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Development of polyembryonic insects: a major departure from typical insect embryogenesis

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Abstract The parasitic wasp *Copidosoma floridanum* represents the most extreme form of polyembryonic development known, forming up to 2000 embryos from a single egg. To understand the mechanisms of embryonic patterning in polyembryonic wasps and the evolutionary changes that led to this form of development we have analyzed embryonic development at the cellular level using confocal and scanning electron microscopy. *C. floridanum* embryogenesis can be divided into three phases: (1) early cleavage that leads to formation of a primary morula, (2) a proliferative phase that involves partitioning of embryonic cells into thousands of morulae, and (3) morphogenesis whereby individual embryos develop into larvae. This developmental program represents a major departure from typical insect embryogenesis, and we describe several features of morphogenesis unusual for insects. The early development of polyembryonic wasps, which likely evolved in association with a shift in life history to endoparasitism, shows several analogies with mammalian embryogenesis, including early separation of extraembryonic and embryonic cell lineages, formation of a morula and embryonic compaction. However, the late morphogenesis of polyembryonic wasps proceeds in a fashion conserved in all insects. Collectively, this suggests a lack of developmental constraints in early development, but a strong conservation of the phylotypic stage.

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Introduction

Most insects are terrestrial organisms that lay their eggs directly exposed to the environment. Adaptations for developing under such conditions include the presence of a strong chorion that protects the embryo from desiccation and an abundant yolk source to supply the nutrients required for development. Associated with this type of egg morphology, most insects undergo syncytial cleavage whereby nuclear division results in several thousand nuclei residing in a common cytoplasm before cellularization. These nuclei then migrate to the egg surface and form a cellular blastoderm. This blastoderm forms a single-layered epithelium overlying the yolk, which forms the primordium for future embryonic development. Future invaginations of the epithelium form the mesodermal and endodermal derivatives of the insect. This process is remarkably conserved among insects, although individual species can vary greatly in the size of the initial embryonic primordium that develops from the blastoderm (reviewed in Sander 1976).

Life history, however, plays a substantial role in shaping early development in many phyla (Strathmann 1985; Wray and Bely 1994; Wray 1995) and extreme modifications in early development have been described in insect taxa whose eggs develop under very different environmental circumstances. One such group are wasps that develop as internal parasites of other arthropods. Among these parasites are polyembryonic species that form anywhere from two to as many as two thousand individuals from a single egg (reviewed in Ivanova-Kasas 1972; Strand and Grbić 1997). Embryogenesis of polyembryonic wasps differs significantly from that of other insects, as would be expected if thousands of progeny are to develop clonally from a single egg. For example, unlike most insects, polyembryonic species undergo holoblastic cleavage such that embryogenesis proceeds in a

cellularized environment from an early stage (Grbić et al. 1996a). Another interesting feature associated with insect polyembryony is that the embryonic mass increases in size during the course of development (see Ivanova-Kasas 1972). This developmental trait is associated with mammals (Davidson 1990; Gurdon 1992) and is thought to occur rarely, if at all, in other metazoans. Since polyembryony in insects has arisen exclusively in endoparasitic taxa, it is likely that developmental adaptations associated with an endoparasitic lifestyle have resulted in conditions that favored the evolution of polyembryony (Strand and Grbić 1997).

Polyembryony in parasitic wasps was first described using light microscopy at the beginning of the century (Silvestri 1906; Patterson 1921; Leiby 1922), and more recently by Baehrecke and Strand (1990), Grbić et al. (1992) and Baehrecke et al. (1993). The polyembryonic wasp, *Copidosoma floridanum*, represents the most extreme form of polyembryony reported to date in the literature. This species typically produces up to 2000 embryos from a single egg (Grbić et al. 1992; Baehrecke et al. 1993). *C. floridanum* oviposits its egg into the egg stage of the moth *Trichoplusia ni*. The wasp egg is small (60 μm), devoid of yolk, and undergoes complete cleavage to form a single embryo (Grbić et al. 1996a). After the host egg hatches, the *C. floridanum* embryo proliferates in the host larva to form thousands of embryos. When the host larva reaches its penultimate (fourth) instar, these embryos initiate morphogenesis and ultimately develop into adult wasps. How embryonic polarity is established in each of these 2000 clonal progeny following such massive cell division remains unknown.

Despite the fact that early development of *C. floridanum* proceeds in a cellularized environment, genes that regulate the late stages of embryonic patterning are expressed in a conserved periodic pattern relative to *Drosophila* (Grbić et al. 1996a, reviewed in French 1996). The mechanism by which these conserved genes establish their periodicity within individual embryos, when any recognizable remnant of the original egg polarity is lost, remains unknown.

Polyembryony also poses an interesting problem in the evolution of developmental patterning mechanisms. Polyembryony has likely evolved four independent times within the Hymenoptera, and in each case polyembryonic species are fairly closely related to monoembryonic species (circa 40 million years divergence time; see Strand and Grbić 1997). This repeated and fairly rapid evolution of such an extreme modification of development suggests that the changes required to make the transition to polyembyrony might be discovered by comparing the morphogenesis and patterning mechanisms of different species of Hymenoptera. It is not possible to reconstruct a complete course of polyembryonic development from the existing literature. To understand the mechanisms regulating embryonic patterning in polyembryonic wasps and the evolutionary changes that have led to polyembryony requires detailed information of the cellular events that occur during embryogenesis. Here we characterize the development of *C. floridanum* on a cellular level using a combination of confocal and scanning electron microscopy (SEM).

Materials and methods

T. ni were reared on an artificial diet at 27°C and a photoperiod of 16 h light:8 h dark as described previously (Baehrecke and Strand 1990). Staging of parasitized *T. ni* and *C. floridanum* embryos followed established criteria (Strand et al. 1991; Baehrecke et al. 1993; Grbić et al. 1996a). Host eggs parasitized by *C. floridanum* hatch in 3 days and the parasitized host larva develops through five instars over a 13-day period. The first to fourth instars of the host are each 2 days in duration, whereas the fifth instar is 5 days long. To characterize early embryogenesis in *C. floridanum*, wasp eggs were dissected from host eggs at 1-h intervals and transferred to an observation chamber filled with TC 100 medium (JRH Scientific) supplemented with 10% fetal bovine serum (HyClone). Subsequent stages of *C. floridanum* embryogenesis were collected by dissecting host larvae at selected intervals in each instar. All embryos were thereafter observed using a Nikon Diaphot inverted microscope equipped with Hoffman optics.

Wasp eggs were treated with 100 μg/ml of proteinase K (Promega) for 1 min to remove the outer egg envelope. Phalloidin (Molecular Probes) was dissolved in 1.5 ml of methanol according to the manufacturer's instructions. A working solution was made by first evaporating 25 μl of the phalloidin-methanol stock solution in the dark in a microfuge tube and then resuspending the phalloidin in 200 μl of phosphate buffered saline (pH 7.4; PBS; Grbić et al. 1996a). Eggs were fixed in 4% formaldehyde in PBS for 20 min followed by permeablization in PBT (PBS-Triton; 0.5% Triton) for 30 min. Embryos were transferred into a 1:3 phalloidin:PBT solution overnight at 4°C. Embryos were washed twice for 30 min in PBT, rinsed in PBS and then transferred to 80% glycerol supplemented with isopropylgallate to retard fading of fluorescent dyes. Embryos stained with phalloidin as described above were also incubated in chromomycin (20 μg/ml in PBS) and 10 mM $MgCl_2$ overnight. After 3–4 h of infiltration in glycerol, embryos were mounted on microscope slides. Nuclei were stained with TOTO (Molecular Probes), according to the manufacturer's protocol.

Embryos that ultimately develop into adult wasps are called reproductive larvae (Grbić et al. 1992; see Results section below for more detail). Individual reproductive larvae are identifiable at 20 h of the host's fourth instar (0% development) and complete embryogenesis at 50 h of the host's fifth instar (100% development; Baehrecke et al. 1993). During this period embryos were collected at 5-h intervals. Half of each sample was prepared for SEM while the other half was stained as described above and examined with a Biorad 6000 confocal microscope. Each sample corresponded to 7.5% of the total developmental time. However, because considerable overlap occurred between each sample, we were able to examine much shorter intervals of development. For SEM, embryos were fixed in 2.5% glutaraldehyde overnight at 4°C, briefly sonicated in PBS and hand dissected with glass needles. Embryos were dehydrated through a graded series of ethanol, critical point dried, gold coated and examined with a Hitachi 570 SEM.

Results

Below, we describe the embryogenesis of *C. floridanum*. In order to follow the developmental events associated with polyembryony, it is critical for the reader to understand the relationship between developmental stages of the wasp and developmental stages of its host, *T. ni*. To facilitate this process, we summarize the major embryological events described below in relation to absolute

Table 1 Development of *Copidsoma floridanum* in relation to days (AEL into the host egg) and stage of development of *Trichoplusia ni*

Days (AEL)	Host stage (*T. ni*)	Wasp stage (*C. floridanum*)
1–2	Egg	Egg cleavage, formation of a primary morula.
3–4	First instar larva	Primary morula transforms into the polymorula. Proliferation of cells within proliferative region of the polymorula. Morphogenesis and emergence of precocious larvae from the precocious region of the polymorula.
5–6	Second instar larva	Continued proliferation of cells within the proliferative region of the polymorula. Continued morphogenesis and emergence of precocious larvae from the precocious region of the polymorula.
7–8	Third instar larvala	
9	Fourth instar larva	Cessation of cellular proliferation and the morphogenesis of precocious larvae.
10	Fourth instar larva	Initiation of morphogenesis in reproductive larvae (0% development). Compaction of embryonic primordia and formation of blastula stage embryos.
11	Fifth instar larva	Dorsal furrow and germband formation.
12	Fifth instar larva	Germband uncoils and completes segmentation (100% development).
13	Fifth instar larva	Eclosion of reproductive larvae.
14–30	Fifth instar larva	Consumption of host by reproductive larvae (3 instars), pupation of reproductive larvae, emergence of adult wasps.

age (in days after egg laying (AEL) of the wasp egg) and host stage in Table 1.

Early cleavage is total and results in formation of a primary morula

Immediately after oviposition into the host egg, the wasp egg was pear-shaped with a clear polarity. Both the polar body nucleus and the zygote nucleus were located at the narrow end of the egg. In most insect embryos the anterior egg pole can be defined as the end from which the head of the embryo develops. However, in *Copidosoma* it was not possible to determine the relation of polarity of the egg to the polarity of the resultant embryos. In all previous publications on polyembryonic development, the narrow end of the egg has been referred to as the anterior pole and we will follow that convention here. Immediately after oviposition the zygote nucleus separated from the polar body nucleus (Fig. 1A). The zygote nucleus then migrated to the posterior pole of the egg along with the oosome, an organelle implicated in germ cell determination in other hymenopterans (Fig. 1B; Silvestri 1906; Hegner 1914; Patterson 1921; reviewed in Jeffery 1983). Note that in male hymeopteran embryos, the zygote nucleus is haploid, while in females it is diploid. The oosome (Fig. 1B, open arrowhead) does not stain with either nuclear dye or phalloidin and appears as a dark circular area when viewed by confocal microscopy.

Two hours AEL the *C. floridanum* egg initiated first cleavage (Fig. 1C). At this time the oosome associated with one of the two zygote nuclei and segregated into one blastomere following cleavage (Fig. 1C, open arrowhead). Cleavage furrows initiated from the posterior midline of the egg and extended laterally to one third of the egg length, forming two equal sized blastomeres. This cleavage furrow was incomplete, as revealed by gaps in cortical actin staining (Grbić et al. 1996a). This incomplete division leaves an undivided anterior "polar region" where the polar bodies reside (Fig. 1D; Silvestri 1906). Unlike the polar bodies produced in the eggs of most other insects, the polar bodies in *C. floridanum* do not degenerate (Fig. 1D, arrowhead).

The second cleavage began 3 h AEL and is unequal. One blastomere forms both a large and a small daughter blastomere, while the other forms two blastomeres of equal size (Fig. 1E). The small blastomere (arrow) was the first cell to form a complete cleavage furrow and become dye-uncoupled from the rest of the embryo (Grbić et al. 1996a). In addition, the oosome always segregated to the small blastomere. From this point on it was not possible to follow the fate of the oosome.

During the third egg cleavage, the large blastomeres divided synchronously at 3.5 h AEL, whereas the small blastomere cleaved at 4 h AEL (Fig. 1F). Subsequent cleavage events were asynchronous. While progeny of the large blastomeres maximize contact with each other, the two daughter cells arising from the small blastomere remain rounded (Fig. 1F, arrows).

Formation of an enveloping extra-embryonic syncytial layer

In contrast to the cellularized region, which gave rise to the embryo proper, the nuclei of the polar region divided without cytokinesis to form a multinucleated syncytial cell at the anterior of the egg (Fig. 1F, arrowhead).

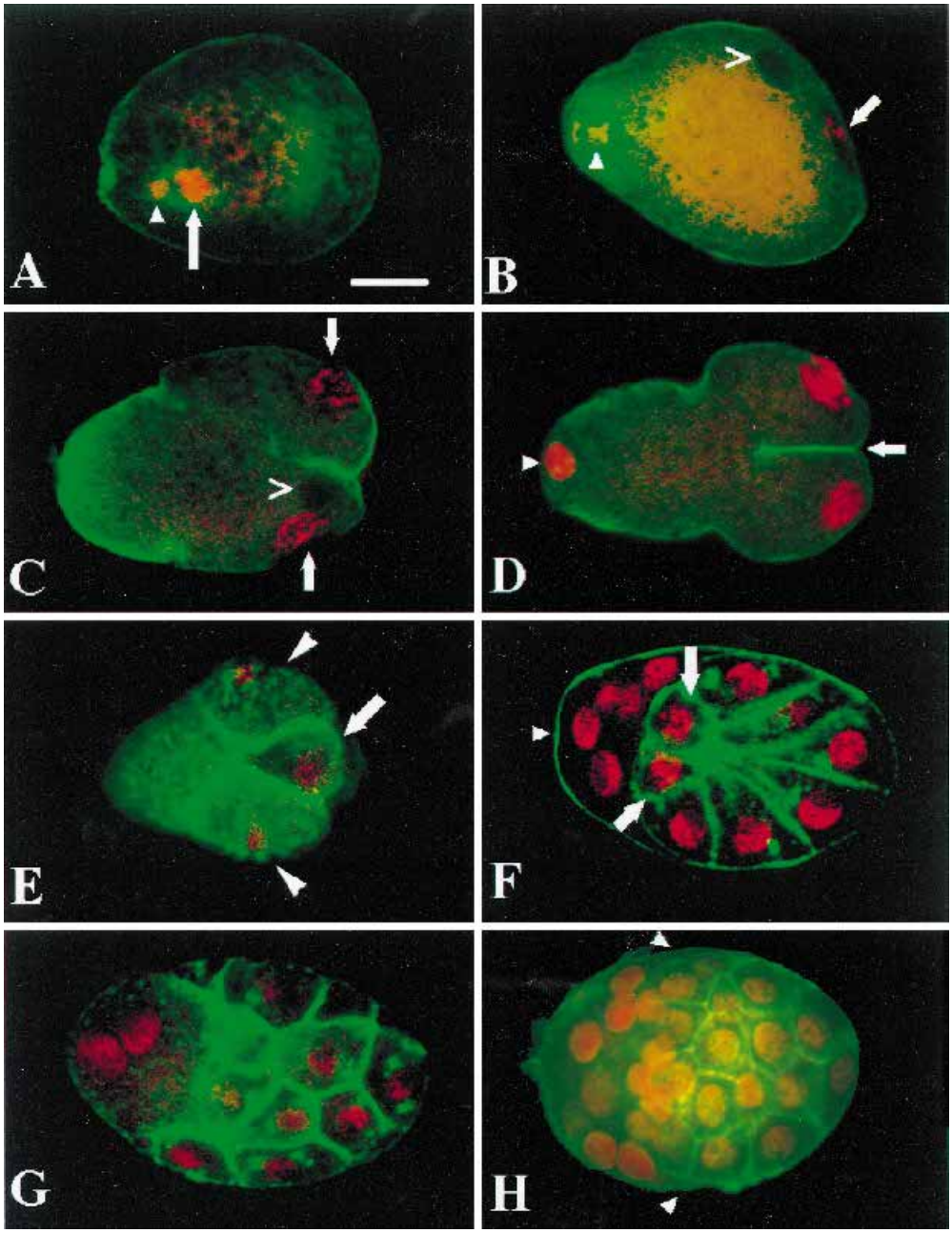

Fig. 1A–H Early cleavage of the *Copidosoma floridanum* embryo. Confocal images of phalloidin (*green*) and nuclear TOTO (*red*)-stained embryos. **A** The uncleaved egg of *C. floridanum* immediately after oviposition (*scale bar* 26 μm; scale bar is the same for all panels). The *arrowhead* marks the polar body and the *arrow* marks the pronucleus. **B** Migration of the pronucleus and oosome to the posterior of the egg. The polar body is marked by the *arrowhead*, oosome with the *open arrowhead* and nucleus with the *arrow*. **C** The initiation of cleavage of the *C. floridanum* egg. Note that the oosome (*open arrowhead*) becomes associated with one of the nuclei (*arrows*). **D** Cleavage furrow extends to one third of the egg. The *arrow* marks the cleavage furrow and the *arrowhead* the polar body. **E** The second egg cleavage. One blastomere gives rise to two equal sized blastomeres (*arrowheads*) and its sister blastomere forms one small (*arrow*) and one large blastomere. The edge of the fourth (large) blastomere is visible *at the right* (*beneath the arrow*); it nucleus is out of focus. **F** The third cleavage of the *C. floridanum* egg. Progeny of the small blastomere are marked by *arrows*. The polar body nucleus has divided several times within the polar syncytium (*arrowhead*). **G** The fourth cleavage. **H** Growth of the polar syncytium. *Arrowheads* mark edges of the polar syncytium as it initiates envelopment of the cellularized part of the embryo. Note the absence of visible actin accumulations in the polar syncytium. Anterior is *to the left* in all images

This cell will form an extra-embryonic syncytial covering of the developing embryonic primordium. After the fourth cleavage the syncytial cell began to envelop the cellularized part of the embryo by migrating around the perimeter of the cellularized area (Fig. 1G, H). This syncytial cell undergoes a remarkable change in shape: it originally forms a cap at the anterior pole of the egg and ultimately expands to cover the entire embryo. Surprisingly, during the onset of its migration over the embryo, there were no visible accumulations of f-actin, as might be expected for a cell that was crawling over the remainder of the embryo (Fig. 1G, H). At this point the embryo ruptured from the chorion and from this point onward was no longer constrained to a regular shape (Fig. 1H).

As the polar syncytium envelops the embryo proper, the shape of the embryo itself changes from its formerly regular ovoid shape to an elongate and irregular form (Fig. 2). This change in shape of the embryo began when the extra-embryonic syncytial cell was halfway around the embryo (Fig. 2A). Interestingly, at this time accumulations of f-actin became detectable at a single point of the leading edge of the syncytial cell (Fig. 2B; also visible at the bottom of Fig. 2A). Embryonic cells positioned between 40 and 60% of the length of the embryo began to change their shape, and became elongated and spindle-shaped (Fig. 2C, D). Phalloidin staining revealed that these elongated cells form cytoplasmic protrusions suggestive of active movements. No cell division could be detected at this stage (data not shown).

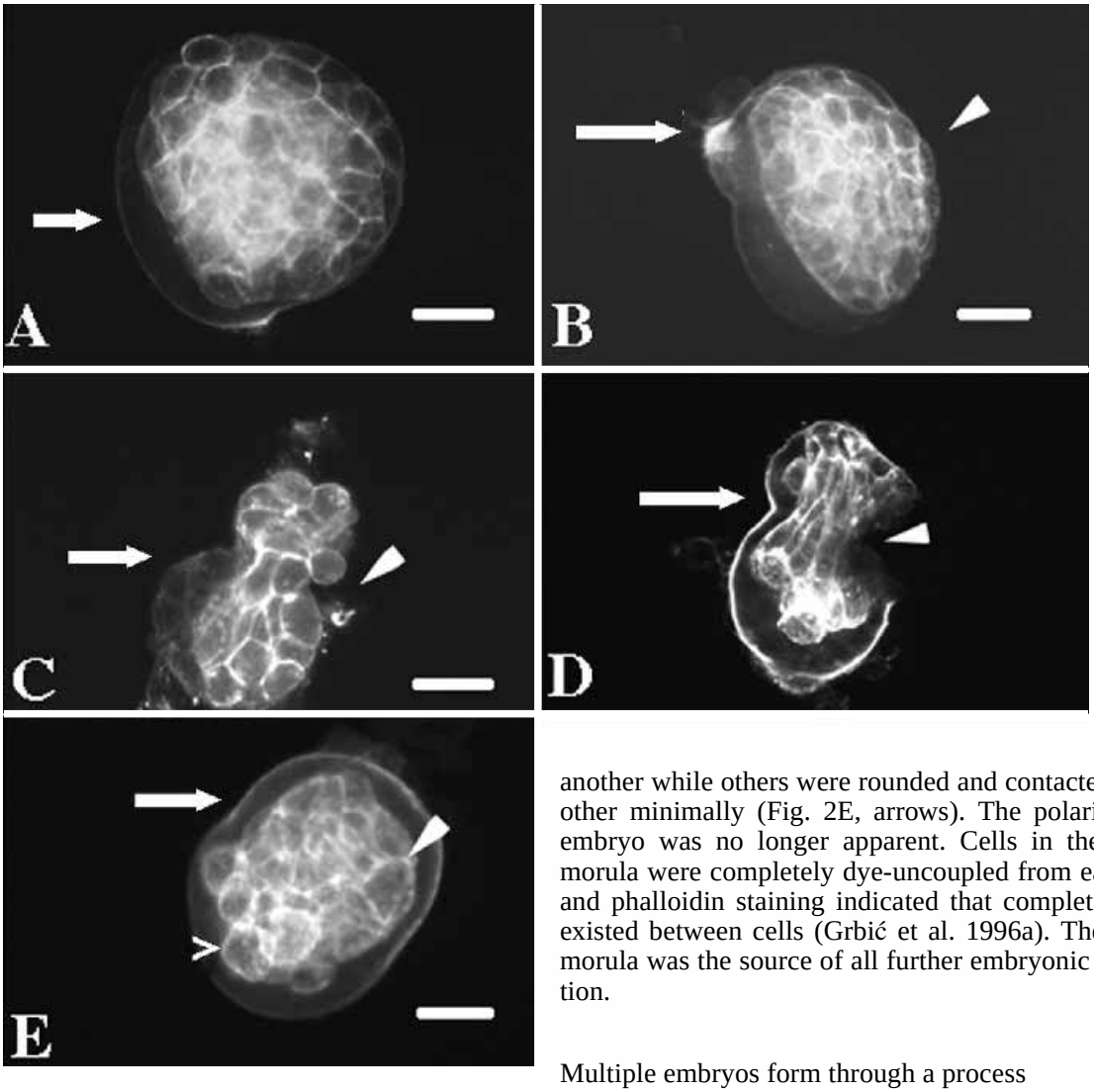

Fig. 2A–E Formation of the primary morula. Confocal images of phalloidin-stained embryos. In all panels the *arrows* indicate the extra-embryonic syncytium and the *arrowheads* mark embryonic cells. **A** Extra-embryonic syncytium (*arrow*) surrounds half of the cellularized region (*scale bar* 40 μm). **B** The extra-embryonic syncytium initiates a series of movements to envelope the cellularized part of the embryo (*arrowhead*). Note actin acumulation in the leading edge of the extra-embryonic syncytium (*arrow*, *scale bar* 43 μm). **C** This leading edge extends over the embryo proper (*arrow*). Embryonic cells in the middle of the cellular mass begin to elongate, while cells at either end maintain a rounded shape (*arrowhead*, *scale bar* 45 μm). **D** The embryonic cells elongate and become spindle-shaped as the extra-embryonic syncytium nearly surrounds the embryo (*scale bar* 43 μm). **E** The primary morula (24 h AEL). The extra-embryonic syncytium fully surrounds the morula (*arrow*). Embryonic cells with rounded morphology are marked by the *open arrowhead*; the *arrowhead* marks elongated and interdigitated embryonic cells (*scale bar* 43 μm)

After 24 h the polar syncytium completely enveloped the future embryonic cells to form a primary morula, which again assumed the shape of a regular ovoid (Fig. 2E). The spindle-shaped cells had disappeared, and the primary morula consisted of a heterogeneous population of cells. Some cells showed close attachments to one another while others were rounded and contacted one another minimally (Fig. 2E, arrows). The polarity of the embryo was no longer apparent. Cells in the primary morula were completely dye-uncoupled from each other, and phalloidin staining indicated that complete furrows existed between cells (Grbić et al. 1996a). The primary morula was the source of all further embryonic proliferation.

Multiple embryos form through a process of proliferation and subdivision

At 48 h AEL, the enveloping syncytial cell began to invaginate into the primary morula (Fig. 3A). This partitions the embryonic cells of the primary morula into smaller clusters, each surrounded by a portion of the original syncytial cell. From this stage through the four larval instars, the embryo is referred to as a proliferating morula. This proliferation followed by partitioning continued until an assemblage of morulae, called the polymorulae, was formed (Fig. 3B).

The number of morulae present and the overall size of the polymorula increased during the host first to third instars (Fig. 3B–D; Table 1). Individual cells within each morula formed loose clusters consisting of several hundred rounded cells which showed no surface specializations or filopodia (Fig. 3D) and exhibited only limited areas of cell-cell contact. Nuclear staining revealed a random pattern of embryonic cell division in each morula. No apparent regions of increased mitotic activity across the polymorula were detectable (not shown).

All new morulae produced during proliferation arose from the invagination of the enveloping syncytial cell

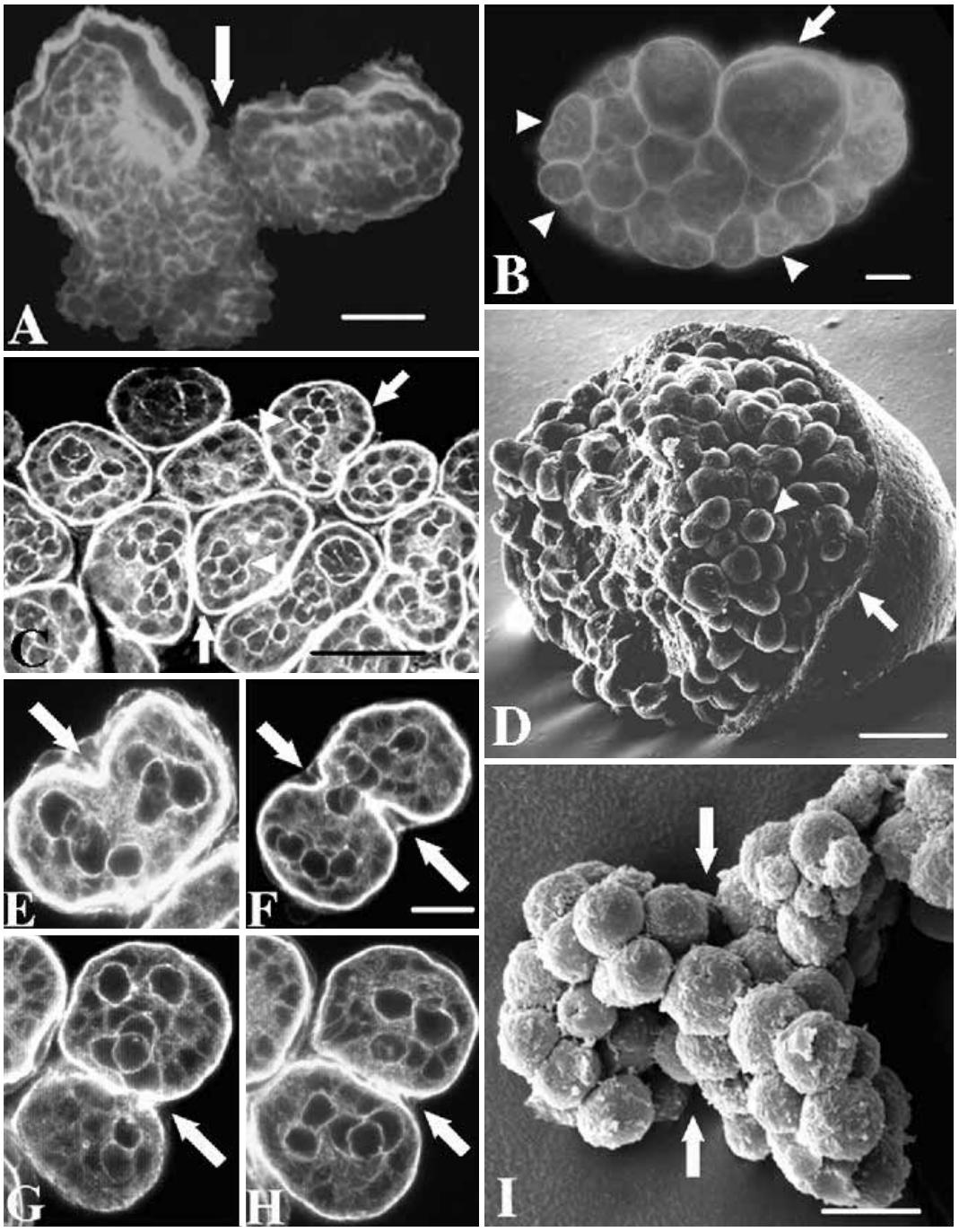

Fig. 3A–I Proliferative phase of development. Scanning electron micrographs and confocal images of phalloidin stained embryos. **A** Splitting of the primary morula. Ingrowth of the polar syncytium, which separates newly formed morulae, is marked by the *arrow* (*scale bar* 32 μm). **B** A polymorula dissected from the host first instar larva (*scale bar* 150 μm). The region of the polymorula undergoing proliferation is marked by the *arrowheads* and the region undergoing morphogenesis is marked by the *arrow*. **C** Higher magnification of the proliferating region of a similarly staged polymorula (*scale bar* 30 μm). Numerous proliferative morulae are surrounded by the extra-embryonic syncytium (*arrow*). The *arrowheads* mark the cells within an individual proliferative morulae. **D** SEM of a single morula extracted from a polymorula of the same stage of development as shown in **B** and **C**, then fractured in half. The extra-embryonic syncytium is marked by the *arrow* and individual embryonic cells by the *arrowhead* (*scale bar* 9 μm). **E** The initiation of partitioning of a morula. Two cell clusters are being separated by ingrowth of the enveloping syncytium (*arrow*). **F** Invagination of the enveloping syncytium (*scale bar* 10 μm; E–H are all depicted at the same magnification). The syncytium grows from opposite sides (*arrows*). **G** Fusion of the extra-embryonic syncytium during the separation of cell clusters (*arrow*). **H** Complete separation (*arrow*) of cell clusters results in formation of two morulae. **I** SEM of cell clusters undergoing separation (*scale bar* 5.5 μm). The extra-embryonic syncytium has been completely removed. Embryonic cells within each cluster are rounded and adhere to one another, while adjoining clusters barely contact one another. *Arrows* mark where the enveloping syncytium would invaginate and separate the two cell clusters

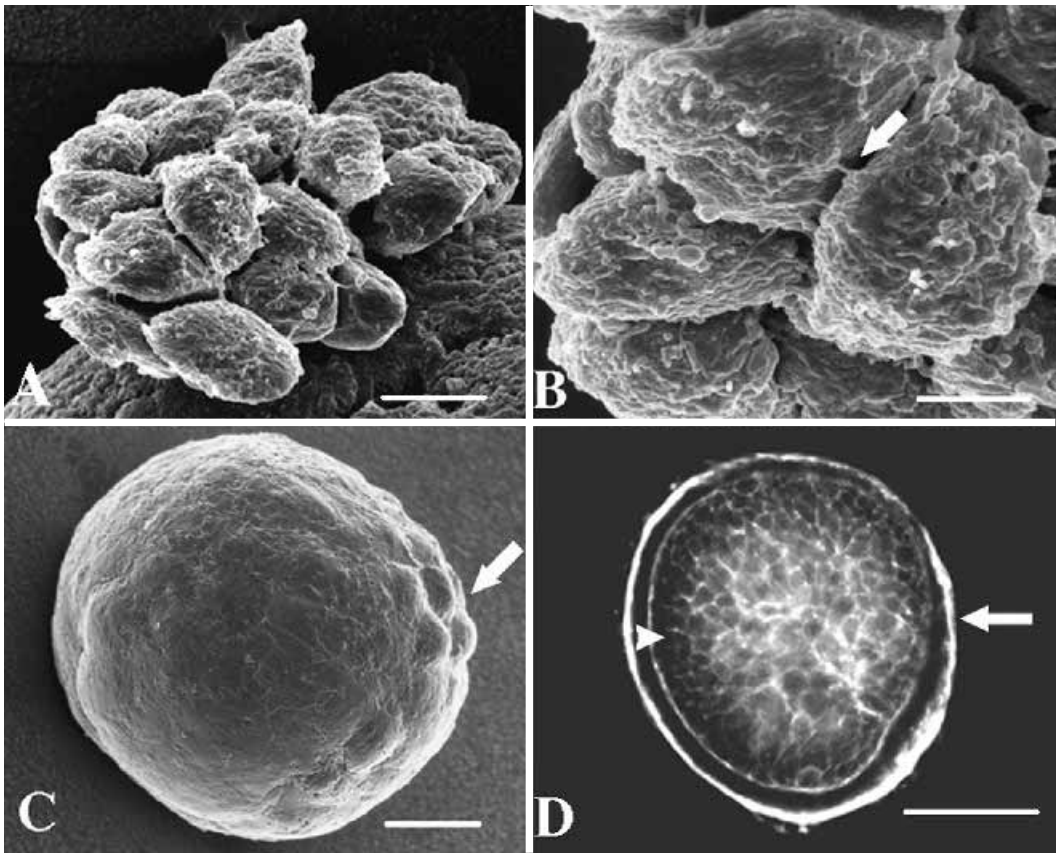

Fig. 4A–D Primordium formation in reproductive embryos. Scanning electron micrographs and confocal images of phalloidin-stained embryos. **A** Initiation of embryonic primordium formation (0% development; *scale bar* 5 μm). Cells are acquiring an elongate shape, with filopodia-like extensions. **B** High magnification of cells forming the embryonic primordium (*scale bar* 1.7 μm). Cells adhere tightly . Filopodia are marked by the *arrow*. **C** Compaction of the embryonic primordium (*scale bar* 15 μm). The cluster of rounded cells marks the posterior of the embryo (*arrow*). **D** Compacted primordium (*arrowhead*) enveloped by the extra-embryonic syncytium (*arrow*; 7.5% development; *scale bar* 30 μm)

which partitions the embryonic cells into smaller, physically separated clusters. This process was initiated with the ingression of the syncytial cell in the approximate center of the morula, separating the morula cells into two loosely connected clusters (Fig. 3E, F). Phalloidin staining revealed that the leading edge of the enveloping cell accumulates actin filaments, typical of a normal cleavage furrow separating two cells in monoembryonic insects (Fig. 3E, F). The ingression resulted in the formation of two new proliferating morulae (Fig. 3H). It is possible that some other cell-intrinsic mechanism also contributes to the separation of morulae, as in some preparations the embryonic cells appear to have separated into two clusters prior to the ingression of the syncytial cell (Fig. 3E). These cells remained rounded and did not show any evidence of shape change that might be associated with active movements. This process repeats itself an undetermined number of times during the first, second and third host instar. Moreover, continued subdivision of individual morulae by this process resulted in progressively smaller numbers of embryonic cells per morula (Fig. 3I).

Regional specialization of the polymorulae

By the time the host first instar larva hatches from its egg, the polymorula is usually located in the thoracic region of the host's hemocoel, and is attached to tracheal branches or larval hypoderm by the extra-embryonic syncytial cell (Strand and Grbić 1997). Polymorulae dissected from first instar host larvae exhibit two regions of specialization. One region contains embryos undergoing morphogenesis: the "precocious" region (Fig. 3B, arrow), while the other region contains morulae that show no signs of undergoing morphogenesis and are continuing to proliferate: the "proliferative" region (Fig. 3B, arrowheads).

The embryos that undergo morphogenesis during the host's first to fourth instar develop into precocious larvae (Grbić et al. 1992). The morphology and behavior of precocious larvae differs from the approximately 2000 reproductive larvae that develop from the proliferative region of the polymorula (see below and Table 1). The two regions of specialization in the polymorula persist through the fourth host instar, resulting in a total of 50–100 precocious larvae (Table 1; Grbić et al. 1992). The details of morphogenesis of precocious larvae will be reported elsewhere. Development of precocious larvae ceases during the host's fourth instar when all presump-

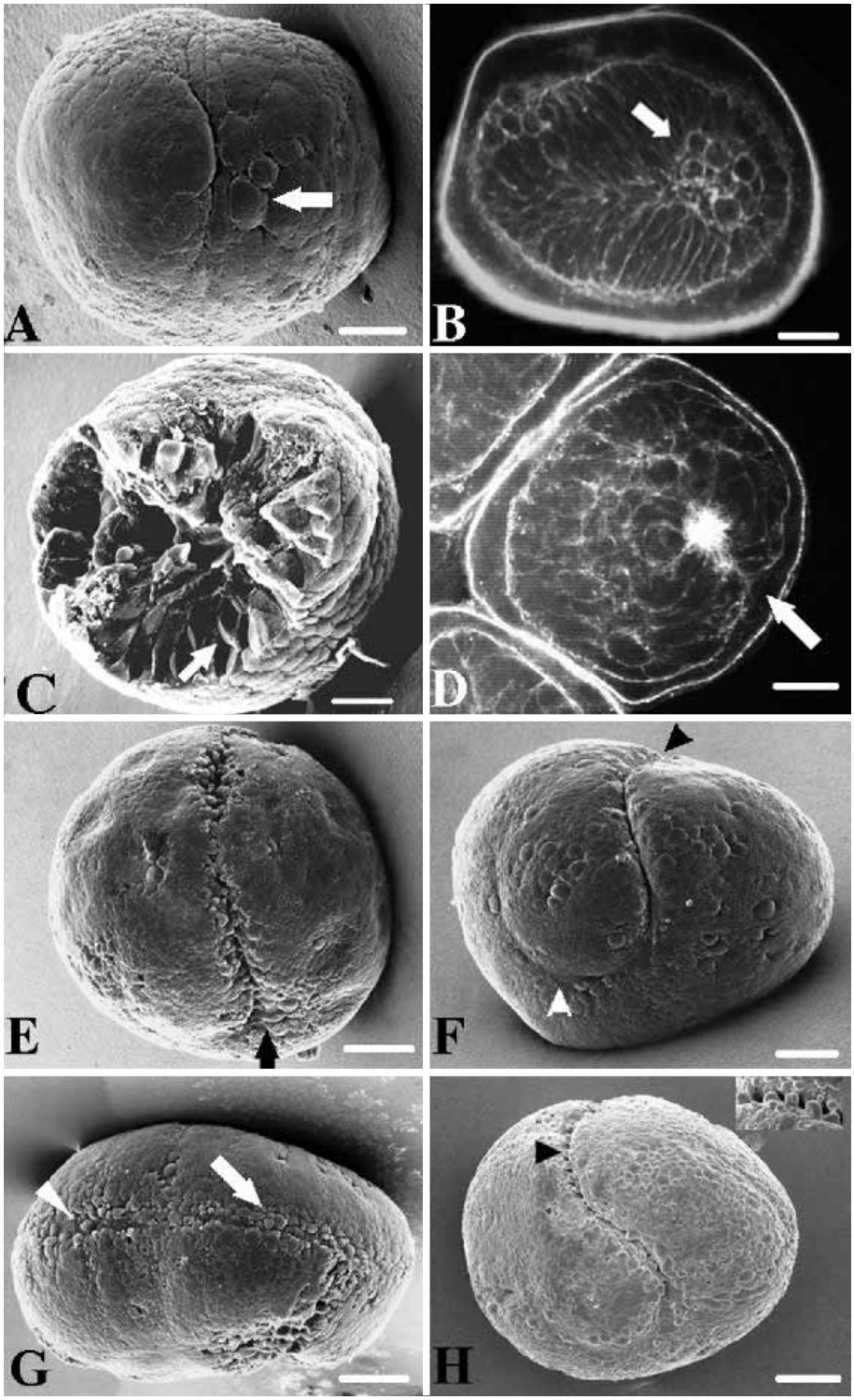

Fig. 5A–H Morphogenesis of *C. floridanum* embryos. Scanning electron micrographs and confocal images of phalloidin-stained embryos. **A** Elongation of the embryonic primordium. Sinking rounded cells of the posterior cluster are marked by the *arrow* (15% development; *scale bar* 6.5 μm). **B** A deeper optical section of the same developmental stage. The posterior cluster (*arrow*) is located dorsally (*scale bar* 6.5 μm). **C** Fractured blastula stage that reveals the tightly interdigitated cells present immediately prior to morphogenesis and the absence of a blastocoel or yolk cavity (*scale bar* 8 μm). **D** Formation of transverse dorsal furrow (lateral view). Phalloidin staining showing actin accumulation in the cells forming the dorsal furrow (22.5% development; *scale bar* 6.5 μm). Note that this was previously mistakenly identified as the ventral furrow (Baehrecke and Strand 1990). **E** SEM of a dorsal view of the same stage of development (*scale bar* 10 μm). Cells forming the dorsal furrow form cytoplasmic extensions (*arrow*). **F** Initiation of germ band formation (*scale bar* 12 μm). The *white arrowhead* marks the cephalic region and the *black arrowhead* marks the tip of the tail (30% development). **G** SEM of the ventral side of an embryo undergoing gastrulation. Gastrulating cells are marked by the *arrow* and the median furrow formed after the cells sink into the interior is marked by the *arrowhead* (*scale bar* 10 μm). **H** Completion of gastrulation. Cellular projections are visible on the cells that mark the anterior and posterior extremities of the germ band (*arrowhead*; 37–52% development; scale *bar* 12 μm). *Inset*: high magnification of cellular projections

tive reproductive larvae synchronously initiate morphogenesis (Grbić et al. 1992; Baehrecke et al. 1993).

Morphogenesis of reproductive larvae

Morulae proliferation ceased during the first day of the host's fourth instar (Table 1). By the second day of the host's fourth instar approximately 2000 morulae were present in the polymorula with each morula consisting of approximately 20 cells (Fig. 4A). At this stage of host development, the embryonic cells within each morula changed from a rounded to an elongate morphology with filopodia-like extensions (compare Figs. 3D and 4A). This transition in cell shape marks the initiation of morphogenesis (0% development) and formation of approxi-

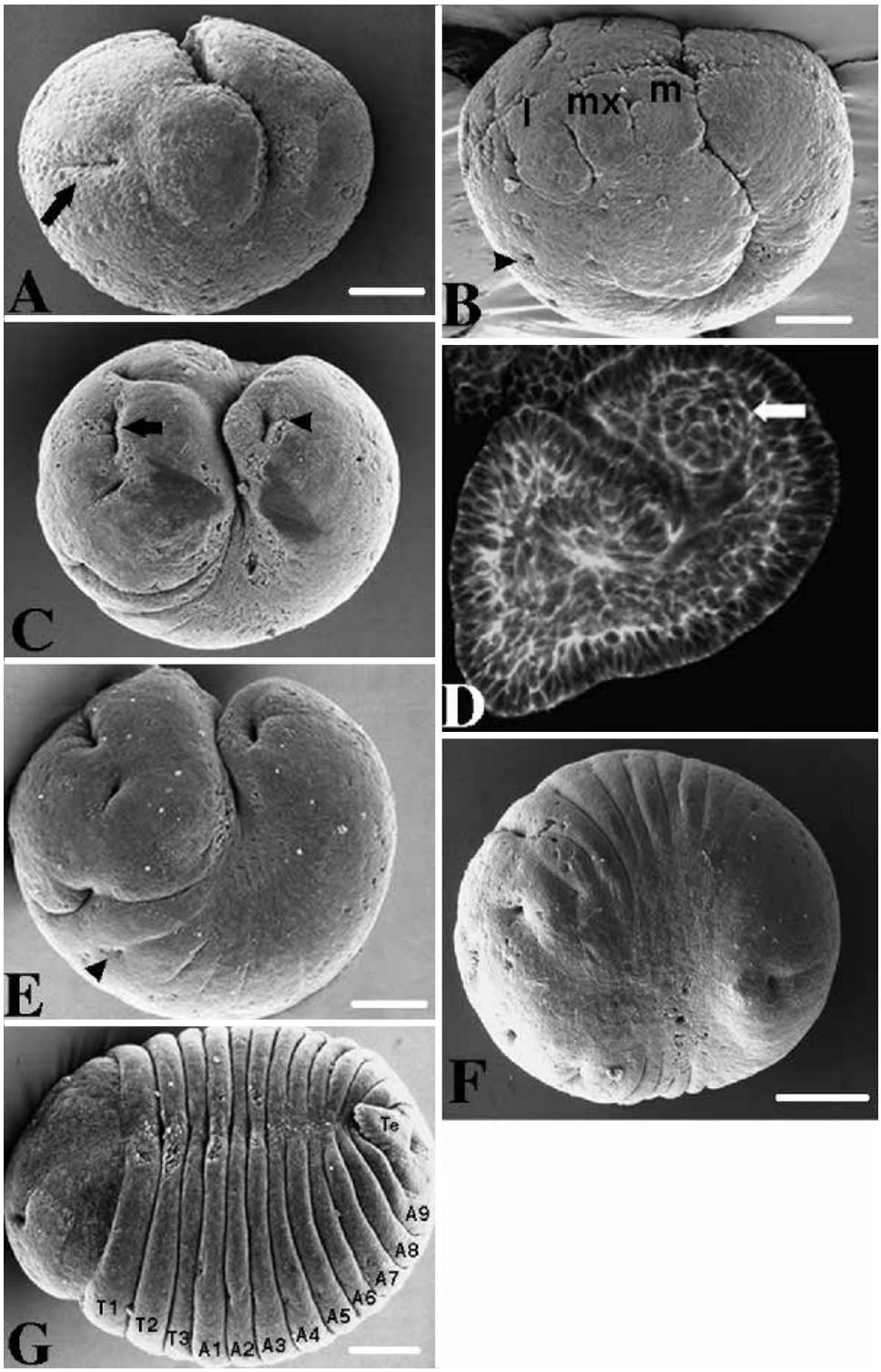

Fig. 6A–G Segmentation of *C. floridanum* embryos. Scanning electron micrographs and confocal images of phalloidin-stained embryos. **A** Onset of visible segmentation. The labial furrow is marked by an *arrow* (60% development). **B** Lateral image of gnathal segments. Mandibular (*m*), maxillar (*mx*) and labial (*l*) segment. Tracheal pits are marked by the *arrowhead*. **C** Dorsal view of the embryo beginning to uncoil. The stomodeum is marked by the *arrow*, proctodeum by the *arrowhead*. **D** Longitudinal optic section through an embryo the same stage as in **C**. The *arrow* points to the posterior cluster of rounded cells. **E** Thoracic segmentation. Thoracic segments are formed in a brief anterior-posterior progression. Note the position of the tracheal pits in relation to the segmental grooves (*arrowhead*). **F** Complete uncoiling of the germband. **G** The completely segmented embryo. Mouthpart segments are involuted and are not visible. Three thoracic segments (*T1–T3*), nine abdominal segments (*A1–A9*) and the telson (*Te*) are marked (*Scale bars* 36 μm)

mately 2000 embryonic primordia. Within each embryo, individual cells extended filopodia toward their nearest neighbor (Fig. 4B). The majority of cells in each embryo adhered tightly to one other, maximizing the area of cell-cell contact, resulting in compaction of the embryonic primordium. The only exception to this was a cluster of cells at the presumptive posterior of the primordium (the posterior cluster) that remain rounded (Fig. 4C). Each embryonic primordium remains enclosed within a separate enveloping syncytium during compaction and all subsequent embryonic events (Fig. 4D).

Embryos next enter the blastula stage. The blastula was completely filled with embryonic cells and lacked a blastocoel cavity typical of vertebrate embryos, or a yolk-filled cavity typical of monoembryonic insects (Figs. 4D, 5B, C). The dorsal and ventral sides of the

embryo quickly became apparent after this stage. The embryonic primordium elongated along the anterior-posterior axis and then bent or buckled to form a dorsal invagination (Fig. 5A). This invagination lead to the formation of a transverse dorsal furrow. After this invagination, the posterior cluster of cells were no longer visible on the external surface of the embryo, and came to lie underneath the presumptive tail of the embryo (Fig. 5A, arrowhead). Phalloidin staining revealed that cells in the posterior cluster remain rounded and in loose contact with the other blastula cells (Fig. 5B). Cells involved in dorsal furrow formation accumulate actin, as detected by staining with FITC-conjugated phalloidin (Fig. 5D). The cells lining either side of this furrow have irregular surfaces with cellular extensions. The cells on the dorsal anterior side of the furrow remain detached from their neighboring cells on the dorsal posterior side of the embryo. The dorsal furrow separates the future head and tail (Fig. 5E).

Following formation of the dorsal furrow, for the first time in their development, the embryos assumed a shape typical of the monoembryonic insect germ anlage. The anlage of each embryo was comprised of bilaterally symetric head lobes followed by a protocorm, or the gnathal and trunk forming region. Each embryo was coiled, with the head region touching the tip of the tail (Fig. 5F). Cells in the cephalic region became rounded and bulged out laterally, outlining the head lobe. A furrow (Fig. 5F, white arrowhead) demarcated the boundary between the head lobe forming region and the remainder of the body.

Head lobe formation was followed by the initiation of gastrulation. In the early phase of gastrulation, parallel rows of cells from the anterior region of the ventral midline become rounded, and separate from the tight epithelium formed by the lateral, non-invaginating cells (Fig. 5G). The rounding of midline cells extended along the ventral midline towards the posterior. The rounded cells ingressed in a brief anterior-posterior progression, leaving behind a flat, shallow gastrulation furrow (Fig. 5G, arrowhead). During the course of gastrulation, nuclear staining did not reveal a distinct proliferation region and mitotic figures were randomly distributed throughout the epidermis (not shown). The cytoplasmic projections along the transverse dorsal furrow remained visible throughout germ band coiling (Fig. 5H, arrowhead; inset: high magnification of cell extensions).

Once the gastrulation furrow reached the posterior of the embryo, segmentation was initiated in the gnathal region (Fig. 6). Cells in the posterior of the gnathal region delaminated and formed a furrow defining the posterior border of the labial segment (Fig. 6A, arrow). Shortly thereafter, furrows delineating the maxillary and mandibular segments formed (Fig. 6B). Simultaneous with gnathal segmentation, cells in future trunk segments sank between the epidermal cells, forming the tracheal pits (Fig. 6B, arrowhead).

The embryo then began to uncoil, a process resembling germband retraction in monoembryonic insects. At the onset of uncoiling, the dorsal cells lost their projections (Fig. 6C). As the embryo uncoiled, the stomodeal invagination, anterior to the presumptive mandibular segment, became apparent as a slit (Fig. 6C, arrow) lying beneath the bulging cells of the presumptive labrum. At the posterior end, the proctodeal invagination was also visible (Fig. 6C, arrowhead). The segment grooves extended dorsally and met at the dorsal midline (Fig. 6C,F,G).

The posterior cluster of cells compacted but did not adhere to any other tissue, forming a ball of cells that remained at the dorsal posterior region of the abdomen (Fig. 6D, arrow). The process of germ band uncoiling changed the shape of the embryo from coiled to “flat”, and clearly separated the head from the tail (Fig. 6E, F). The stomodeal opening shifted more posteriorly and the gnathal region became condensed. Mandibular and maxillar segments disappeared, while the paired cephalic folds formed laterally (Fig. 6E). Finally, the labium sank into a cleft formed by the first thoracic segment.

Condensation of the gnathal region was followed by the segmentation of the thorax and abdomen. Prothoracic segmental bulges appeared first, followed immediately by the formation of the meta- and mesothoracic segments, in a manner typical of long germband embryogenesis. Segmental furrows initially were most prominent in the lateral regions (Fig. 6E). Tracheal pits were always located at the anterior portion of future segments. As uncoiling continued, the abdominal segments formed sequentially in a rapid anterior to posterior progression (Fig. 6F). A complete set of segments ultimately formed, corresponding to three thoracic and nine abdominal segments, plus the telson (Fig. 6G).

Discussion

Embryonic development of *C. floridanum* differs strikingly from most other insects. First, most insect eggs contain large amounts of yolk, and early development proceeds in a syncytium where up to 6000 nuclei reside in a common compartment before cellularization (Schwalm 1988). In contrast, *C. floridanum* eggs lack yolk and complete cellularization of the embryo begins at the second cleavage with all subsequent development proceeding in a cellularized environment (Grbić et al. 1996a). Second, polar bodies usually degenerate in insects and do not participate in embryonic development (Anderson 1972). However, in *C. floridanum* polar bodies form an extra-embryonic syncytium that first surrounds the primary morula and later partitions embryonic cells during proliferation to form increasing numbers of proliferating morulae. Previous studies indicate that the extra-embryonic structures produced by polyembryonic wasps and some endoparasitic, monoembryonic wasps play an important role in uptake of nutrients from the host (reviewed by Tremblay and Caltagirone 1973; Koscielski and Koscielska 1985). Third, the proliferative phase of development that *C. floridanum* undergoes has no counterpart among monoembryonic insects. Fourth,

morphogenesis in a majority of other insects involves extensive folding of the blastoderm (Schwalm 1988). In contrast, rather than forming a “blastoderm” on the surface of the yolk mass, *C. floridanum* embryos form a compact blastula that lacks a blastocoel cavity.

Convergent evolution in response to development in another organism

Comparing embryogenesis of *C. floridanum* with other taxa outside the Arthropoda, we find many analogies with early embryogenesis of mammals. During mammalian embryogenesis, the separation of an extra-embryonic lineage from that of the embryo proper occurs early (Cross et al. 1994). In *C. floridanum* the first cleavage event also separates the future extra-embryonic lineage from the embryonic lineage. The blastocyst stage of mammals arises when undifferentiated cells of the inner cell mass become surrounded by an extra-embryonic trophectoderm. Likewise, the primary morula of *C. floridanum* consists of an extra-embryonic syncytial cell (which in the older literature is referred to as a “trophamnion”; see Silvestri 1906; Patterson 1921) surrounding an undifferentiated mass of cells. In mammals, trophoblast cells are extremely invasive and anchor the placenta to the uterine wall. Similarly, the extra-embryonic syncytium of *C. floridanum* anchors the embryo to host tissues such as trachea and fat body (Strand and Grbić 1997). Finally, the compaction of embryonic cells that occurs during the onset of morphogenesis of *C. floridanum* resembles compaction of the mammalian embryo. Polyembryonic development resulting in a net increase in embryonic mass occurs in the wasp – a developmental feature thought to occur only in mammalian embryos (Davidson 1990; Gurdon 1992). Another coincidence is that the armadillo, (mammal, genus *Dasyphus*) displays obligate polyembryony that is also achieved by the early division of the embryo (Patterson 1927).

Obviously, the embryonic characteristics shared between polyembryonic wasps and mammals have little or nothing to do with ancestry, given the phylogenetic distance between the vertebrates and arthropods. However, the environment in which mammalian and polyembryonic wasp embryos develop share several common features. Mammalian eggs are small and devoid of yolk, and after implantation, embryos take up nutrients from the uterine environment begining at an early stage of development. *C. floridanum* eggs are a similar size to mouse eggs (50 μm) and also lack yolk. The embryos of polyembryonic wasps also develop in intimate association with host tissues, and absorb nutrients by way of the extra-embryonic “membrane” (Koscielski and Koscielska 1985). Therefore, mammmalian and polyembryonic wasp embryos have evolved similar adaptations to a common environment via convergent evolution.

It remains to be seen whether the similarities observed at the cellular level are also reflected at the molecular level. Despite the striking differences between early development of *C. floridanum* and other insects, development from formation of the germband (the phylotypic stage in insects) onward, resembles morphogenesis in other insects. This suggests a strong evolutionary constraint on the phylotypic stage. Embryonic development of *C. floridanum* provides another example of how early phases of embryonic development can be quite variable, without consequences for adult morphology, since adult wasps have a similar morphology to their syncytially developing monoembryonic relatives (see Sander 1976; Raff 1996, for many examples in other animals).

Mechanisms regulating the evolution of polyembryonic development

While accidental polyembryony occurs commonly in animals (Cappe de Bailon 1927; Counce 1968; Kelly 1977; Gardiner and Rossant 1976; Laale 1983), obligate polyembryony is only known to occur in selected species of arthropods and five other phyla (Bell 1982; Hughes and Cancino 1985). Experimental evidence also reveals that individual blastomeres isolated early in embryogenesis have the potential to form complete embryos in sea urchins and frogs (Driesch 1892; Speman 1938). This suggests that if blastomeres are prevented from receiving the cell-cell signalling information that normally restricts their fate, they have the potential to form a whole organism. A similar genetic system, whereby cells are set aside from signals to differentiate and therefore remain in an undifferentiated state capable of continued proliferation, could function in the production of imaginal cells in larvae of a variety of species (Davidson et al. 1995).

The evolution of arthropod polyembryony is consistent with such a scenario. All species of polyembryonic arthropods are insects from two orders: the Hymenoptera (wasps) and Strepsiptera. Both orders are comprised of predominantly endoparasitic species that lay small, yolkless eggs in which cellularization appears to occur immediately (Silvestri 1906; Lieby 1924; Parker 1931; Noskiewicz and Poluszynski 1935). One of the genetic changes underlying the origins of polyembryony could be a delay in the onset of cell-cell interactions involved in differentiation, sufficient to keep the embryonic cells in an undifferentiated and proliferative state.

The development of *C. floridanum*, in particular, is consistent with such a scenario. As in many insects, one of the first zygotic determination events in *C. floridanum* separates extra-embryonic from embryonic cells (see Foe 1989). However, in *C. floridanum*, unlike other insects, this is the only cell-type determination that results in early cellular differentiation. We speculate that the next determination event separates germ cells (or oosome-containing cells) from non-germ cells. Subsequent to this there is a critical event (or events) that occurs in polyembryony that prevents the differentiation of both germ cells and non-germ cells. This mechanism could involve some of the same types of mechanisms that are used to maintain stem and germ cells in other animals.

Two lines of evidence suggest that embryonic cells in *C. floridanum* are in an undifferentiated state during the proliferative stage of embryogenesis. First, their morphology is rounded which is typical of undifferentiated cells (in contrast to cells initiating morphogenesis and formation of the embryonic primordium). Second, we do not detect the expression of several genes known to be involved in initiation of morphogenetic programs and differentiation (*even-skipped*, *engrailed*, *Distalless* and homeotic genes) during the proliferative stage of embryogenesis. However, these genes are expressed during morphogenesis (Grbić et al. 1996a,b; Grbić, unpublished observations).

Other changes we consider important for polyembryonic development include the development of the extraembryonic syncytium into a structure capable of absorbing nutrients from the host. This enables extensive cell proliferation and the net mass increase of the polyembryo. It also plays an essential role in partitioning embryonic cells so that multiple embryos develop. Finally, polyembryonic species must synchronize their own embryogenesis with that of their host.

Mechanisms of *C. floridanum* development

It is unclear how embryonic patterning initiates in *C. floridanum*. Development of other advanced insects like *Drosophila*, *Tribolium*, *Manduca*, and *Musca* begins in a syncytial environment. The patterning cascade also invariably includes a pair-rule phase followed by the expression of segment-polarity and homeotic genes (Akam 1987; Sommer and Tautz 1991, 1993; Patel et al. 1994; Brown et al. 1994; Kraft and Jäckle 1994). Late patterning of the *C. floridanum* germband also utilizes segment-polarity and homeotic genes in a manner consistent with all other studied insects (Grbić et al. 1996a). However, the expression pattern of the pair-rule gene *even-skipped* exhibits a segment-polarity rather than a pair-rule phase.

The major difference between embryogenesis of *C. floridanum* and the aforementioned advanced insects is the absence of a syncytial phase. If early cellularization is a significant feature in the loss of EVE pair-rule pattern in *C. floridanum*, we would expect changes in early patterning to occur in other insects whose embryos cellularize early in development. One such insect is the grasshopper whose embryos also appear to cellularize early (Ho. et al. 1997), and where neither the pair-rule genes *eve* or *ftz* are expressed in a pair-rule pattern (Patel et al. 1992; Dawes et al. 1994). We have similarly found that the embryos of some monoembryonic wasps cellularize early and that EVE is not expressed in a pair-rule pattern (Grbić and Strand 1998). Early cellularization in *C. floridanum*, and possibly other insects, may prevent free diffusion of molecules as large as the maternal coordinate and gap transcription factors involved in early patterning of the *Drosophila* embryo (Grbić et al. 1996a). By analogy with other totally cleaving embryos, early cellularization of insect embryos could also invoke an earlier role for cell-cell interactions than is observed in *Drosophila*.

In spite of the similarity to short germband embryogenesis provided by the absence of a syncytium, our studies clearly show that germband formation and segmentation in polyembryos does not occur in a manner typical of short germband embryogenesis.

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References

Akam M (1987) The molecular basis for metameric pattern in the *Drosophila* embryo. Development 101:1–22

Anderson DT (1972) Embryology and phylogeny of annelids and arthropods. Pergamon Press, Oxford

Baehrecke EH, Strand MR (1990) Embryonic morphology and growth of the polyembryonic parasitoid *Copidosoma floridanum* (Ashmead) (Hymenoptera: Encyrtidae). Int J Insect Morphol Embryol 19:165–175

Baehrecke EH, Aiken JM, Dover BA, Strand MR (1993) Ecdysteroid induction of embryonic morphogenesis in a parasitic wasp. Dev Biol 158:275–287

Bell G (1982) The masterpiece of nature: the evolution and genetics of sexuality. Croom Helm, London

Brown SJ, Parrish JK, Denell RE (1994) Genetic control of early embryogenesis in the red flour beetle, *Tribolium castaneum.* Am Zool 34:343–352

Cappe de Baillon P (1927) La tératologie des insectes. Encycol Entomol 8:1–291

Counce SJ (1968) Development of composite eggs in *Miastor* (Diptera:Cecidomidae). Nature 218:781–782

Cross JC, Werb Z, Fisher SJ (1994) Implantation and placenta: key pieces of the developmental puzzle. Science 266:1508–1518

Davidson EH (1990) How embryos work: a comparative view of diverse mode of cell fate specification. Development 108:365–389

Davidson EH, Peterson KJ, Cameron RA (1995) Origin of bilaterian body plan: evolution of developmental regulatory mechanisms. Science 270:1319–1325

Dawes R, Dawson I, Falciani F, Tear G, Akam M (1994) *Dax*, a locust *Hox* gene related to *fushi-tarazu* but showing no pair-rule expression. Development 120:1561–1572

Driesch H (1892) The potency of the first two cleavage cells in echinoderm development. Experimental production of partial and double formations. In: Willier BH, Oppenheimer JM (eds) Foundations of experimental embryology. Hafner, New York

Foe VE (1989) Mitotic domains reveal early commitment of cells in *Drosophila* embryos. Development 107:1–22

French V (1996) Segmentation (and *eve*) in very odd insect embryos. BioEssays 18:435–438

Gardiner RC, Rossant J (1976) Determination during embryogenesis in mammals. Ciba Found Symposium 40:5–18

Grbić M, Strand MR (1998) Shifts in the life history of parasitic wasps correlate with pronounced alterations in early development. Proc Natl Acad Sci USA 95:1097–1101

Grbić M, Ode PJ, Strand MR (1992) Sibling rivalry and brood sex ratios in polyembryonic wasps. Nature 360:254–256

Grbić M, Nagy LM, Carroll SB, Strand MR (1996a) Polyembryonic development: insect pattern formation in a cellularized environment. Development 12:795–804

Grbić M, Nagy LM, Strand MR (1996b) Pattern duplication in the polyembryonic wasp *Copidosoma floridanum*. Dev Genes Evol 206:281–287

Gurdon JB (1992) The generation of diversity in pattern in animal development. Cell 68:185–199

Hegner RW (1914) Studies on germ cells. J Morphol 26:495–561

Ho K, Dunin-Borkowski OM, Akam M (1997) Cellularization in locust embryos occurs before blastoderm formation. Development 124:2761–2768

Hughes RN, Cancino JM (1985) An ecological overview of cloning in metazoa. In: Jackson JBC, Buss LW, and Cook RE (eds) Population biology and evolution of clonal organisms. Yale University Press, New Haven, CT, pp 153–186

Ivanova-Kasas OM (1972) Polyembryony in insects. In: Counce SJ, Waddington CH (eds) Developmental systems, vol 1. Insects. Academic Press, New York, pp 243–271

Jeffery WR (1983) Maternal RNA and the embryonic localization problem. In: Siddiqui M (ed) Control of embryonic gene expression. CRC Press, Boca Raton, FL

Kelly SJ (1977) Studies of the developmental potentials of 4- and 8-cell stage mouse blastomeres. J Exp Zool 200:365–376

Koscielski B, Koscielska MK (1985) Ultrastructural studies on the polyembryony in *Ageniaspis fuscicollis* Dalm. (Chalcidoidea, Hymenoptera). Zool Pol 32:203–215

Kraft R, Jäckle H (1994) *Drosophila* mode of metamerization in the embyrogenesis of the lepidopteran insect *Manduca sexta*. Proc Natl Acad Sci USA 91:6634–6638

Laale HW (1983) Naturally occuring diblastodermic eggs in the zebrafish, *Brachydanio rerio*. Can J Zool 62:386–390

Leiby RW (1922) The polyembryonic development of *Copidosoma gelechiae* with notes on its biology. J Morphol 37:195–285

Leiby RW (1924) The polyembryonic development of *Platygaster vernalis*. J Agric Res 28:829–839

Noskiewicz J, Poluszynski I (1935) Embryologische Untersuchungen an Strepsipteren. Zool Pol 1:53–92

Parker HL (1931) *Macrocentrus gifuensis* Ashmead, a polyembryonic braconid parasite of the european corn borer. US Dep Agric Tech Bull 230:1–62

Patel NH, Ball E, Goodman CS (1992) Changing role of *even-skipped* during the evolution of insect pattern formation. Nature 357:339–342

Patel NH, Condron BG, Zin K (1994) Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ insects. Nature 367:429–43

Patterson JT (1921) The development of *Paracopidosomopsis*. J Morphol 36:1–69

Patterson JT (1927) Polyembryony in animals. Q Rev Biol 2:399–426

Raff RA (1996) The shape of life. University of Chicago Press, Chicago

Sander K (1976) Specification of the basic body pattern in insect embryogenesis. Adv Insect Physiol 12:125–238

Schwalm F (1988) Insect morphogenesis. Karger, Basel

Silvestri F (1906) Contribuzioni alla conoscenza biologica degli imenotteri parasiti. Biologia del *Litomastix truncatellus* (Dalm.) (2 nota preliminare). Ann Regio ist Super Agrar Portici 6:3–59

Sommer R, Tautz D (1991) Segmentation gene expression in the housefly *Musca domestica.* Development 113: 419–430

Sommer R, Tautz D (1993) Involvement of an orthologue of the pair-rule gene *hairy* in segment formation of the short-germband embryo of *Tribolium* (Coleoptera). Nature 361:448–450

Speman H (1938) Embryonic development and induction. Yale University Press, New Haven

Strand MR, Grbić M (1997) Development and evolution of polyembryonic insects. Curr Top Dev Biol 35:121–160

Strand MR, Goodman WG, Baehrecke EH (1991) The juvenile hormone titer of *Trichoplusia ni* and its potential role in embryogenesis of the polyembryonic wasp *Copidosoma floridanum*. Insect Biochem 21:205–214

Strathmann RR (1985) Feeding and nonfeeding larval developmant and life-history evolution in marine invertebrates. Annu Rev Ecol Syst 16:339–361

Tremblay E, Caltagirone LE (1973) Fate of polar bodies in insects. Annu Rev Entomol 18:421–444

Wray GA (1995) Punctuated evolution of embryos. Science 267:1115–1116

Wray GA, Bely AE (1994) The evolution of echinoderms is driven by several distinct factors. Development Suppl 97–106