti-Engrailed antibody EN4F11, anti-Eve or the anti-Ubx/Abd-A antibody mAbFP6.87 and counterstained with FITC-conjugated phalloidin (green). (A) En stripes are first expressed in the presumptive mandibular and labial (arrow) segments in the extending germband. Assignment of stripes to a particular segment are based on morphological criteria (Grbić et al., unpublished observations). (B) Simultaneous formation of the maxillary (arrow), thoracic and first abdominal stripes. (C) The En pattern in the fully extended germband with staining restricted to three gnathal, three thoracic and nine abdominal segments. Each En stripe is 1-2 cells wide. No expression is detected in the head which correlates with the reduction in larval head morphology of this species. An arrow marks the posterior boundary of the head. (D) The En pattern in a fully retracted germband. (E) An embryo double-labeled with anti-Eve (blue) and anti-En (red) at an early germband stage similar to A. At this time ten Eve stripes are detectable while En expression has just begun in the anterior segments (arrow marks maxillary segment). The En stripes coincide with the anterior Eve stripes with staining appearing pink. (F) The initial expression of Ubx/Abd-A in the extending germband. (G) The Ubx/Abd-A pattern takes on a striped appearance during germband extension. (H) Double-labeling of Ubx/Abd-A (blue) and En (red) in an embryo undergoing germband retraction. Anterior is to the left in all panels. D is a dorsal view after dorsal closure, while all the other images show a lateral view of the embryo. Scale bars, 25 μm.

elongation appears to be generated by a proportional elongation of the entire embryonic primordium, rather than growth from the posterior (Grbić et al., unpublished observations). Finally, the Ubx/Abd-A antigen is expressed in a temporal manner characteristic of other long germband insects (e.g *Manduca sexta* and *D. melanogaster*) with the antigen highlighting the entire putative abdomen (Akam and Martinez Arias, 1985; Nagy et al., 1992). In contrast, antigen is expressed in short germband insects, like the grasshopper, as segments form (Tear et al., 1990; Kelsh et al., 1994).

Conservation of the zootype

Inspired by the general conservation of homeotic gene expression patterns in several divergent metazoans, Slack et al., (1993) proposed that the animal kingdom can be defined by organisms that express a particular pattern of *Hox* genes, called the zootype. The zootype is predicted to be expressed at the phylotypic stage, a conserved developmental stage within each phylum. In all insects examined, the homeotic genes are expressed in roughly conserved domains (the zootype) during the extended germband stage (the phylotypic stage), regardless of the mode of segmentation. It was predicted that the zootype could be secondarily lost in some forms of parasitic development, whose life cycle and morphology are extremely modified (Slack et al., 1993). However, we find both the zootype and phylotypic stage to be conserved in *C. floridanum*. Although *C. floridanum* development prior to the formation of the germband is completely different than any described relative (Grbić et al., unpublished observations), the late embryonic developmental program becomes channeled towards the conserved developmental program, characteristic of the zootype. We conclude that the Ubx/Abd-A expression pattern in *C. floridanum* reflects the preservation of the zootype even in this extreme form of parasitic development.

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