DEVELOPMENTAL DYNAMICS 236:3484–3495, 2007

RESEARCH ARTICLE

Gastrulation in the Spider *Zygiella x-notata* Involves Three Distinct Phases of Cell Internalization

R. Crystal Chaw,[†‡] Emily Vance,[†] and Steven D. Black[*]

The cell movements of gastrulation were analyzed in embryos of the spider *Zygiella x-notata*, using time-lapse video, cell tracing, and improved histology. Cells are internalized near the center of the germ disc in three distinct phases. First, cumulus mesenchyme cells ingress and migrate as a group beneath the superficial layer. Second, mass internalization through a blastopore yields a diffusely organized deep layer. Third, superficial cells accumulate at the center of the germ disc to form the caudal bud. The floor is internalized, and the caudal bud moves over the nascent dorsal field to form the caudal lobe. This pattern of gastrulation differs from the canonical pattern described in the historical literature: (1) the cumulus of *Z. x-notata* is completely formed before any other cells internalize; and (2) the caudal lobe is formed by means of the caudal bud, which is a locus of cell internalization. *Developmental Dynamics 236:3484–3495, 2007.*

Key words: gastrulation; morphogenesis; arthropod; spider; arachnid; chelicerate

Accepted 29 September 2007

INTRODUCTION

Gastrulation is the morphogenetic process that establishes the germ layers and converts the simple polarities of the egg into the more complex organization of the later embryo. As with most other animal embryos, gastrulation in spiders internalizes both prospective endoderm and mesoderm. Classic studies of spider gastrulation showed that the embryo internalizes two distinct populations of cells. First, internalization of a portion of the blastoderm creates a small region comprising multiple cell layers, which is called the "primitive plate" (synonymous with "anterior cumulus" and sometimes with "primary thickening"). Associated with the primative plate is a small second population of cells that forms the "cumulus" (synonymous with "posterior cumulus"). The internalized cumulus mesenchyme cells migrate as a group underneath the blastoderm and travel to the edge of the germ disc. Continued internalization of prospective mesoderm and endoderm cells at the center of the germ disc enlarges the primitive plate during and after cumulus migration. This general pattern is said to be followed by most araneomorph spiders and is considered the canonical model of spider gastrulation (see reviews by Anderson, 1973; Foelix, 1996).

Both historical and modern literature on spider development highlight the importance of the second population of internalizing cells, the cumulus mesenchyme. Holm (1952) used transplantation experiments in *Agelena labyrinthica* to show that a secondary axis can be generated by moving the cumulus to another area of the germ disc. Akiyama-Oda and Oda (2003) found that the cumulus of *Achaearanea tepidariorum* (*Achaearanea* has been transferred to the genus *Parasteatoda* by Saaristo [2006]) is a site of

The Supplementary Material referred to in this article can be found at http://www.interscience.wiley.com/jpages/1058-8388/suppmat
Kleinholtz Biological Laboratories, Department of Biology, Reed College, Portland, Oregon
[†]Drs. Chaw and Vance contributed equally to this work.
[‡]Dr. Chaw's present address is Department of Integrative Biology, University of California, Berkeley, CA 94720.
[*]Correspondence to: Steven D. Black, Kleinholtz Biological Laboratories, Department of Biology, Reed College, 3203 S.E. Woodstock Blvd., Portland, OR 97202. E-mail: sblack@reed.edu

DOI 10.1002/dvdy.21371
Published online 9 November 2007 in Wiley InterScience (www.interscience.wiley.com).

decapentaplegic (dpp) signaling. The epithelial cells that overlie the path of cumulus migration extend cytonemes to the cumulus and respond to the dpp signaling by expressing phosphorylated mothers against dpp (pMAD). In response to cumulus migration and dpp signaling, the embryonic germ disc breaks radial symmetry. The point on the germ disc periphery opposite the endpoint of cumulus migration becomes the anterior pole of the germ band, and the epithelial cells that overlie the path of cumulus migration become the dorsal extraembryonic area. Dpp-depleted embryos do not break radial symmetry, and show a phenotype of ventralized segmented rings (Akiyama-Oda and Oda, 2006). Past and present studies establish the cumulus as an important site of cell–cell communication that establishes embryonic axis polarity.

A recent study by Oda et al. (2007) further characterized the gastrula of *A. tepidariorum* by examining the expression patterns of *forkhead*, *twist*, and *dpp*. Prospective cell fates were inferred from expression patterns and from positions of cells, primarily from fixed embryos. Oda et al. were able to present a detailed picture of the organization of the gastrula. First, putative endoderm (including cumulus mesenchyme) ingresses at the center of the germ disc; then, putative endoderm and mesoderm internalize at the periphery of the germ disc; finally, additional putative mesoderm internalizes at the center by means of a pit (Akiyama-Oda and Oda, 2003). Although the actual fates of the various cell populations are not known at this time, the studies by Oda et al. clearly demonstrate that specific regions of the embryo can be defined very early and that the cells can be identified by in situ hybridization during subsequent development. The early internalization of central, noncumulus cells seen by Oda et al. can be interpreted as the initial formation of the primitive plate, and is thus consistent with the canonical model. What is unexpected is that most of the endoderm and mesoderm in *A. tepidariorum* seems to originate from the periphery. Classic accounts of spider gastrulation do not recognize the peripheral region as an area of significant cell internalization, although Montgomery's (1909) histological study of *A. tepidariorum* (then called *Theridion* or *Theridium tepidariorum*) noted some vitellophages internalized at the periphery. The later internalization of central cells to form the interior of the caudal lobe is probably consistent with the canonical model, because Holm (1952) recognized that internalization of cells at the center of the germ disc continued throughout gastrulation (see also the *Analpol* of Holm, 1940). To summarize, gastrulation of *A. tepidariorum* embryos departs from the canonical model by having large-scale internalization at the periphery of the germ disc.

The difference in gastrulation movements between *A. tepidariorum* and the canonical model suggest to us that the model is in need of reevaluation. Indeed, the model is based primarily on analyses of fixed material and Holm's (1952) cell-marking experiments of *A. labyrinthica*. Thus, we have used modern methods to study gastrulation in a spider embryo. We analyze the cell movements of gastrulation at the cellular level using time-lapse video, cell tracing, and improved histological methods. The species studied, *Zygiella x-notata* (family Araneidae), is an araneoid spider as is *A. tepidariorum* (family Theridiidae). *Z. x-notata*'s pattern of gastrulation differs from the canonical pattern by exhibiting a heterochronic shift in cumulus formation relative to formation of the primitive plate, and by using an unusual mechanism to internalize putative mesoderm of the caudal lobe. The pattern of cell movements also differs fundamentally from that described for *A. tepidariorum*. Our analysis of the morphogenetic movements of the *Z. x-notata* gastrula suggests that there is more variation in gastrulation in spider embryos than has been previously appreciated.

RESULTS AND DISCUSSION

Early embryos of *Zygiella x-notata* undergo three phases of cell internalization, which we designate gastrulation I, II, and III. Signal stages are depicted in Figure 1, and the timing of these stages is given in Figure 2. Supplementary Movie S1, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat, shows time-lapse of *Z. x-notata* development beginning with stage C. Before gastrulation, the embryos follow the general pattern described for entelegyne spiders (Holm, 1952); the 700-μm embryos undergo rounds of nuclear division in what appears to be a common cytoplasm (Fig. 1A). Then, most if not all energids migrate to the periphery (Fig. 1B). A blastoderm forms and the embryo contracts to form the perivitelline space. At the beginning of contraction, the blastoderm is uniform; by the end of contraction, a small white spot has appeared at one side of the blastoderm. This spot is a cluster of cells that marks the point of blastopore formation (Fig. 1C). As more cells accumulate locally at the blastopore, most of the other blastoderm cells migrate to the same hemisphere to form the germ disc. The blastopore is at the center of the germ disc.

Gastrulation I

This phase begins with the formation of the blastopore (equivalent to the "primitive pit" and the "primitive groove" of Holm [1940, 1952]) and involves ingression of fewer than 20 blastoderm cells. The primary function of gastrulation I appears to be the internalization of cumulus mesenchyme cells. By tracing cells in time-lapse, it is possible to identify the ingressing cells and their points of ingression. A typical embryo is shown in Figure 3, with the cells that will ingress during gastrulation I false-colored red. Figure 3 shows that the central blastoderm region internalizes first. These cells include the cumulus mesenchyme cells, which will migrate as a group (white arrowhead) underneath the surface blastoderm. Cells further away from the blastopore are false-colored a variety of colors in Figure 3, and they can be seen to converge on the central region without internalizing (see Supplementary Movie S2, representative of N = 11 embryos analyzed by time-lapse). Although the majority of ingressing cells form the cumulus (comprising 8–12 cells, N = 6 embryos examined in serial sections), a small number of other blastoderm cells ingress individually very near or with the cumulus, and these can be seen migrating beneath

the superficial layer in time-lapse. From our time-lapse observations and sections, we were unable to discern a clear pattern of migration for these other ingressing cells. They are probably newly formed vitellophages, similar to those described for *A. labyrinthica* (Holm, 1952), *Latrodectus mactans* (Rempel, 1957), and *A. tepidariorum* (Montgomery, 1909). Vitellophages may ingress at multiple points in these spiders, but appear to ingress near the blastopore in *Z. x-notata*. At this stage, we typically find cells in the yolk only if they are close to the blastopore, although in some embryos we have found two or three cells deep within the yolk. In section, the ingressing cumulus mesenchyme cells are apically constricted (Fig. 4A,B), similar to the bottle cells of the amphibian blastopore. The apically constricted cumulus cells can also be seen externally (they are the unlabeled central cells in Fig. 5A), whereas the surrounding cells retain an unconstricted shape. The bottle morphology suggests an active role on the part of the internalizing cumulus cells. The blue arrowheads in Figure 4B identify probable vitellophages. Internalization of cells during gastrulation I occurs at or near the blastopore in *Z. x-notata*; no internalization occurs at other regions.

Once internalized, the cumulus mesenchyme cells migrate posteriad as a group underneath the superficial layer, as described for other species (Holm, 1940; Akiyama-Oda and Oda, 2003). The migration can be easily seen in time-lapse (see white arrowhead in Fig. 3 and Supplementary Movie S2). Cumulus cell internalization and cumulus migration take approximately 26 hr (Fig. 2). The migrating cumulus mesenchyme cells in *Z. x-notata* appear vacuolated (likely a result of our fixation protocol) and are shown in Figure 4C,D. Scanning electron micrographs of *A. tepidariorum* embryos by Akiyama-Oda and Oda (2003) show that the superficial cells overlying the cumulus interact with it by extending cytonemes, and a similar morphology is revealed at the histological level in *Z. x-notata*—superficial cells contact the cumulus by means of long, narrow protrusions (green arrowhead, Fig. 4D). These protrusions are likely to effect cell–cell communication between the cumulus mesenchyme cells and overlying superficial cells of the germ disc, as suggested by Akiyama-Oda and Oda (2003) for *A. tepidariorum*. Up to this point, gastrulation in *Z. x-notata* differs from the canonical model by internalizing only cumulus mesenchyme and a few vitellophages; no primitive plate is formed. In *A. tepidariorum*, all the early ingressing cells express forkhead and include cumulus mesenchyme and additional cells designated central endoderm (Oda et al., 2007; see also Montgomery, 1909, whose sections show a considerable number of cells internalized before formation of the "cumulus posterior"). Other araneomorph spiders show internalization of a large number of noncumulus cells during this early period, for example, *Latrodectus mactans* (Theridiidae; Rempel, 1957), *Torania variata* (Sparassidae; Ehn, 1963), *Cupiennius salei* (Ctenidae; Seitz, 1966), and *A. labyrinthica* (Agelenidae; Holm, 1952).

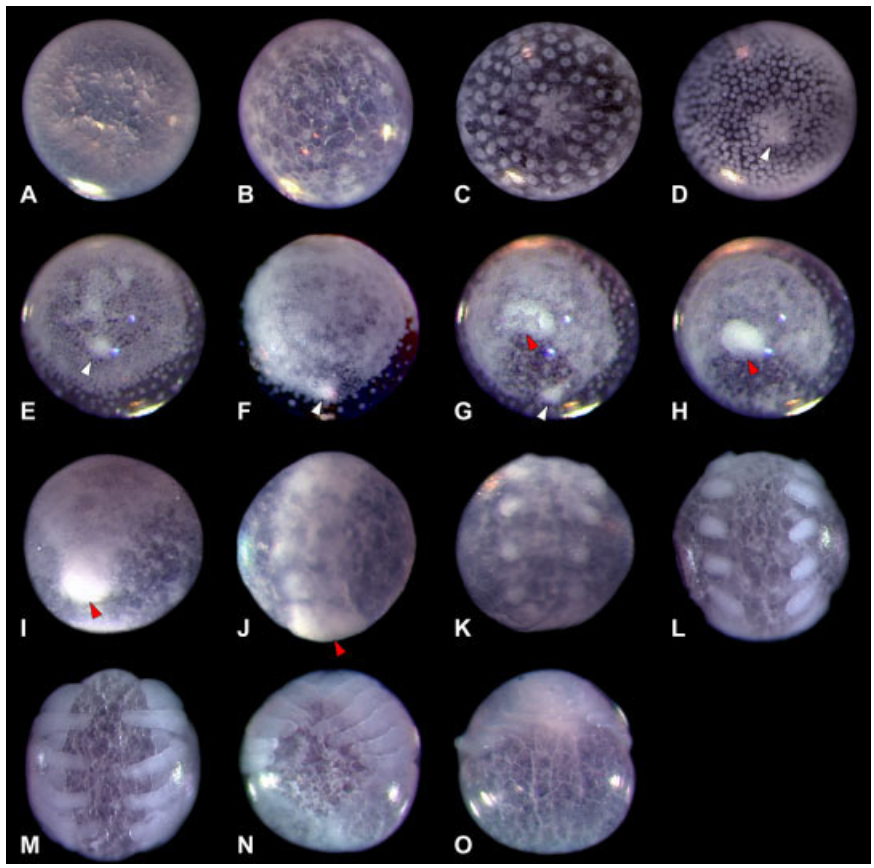

Fig. 1. Normal development. Embryos oriented posterior down in all panels. A–H: View of germ disc side. I–O: Ventral view. **A:** Prenuclear migration. Embryo appears as a mass of yolk spherules. **B:** Nuclear migration. **C:** Gastrulation I begins with cumulus formation; contraction of blastoderm. **D:** Cumulus migration begins (white arrowhead); germ disc apparent. **E:** Gastrulation II (mass internalization) begins. **F:** Cumulus migration ends; continued mass internalization has produced a dispersed deep layer of cells. **G:** Gastrulation III begins with caudal bud formation (red arrowhead); dorsal field begins to form. **H:** Caudal bud complete; dorsal field expansion. **I:** Caudal bud movement, dorsal field expansion continues. **J:** Germ band formation, segmentation apparent. **K:** Appendage bud formation. **L:** Inversion begins: ventral sulcus appears along ventral midline; appendage buds elongate. **M:** Mid-inversion. **N:** Inversion complete, ventral closure begins. **O:** Ventral closure complete. Original magnification, ×54. Supplementary Movie S1 shows development of a single embryo from germ disc contraction to hatching.

Gastrulation II

A second phase of cell internalization begins as the cumulus mesenchyme cells are migrating. Two things set gastrulation II apart from cumulus formation in *Z. x-notata*. First, gastrulation II involves many more cells and must be the major source of cells for the mesoderm and endoderm of the prosoma. Second, the two phases be-

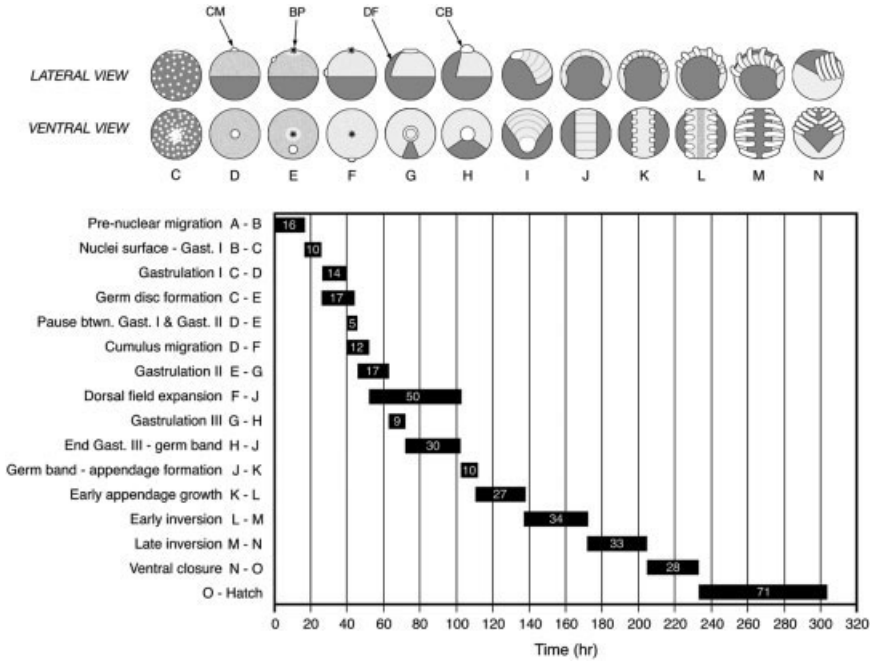

Fig. 2. Timing of normal development at room temperature. Horizontal bars show duration of each stage; numbers in bars indicate duration in hours. Each bar represents the average of at least two embryos from different egg sacs (range = 2–11 embryos). CM, cumulus; BP, blastopore of gastrulation II; DF, dorsal field; CB, caudal bud.

gin separately; there is a definite pause (approximately 5 hr) between cumulus formation and the beginning of gastrulation II (Fig. 2). This pause can be seen as a period of minimal cell internalization while the cumulus is migrating (for example, in the 4 hr spanned by Figure 3D,E, fewer than five cells in this embryo are internalized in the central region). The pause is then followed by mass ingression through a central blastopore (Fig. 5 and Supplementary Movie S3). Gastrulation II is the source for much of the presumptive mesendoderm and does not begin until after the fully formed cumulus begins to migrate.

Cells internalize during gastrulation II via a blastoporal region that forms in the center of the germ disc. The blastopore cannot be seen easily in living embryos, as has been remarked for some other species (e.g., *L. mactans*, Rempel, 1957; *A. tepidariorum*, Akiyama-Oda and Oda, 2003); however, our time-lapse videos and histological sections clearly demonstrate blastoporal function and morphology. Time-lapse analysis shows a pattern of movement of superficial cells to the central region. This is the same area of the germ disc at which the presumptive cumulus mesenchyme cells ingressed. This finding is shown in Figure 5 by the central position of both the presumptive cumulus cells and, hours later, the area in which other superficial cells ingress during gastrulation II. In Figure 5A, the presumptive cumulus mesenchyme cells are already apically constricted (unlabeled cells at center). Some cells that will internalize during gastrulation II are false-colored to reveal their progress to the blastoporal region and subsequent internalization. Not all cells internalize at exactly the same point. Internalization of these cells begins approximately at Figure 5G and extends beyond Figure 5L; eventually even the turquoise-colored cells will ingress (see Supplementary Movie S3). The region of the superficial layer that will internalize during gastrulation II can be approximated by a circle that includes the turquoise-colored cells in Figure 5. This circle corresponds spatially with the region of presumptive mesendoderm in Holm's (1952) fate map of an earlier stage *A. labyrinthica*. The period of gastrulation II in *Z. x-notata* is approximately 17 hr, ending when the caudal bud forms (see below), although there is some variation between embryos (N = 11 embryos analyzed by time-lapse).

Histological sections of embryos during gastrulation II show a small blastopore at the center of the germ disc, complete with apically constricted cells (Fig. 6A,B). These cells appear less elongated than the more classic bottle cells seen in gastrulation I. Early in gastrulation II, the cumulus mesenchyme cells are midway through migration, and only a few cells have internalized at the blastopore. Figure 6A,B shows sections through the early blastopore some distance from the cumulus (Stage E, same embryo as pictured in Fig. 4C,D). Together, both figures show that early in gastrulation II, the only cells below the superficial layer are next to the blastopore (with the exception of cumulus mesenchyme cells and vitellophages). As gastrulation II continues, time-lapse and histology show increased cell internalization. The movies show movement of superficial layer cells toward the central region, and migration of internalized cells centrifugally away from the blastopore (see Supplementary Movies S4 and S5). The direction of movement of internalized cells toward the periphery of the germ disc is opposite that described for the same stage of *A. tepidariorum* (Oda et al., 2007). In *A. tepidariorum*, the peripheral rim of the germ disc is said to be a second site of cell internalization—both peripheral forkhead-positive cells (designated endoderm, "pEND") and forkhead- and twist-positive cells (designated mesoderm, "pMES") are found in a deep layer at the rim. Moreover, migration of these internalized cells is thought to be in a *centripetal* direction, toward the center of the germ disc. A smaller number of twist-positive cells also internalize at the center of the germ disc ("cMES"), at a later stage. Careful analysis of our movies and histological sections show that, unlike *A. tepidariorum*, all the cells of the deep layer in *Z. x-notata* originate near the center of the germ disc. Sections near the end of gastrulation II show the continued presence of a blastopore (Fig. 6C,D). The blastopore can span several sections. At this stage of gastrulation, there is now a diffuse deep layer of cells underlying the

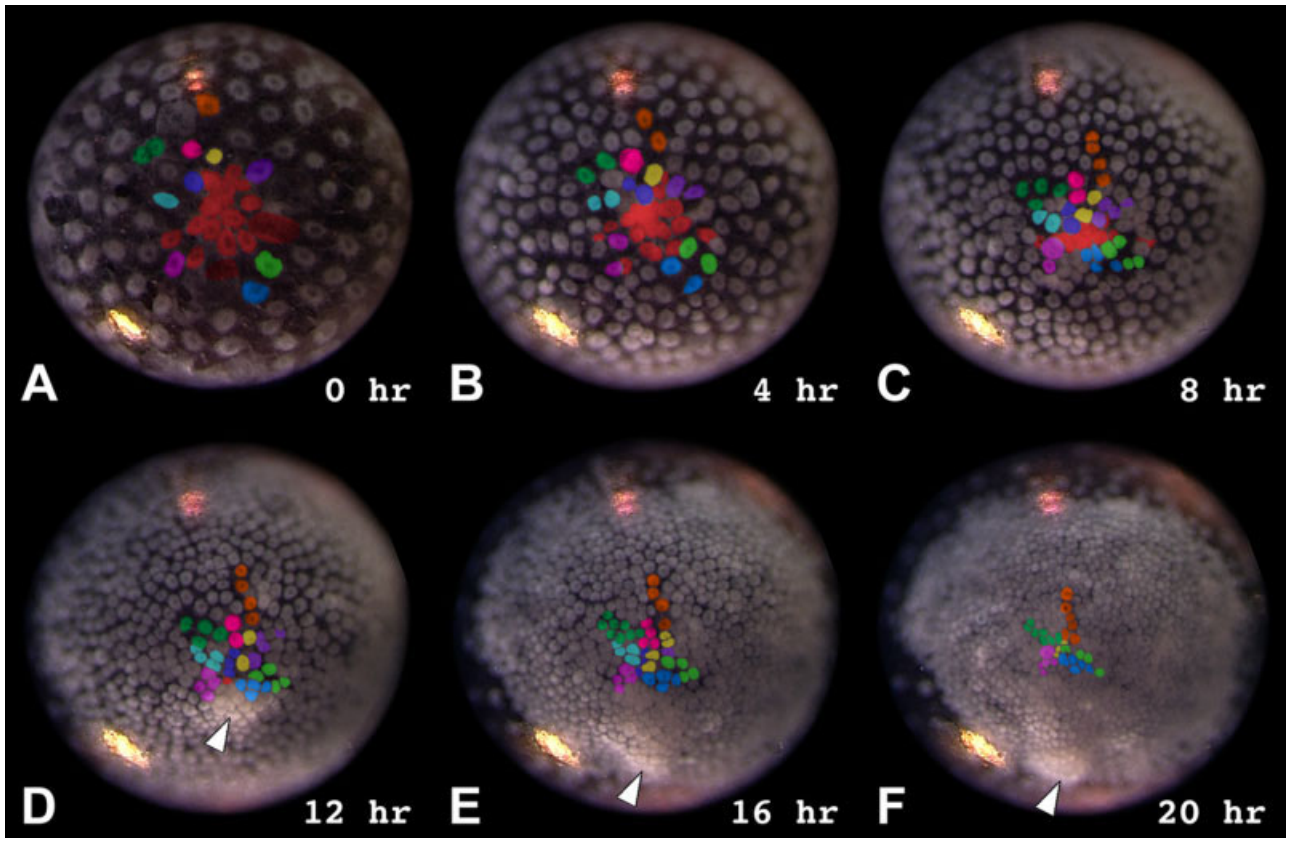

Fig. 3.

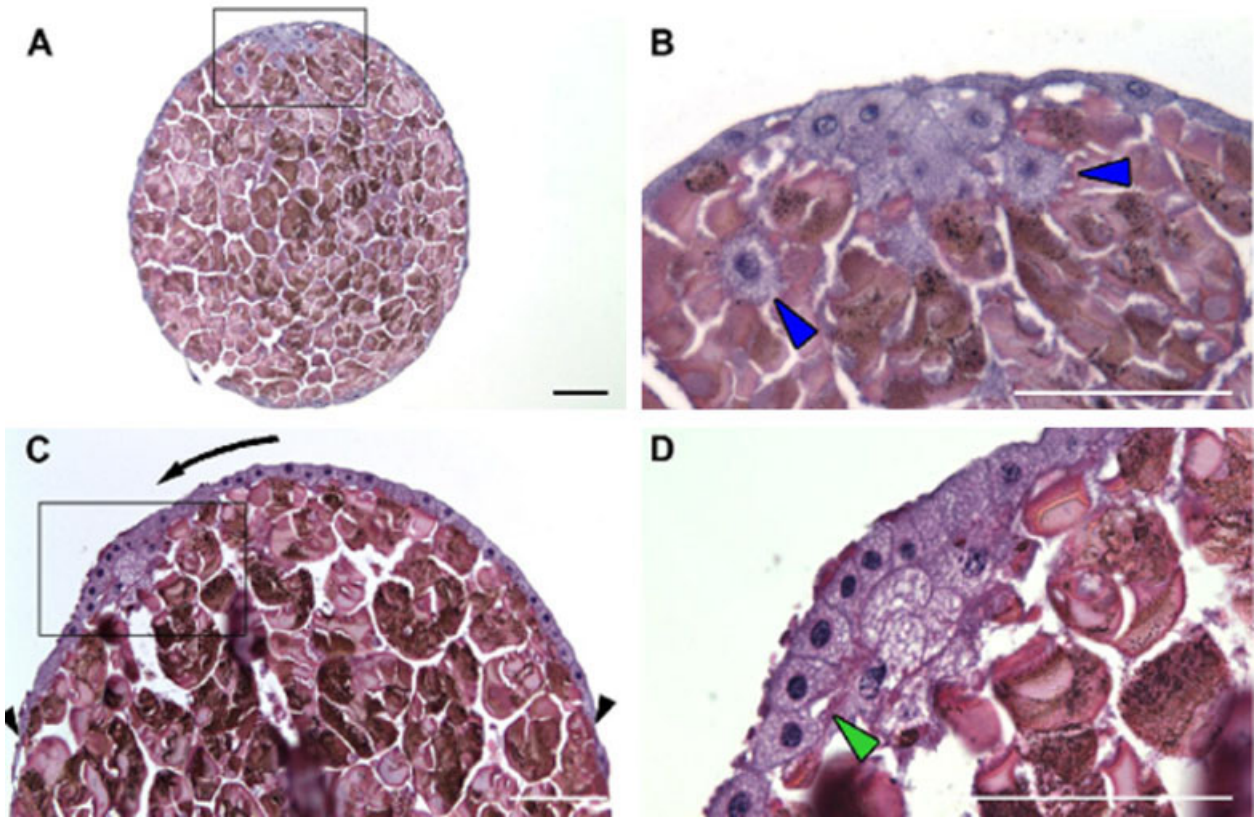

Fig. 4.

germ disc. These cells can be seen in sections adjacent to the blastopore and at some distance from it (green arrowheads in Fig. 6C,E). Note that the only deep cells at the periphery are cumulus mesenchyme cells (adjacent to blue arrow, Fig. 6C). Analysis of serial sections reveals no accumulation of other deep cells at the periphery, which is consistent with an absence of cell internalization at the rim. The deep layer can be seen in the external view as well; a comparison of Figure 1E (start of gastrulation II) and Figure 1F (near end of gastrulation II) shows that the germ disc of the later stage has become more opaque, which correlates with the increased number of internal cells shown by histology.

In many other spiders, cumulus formation is preceded by cellular ingression through a blastopore to form the "primitive plate" or "primary thickening," which is an area of the early germ disc with multiple cell layers (e.g., *A. labyrinthica* [Holm, 1952], *Torania variata* [Ehn, 1963], *Cupiennius salei* [Seitz, 1966]). The cumulus forms from this area and is described as a further accumulation of cells, a bulge on the primitive plate, or as a larger thickening that appears on one side of the primitive plate. In *Z. x-notata*, the internalization of cumulus mesenchyme cells occurs both first and separately from the gastrulation of the rest of the mesendoderm. There is no primitive plate that forms before the cumulus. Figure 4 shows that the only deep layer cells of the early gastrula are the cumulus mesenchyme cells. The primitive plate as described for other spiders thus represents relatively early internalization of some of the presumptive mesendoderm. The minimal internalization of mesendoderm before cumulus formation in *Z. x-notata* may represent a heterochrony of gastrulation phases compared with the pattern historically described for many other spiders.

Gastrulation III

The third round of gastrulation in *Z. x-notata* involves a distinctly different mechanism that forms a structure we designate the caudal bud. Near the end of gastrulation II, the pattern of cell behavior changes. Superficial cells continue to migrate toward the center of the germ disc, but instead of internalizing, many cells remain on the surface and pile up around the blastoporal region. As cells continue to layer, the mass forms a distinct volcano-like shape (Fig. 7). The central floor of the volcano is internalized as superficial cells of the rim move toward the center, over the floor (three such superficial cells are false-colored red in Figure 7A–C to show convergence to the central region). Cell-shape changes observed in time-lapse (D. Rasmussen, unpublished results) suggest that closure of the "mouth" may occur through a purse-string–like constriction of the cells of the rim as in *Drosophila* dorsal closure (Kiehart et al., 2000). It is also possible that the lateral edges of the volcano are pushed together over the central floor by cell crowding, or that some combination of constriction and pushing results in internalization of the floor. In section, the mouth of the mid-stage caudal bud is distinct (Fig. 7E). Cells of the floor also leave the superficial layer by ingression (D. Rasmussen, unpublished results). There is variation in the size of the caudal bud—for example, the embryo depicted in Figure 7 A–D has a larger caudal bud than the embryo in Figure 8—but in all cases, the caudal bud undergoes the same cellular movements to internalize the central floor (N = 12 embryos analyzed by time-lapse. Supplementary Movie S5 shows a relatively large caudal bud, and Movie S6 shows a relatively small caudal bud). Gastrulation III ends with the closure of the "mouth," at which point the caudal bud begins to move posteriad along the midline. This typically occurs as the dorsal field (see below) expands beyond approximately 100 degrees of arc. Sections of the caudal bud at this stage show cuboidal cells along the leading edge that appear tightly organized (Fig. 7F), and the impression from time-lapse is that the leading edge is more sharply defined than the trailing edge (see Supplementary Movie S6). Behind the caudal bud is an additional layer of cells. Time-lapse analysis and our preliminary labeling experiments with 1,1′, di-octadecyl-3,3,3′,3′,-tetramethylindo-carbocyanine perchlorate (DiI) suggest that the caudal bud becomes part of the posterior-most part of the embryo, with labeling observed in both superficial and deep layers.

The caudal bud is probably the precursor in *Z. x-notata* to the posterior growth zone. The posterior growth zone is the region of the germ band from which the posterior segments arise and is a feature typical of short- and intermediate-germ band insects, many other arthropods including spiders, and some annelids (Anderson, 1973; Davis and Patel, 2002; Stollewerk et al., 2003; Schoppmeier and Damen, 2005; Oda et al., 2007; de Rosa et al., 2005). The early caudal lobe of *A. tepidariorum* has been shown by Oda et al. (2007) to form in a similar place and time as the caudal bud. Putative mesoderm cells in the early caudal lobe of *A. tepidariorum* express twist and internalize in small groups. However, the caudal bud of *Z. x-notata* is distinguished from the caudal lobe of *A. tepidariorum* because it is more tightly organized and forms the distinct "mouth" while internalizing the central floor. The caudal bud is seen to move in a posterior direction before the germ band is formed, passing rapidly across the dorsal field (see Supplementary Movies S5 and S6). This posteriad movement takes only some 9 hr, whereas the formation of the posterior segments continues for long afterward, presumably by a

Fig. 3. External view of gastrulation I. The cumulus forms by ingression of superficial cells at the center of the germ disc. The cells that ingress during gastrulation I are colored red. Stills 4 hr apart. Embryo oriented posterior down. **A:** Gastrulation begins with congregation of cells at center. **B,C:** Internalization of cells. **D,E:** Internalized cumulus mesenchyme cells (white arrowhead) migrate posteriad beneath the superficial layer. **F:** Cumulus reaches margin of germ disc. Gastrulation II begins as cells are internalized at the center of the germ disc (e.g., pink, purple). Original magnification, ×54. Supplementary Movie S2 shows gastrulation I (and beginning of gastrulation II) with false-colored cells.

Fig. 4. Histology of gastrulation I: cumulus formation and migration. **A,B:** Ingression of cells at the center of the germ disc to form nascent cumulus (stage C); blue arrowheads point to putative vitellophages. **C,D:** Cumulus in mid-migration (stage E). Parasagittal section, slightly oblique. Curved arrow shows direction of migration of cumulus; black arrowheads indicate margin of germ disc. Inset shows that cumulus mesenchyme cells are vacuolated and have contact with superficial cells; green arrowhead points to one of the superficial cells with an elongated process in contact with cumulus mesenchyme cells. Others are visible in the figure. Scale bar = 100 μm.

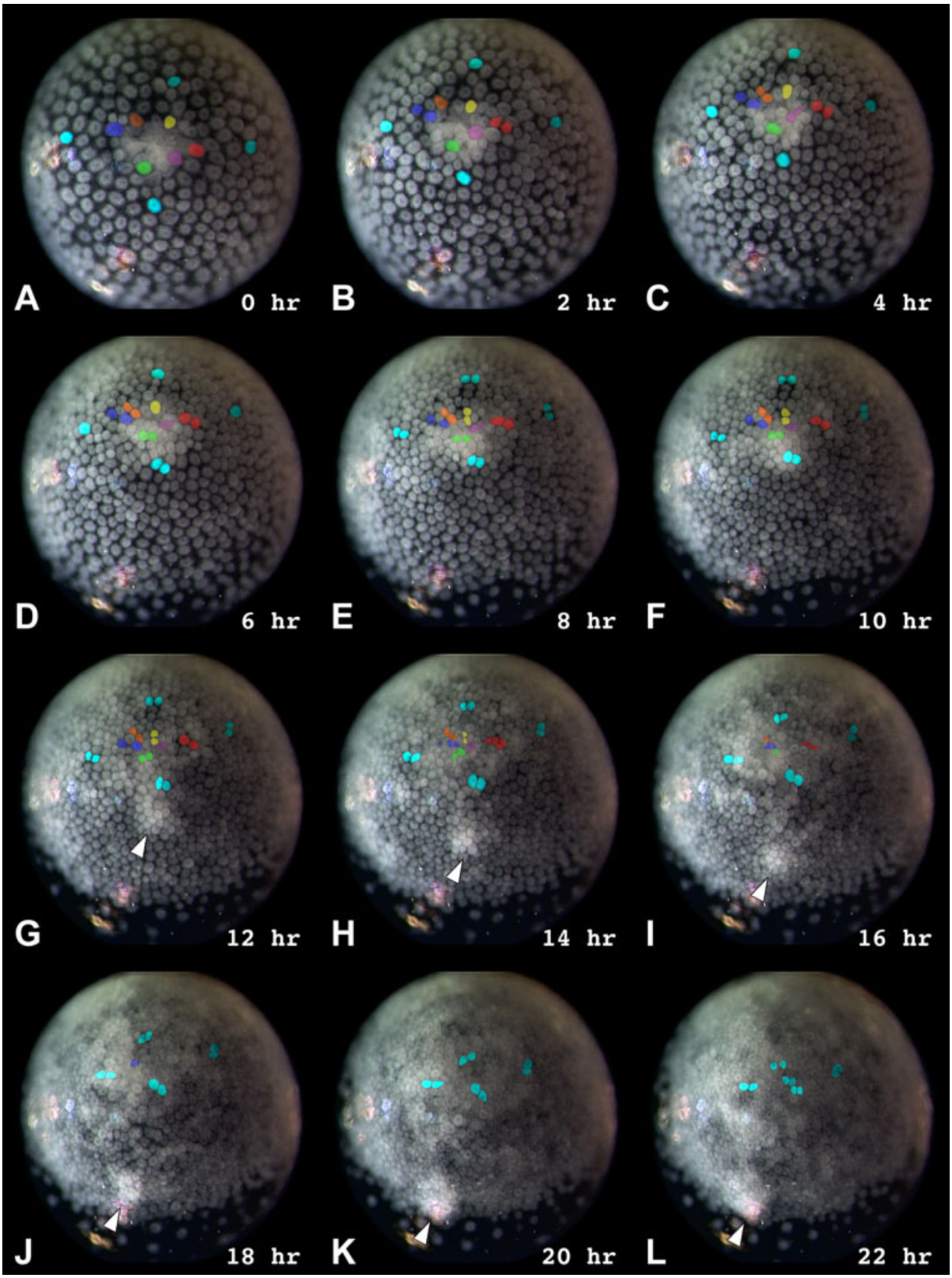

Fig. 5. External view of gastrulation II. The blastopore will form at the site of the blastopore for gastrulation I. Mass internalization begins only after the cumulus has formed. Some superficial cells that will internalize during gastrulation II are false-colored. Cumulus cells are not colored. Stills 2 hrs apart. Embryo oriented posterior down. **A–D:** Cumulus formation (stage C). **E–K:** Cumulus migration (stages D–F). **G–I:** Early mass internalization (stage E). **J–L:** Continuing mass internalization (stage F). Original magnification, ×57. Supplementary Movie S3 shows gastrulation II with false-colored cells; Supplementary Movie S4 shows movement of superficial cells to blastopore; and Supplementary Movie S5 shows centrifugal movement of internalized cells.

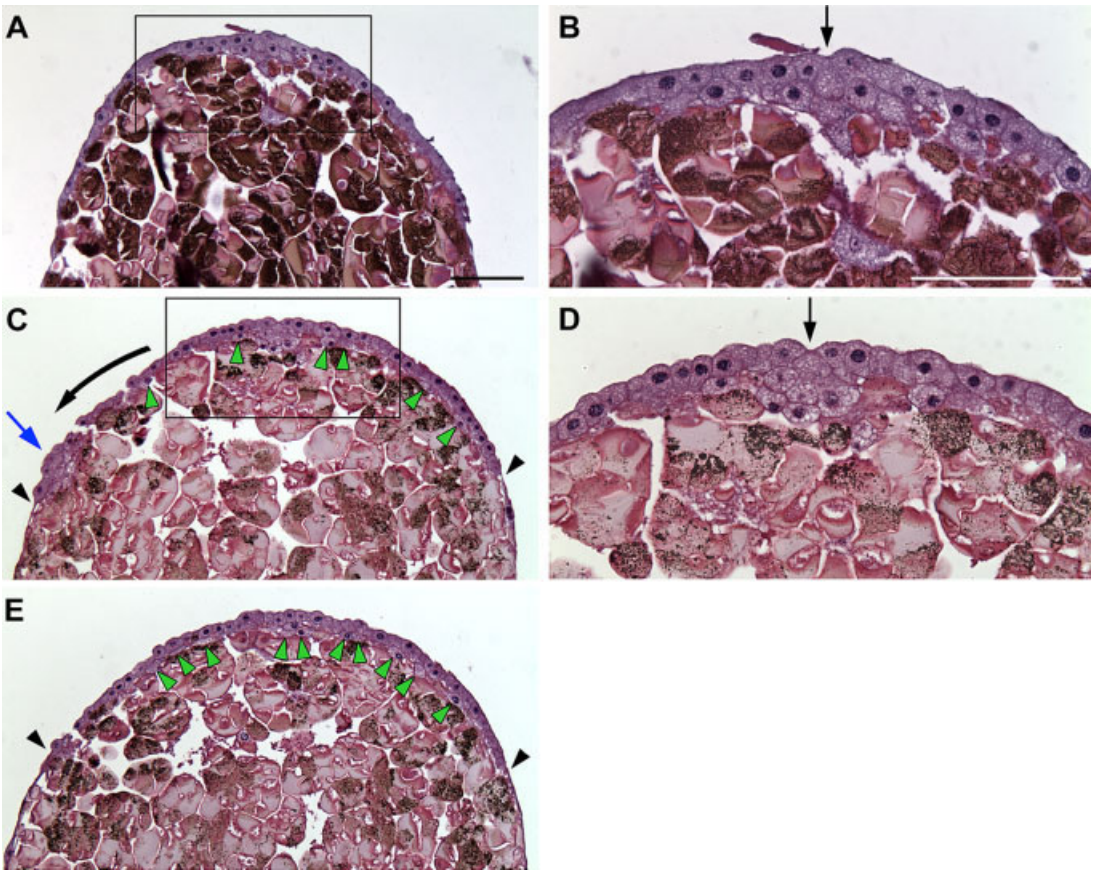

Fig. 6. Histology of gastrulation II: mass internalization through blastopore. Parasagittal sections, slightly oblique. **A,B:** Beginning of mass internalization (stage E). Central blastopore is indicated by black arrow. Cumulus is in mid-migration but is not visible in this section. This is the same embryo as in Figure 4C,D. Not much internalization has occurred by this stage—the only cells in the deep layer are beneath the blastopore or are associated with the cumulus. **C,D:** Cumulus end-migration stage (stage F). Cumulus indicated by blue arrow and its direction of migration by heavy black arrow. Black arrowheads indicate margin of germ disc. Note increased number of cells in diffusely organized deep cell layer (green arrowheads). Blastopore indicated by black arrow in D. **E:** Section from same embryo as C, D showing large number of internalized cells 24 μm away from the blastopore. Scale bar = 100 μm.

mechanism that does not involve de novo internalization of cells. During the posterior segmentation process, it is likely that the cells derived from the caudal bud function as a more typical posterior growth zone, which is known for spiders generally (Anderson, 1973; Stollewerk et al., 2003; Schoppmeier and Damen, 2005). We speculate that the early caudal bud of *Z. x-notata* contains a significant number of presumptive mesoderm cells and that these cells express mesodermal markers such as twist at high levels. A caudal bud-like structure may have been observed in other species of spiders. Anderson (1973) posits that several older studies of liphistiid (*Heptathela kimurai*, Yoshikura, 1955), mygalomorph (*Atypus karschi*, Yoshikura, 1958; *Conothele*, Crome, 1963; *Ischnocolus*, Schimkewitsch and Schimkewitsch, 1911), and haplogynid embryos (*Segestria bavarica* and *S. sonoculata*, Holm, 1940) show a similar structure. Consulting the original sources reveals, however, that with the possible exception of *H. kimurai* (see Yoshikura's Fig. 16C [Yoshikura, 1955]), the structures in the other embryos resemble the denser regions of adjacent segments of the germ band rather than the highly organized caudal bud of *Z. x-notata*. Neither is the caudal structure in the other species said to move across the germ disc; presumably extension of the germ band and creation of the posterior segments drive the separation of anterior and posterior poles.

Dorsal field formation begins as the caudal bud forms, at the end of gastrulation II. The dorsal field is an expanding area of superficial cells in the presumptive dorsal region of the germ disc, through which yolk is visible. As with the caudal bud, the dorsal field begins to form after the cumulus has reached the edge of the germ disc. From histology and time-lapse, the dorsal field in *Z. x-notata* seems to arise from thinning and expanding of superficial germ disc cells of the dorsal region (Fig. 8, Supplementary Movie S7). This expansion begins before the caudal bud begins to migrate. Dorsal field cells come to surround the posterior germ band on three sides, as can be seen in Figure 8. Holm (1952) dyed the surface of an *A. labyrinthica* gastrula in similar positions, and observed the same spreading of marks laterally, to end up on the sides of the posterior germ band. He suggested that the dorsal field arose as a result of the outer cell layer being pushed transversely and a deep layer emerging, presumably by movements of mesendoderm cells, or perhaps from

the cumulus mesenchyme cells. Holm thought that much of the dorsal field originated from the cumulus. In *Z. x-notata*, when cumulus mesenchyme cells reach the margin of the germ disc, they dissipate and appear to wander beneath the dorsal field cells. We were unable to determine a consistent pattern of migration or the ultimate fate of the cumulus cells. The expansion of the superficial layer of the dorsal field is probably due to the spreading and movement of the superficial cells themselves, as suggested by the cell tracings in Figure 8. Dorsal field expansion takes approximately 50 hr, at which point the germ band is elongated.

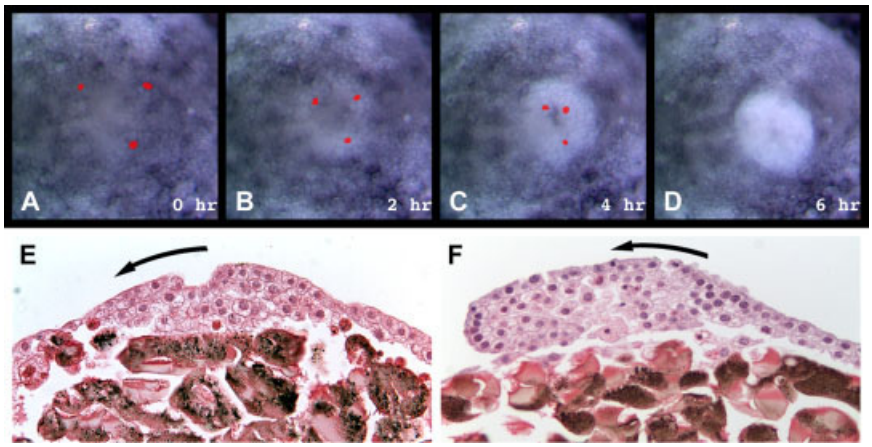

Fig. 7. Gastrulation III. Caudal bud formation. Close up of center of germ disc, prospective dorsal field at lower right. Three cells on the rim of the caudal bud are false-colored red. Stills 2 hr apart. **A:** Accumulation of cells in the center of the germ disc. **B:** Convergence of central cells makes the caudal bud visible as a volcano-like structure. **C:** Continued convergence narrows the mouth of the caudal bud, which is now several cells thick. **D:** Mouth has closed; caudal bud beginning its posteriad movement (cells cannot be traced at this point). **E,F:** Sagittal sections; direction of movement of caudal bud is indicated by black arrow. C,E: stage G. F: Caudal bud during movement, stage I. Original magnification, ×64. Supplementary Movies S5 and S6 show gastrulation III and movement of caudal bud from different perspectives.

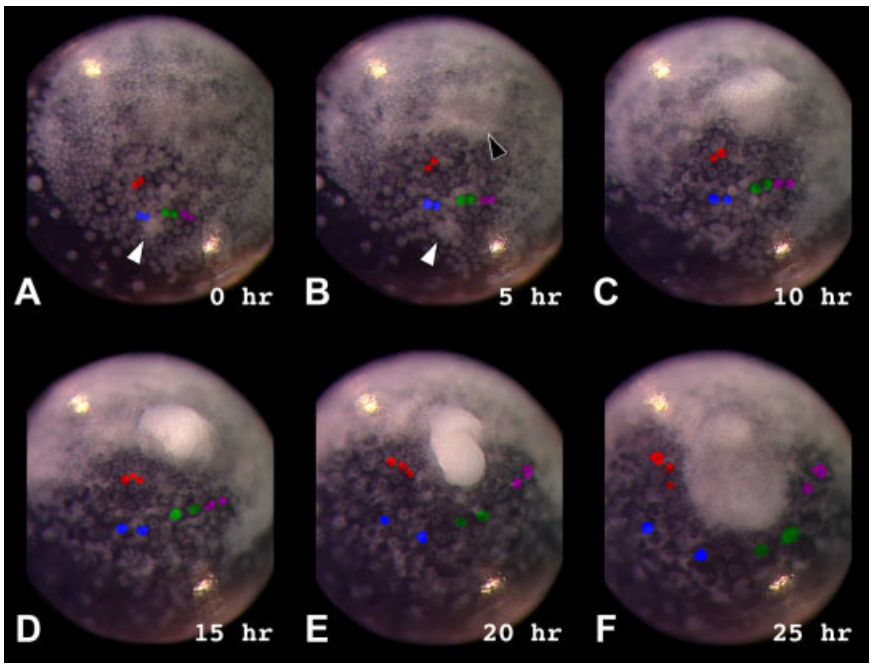

Fig. 8. Dorsal field expansion. Some prospective dorsal field cells in the superficial layer were false-colored and traced. Stills 5 hr apart. Embryo oriented posterior down. White arrowhead indicates cumulus. Black arrowhead indicates forming caudal bud. **A:** Cumulus reaches posterior margin of germ disc (stage F). **B:** Dorsal field emerges, and caudal bud begins to form (stage G). **C–F:** As the cumulus dissipates and the caudal bud forms and migrates, dorsal field cells expand in area, become thin so that yolk is visible underneath, and move away from each other (stages H, I). Supplementary Movie S7 shows dorsal field expansion with false-colored cells. Original magnification, ×54.

In summary, our study finds that *Z. x-notata* embryos internalize cells at a central blastoporal region in three phases. Internalization of cumulus mesenchyme occurs first and is followed after a pause by internalization of a large number of cells. The latter cells must be prospective mesoderm and endoderm based on their number and the fact that they constitute a de novo layer of cells underneath the superficial layer of the germ disc. The third phase of gastrulation internalizes putative mesoderm to form the caudal bud, which is the likely precursor to the posterior growth zone.

In *Z. x-notata* a "primitive plate," that is, a central area of the germ disc with multiple cell layers, does not form before the cumulus nor does the cumulus separate from such an area. The sequence of cell internalization in *Z. x-notata* is thus reversed from the pattern thought to be typical for spider development (Anderson, 1973; Foelix, 1996). However, because many of the older studies relied on interpretation of sectioned embryos rather than combining histological and time-lapse analyses, it is possible that the development of the "typical spider" is not so different than the pattern we describe for *Z. x-notata*. For example, perhaps in other species cumulus mesenchyme cells also internalize first, but delay their migration until other mesendodermal cells begin to ingress (and the primitive plate becomes visible). This would be consistent with Holm's (1952) fate map of the superficial layer of *A. labyrinthica*, in which the presumptive cumulus is at the center of the blastoderm surrounded by presumptive mesendoderm. The cumulus would form from some of the first cells to internalize.

During gastrulation II in *Z. x-notata*, the direction of movement of cells after internalization is centrifugal; cells move from the central blastoporal region toward the peripheral rim

of the germ disc. This is the opposite direction than that seen in *A. tepidariorum* embryos, which internalize the bulk of the putative endoderm and mesoderm at the periphery (Akiyama-Oda and Oda, 2003, 2006; Yamazaki et al., 2005; Oda et al., 2007). This difference suggests that the two species have markedly dissimilar fate maps, despite being representatives of the same superfamily, Araneoidea, with common ancestry estimated at approximately 150 MYA (Ayoub et al., 2007).

Caudal bud-like structures may have been observed in other spiders (Anderson, 1973), and the presence of a pit in the prospective caudal lobe of *A. tepidariorum* may indicate cell internalization (Akiyama-Oda and Oda, 2003). However, the caudal bud of *Z. x-notata* is distinct because of its mode of formation and demonstrated internalization of cells by overgrowth of the superficial layer; its rapid movement across the dorsal field; and the cuboidal cells that appear highly organized along its leading edge.

The basic pattern of gastrulation seen in *Z. x-notata*, despite occurring in three phases, resembles that historically described for other species of araneomorph spiders. In general, araneomorphs internalize two distinct populations of cells: the cumulus and the general mesendoderm. With the exception of the pattern found in *A. tepidariorum*, gastrulation occurs at a central, blastoporal region on the ventral side of the embryo. Additionally, the araneomorphs internalize most of their mesendoderm in one continuous phase as opposed to, for example, the insects, where internalization of mesoderm and endoderm has been uncoupled in both space and time (Roth, 2004). The stomodeum and proctodeum of spiders typically originate as separate invaginations later in development, but the bulk of the endoderm is thought to internalize with the mesoderm during gastrulation (Holm, 1952; Anderson, 1973). The caudal bud of *Z. x-notata*, which we assume to be a derived feature, is unique in that it adds a third phase to the process of gastrulation, but remains in line with the spatial pattern of most araneomorphs because cell internalization still occurs in a region central to the germ disc, on the ventral side of the embryo.

If a cumulus–mass internalization pattern of gastrulation can be thought to represent that of araneomorph spiders generally, how does this pattern compare with that of other chelicerate taxa? Few pertinent embryological data exist for many chelicerate taxa and comparisons are further constrained by differences in methodology. Still, to our knowledge, Opiliones is the only other arachnid order that has a posterior cumulus (function unknown, Holm, 1947). A cumulus has been tentatively identified in embryos of an ovoviviparous scorpion, but its position vis-a-vis the anterior–posterior axis needs confirmation (Abd-el Wahhab, 1954). A cumulus is absent in pseudoscorpions and the Acarina; there are no reliable data for the other chelicerate orders (Anderson, 1973). Gastrulation in other arachnid groups seems to follow the pattern seen in the basal liphistiid spider *H. kimurai* (Yoshikura, 1955). Specifically, a ventral blastoderm is formed and gastrulation is thought to proceed through a linear blastoporal groove; no posterior cumulus is seen. Scorpions show a variation on this pattern; gastrulation is said to proceed by proliferation from a ridge on the blastoderm without formation of a blastoporal groove (Abd-el Wahhab, 1954). In the most basal living chelicerates, the Xiphosura or horseshoe crabs, gastrulation also proceeds through a ventral blastoporal groove. Interestingly, a posterior cumulus (function unknown) is thought to exist in three of the four species (Sekiguchi et al., 1988). Thus, to generalize for the sake of discussion, the chelicerate gastrulation pattern appears to be ingression of mesoderm and endoderm through a ventral blastoporal groove. The two-step gastrulation pattern involving a cumulus and mass internalization of mesendoderm that is seen in araneomorph spiders is not necessarily typical of other chelicerates but could be similar to the pattern in the horseshoe crabs.

Gastrulation in other arthropods typically shows more separation of presumptive mesoderm and endoderm in both space and time. The *Drosophila* pattern of separate internalization of mesoderm at the ventral furrow and endoderm by means of anterior and posterior midgut invaginations holds in general for other insects (Roth, 2004). This finding is clearly different from the chelicerate pattern, where much of the endoderm is internalized more or less continuously with the adjacent mesoderm. In crustaceans, the pattern of gastrulation is more variable. Of the species reviewed by Gerberding and Patel (2004), those that have a pause during gastrulation appear to follow two general patterns, although exceptions exist in each group. The non-malocostracans generally follow a pattern more similar to insects, wherein mesoderm and endoderm move to the interior separated by space, time, or both. Malocostracans have more examples showing internalization of a small number of cells (mesendoderm precursors, vitellocytes, or germ cells) followed by internalization of adjacent mesendoderm. There remains much controversy regarding which group and what mode of gastrulation is ancestral. Gastrulation in Myriapoda is not well characterized and appears to occur by ingression and proliferative mitosis from a blastodisc (Heymons, 1901, as cited in Anderson, 1973).

Finally, a comparison with some species of the phylum Onychophora is interesting because of its phylogenetic position. Onychophorans are generally considered a closely related outgroup to the arthropods (Giribet et al., 2001; Brusca and Brusca, 2002). We will focus on the development of onychophoran eggs with significant amounts of yolk, as these are thought to be the least derived (Anderson, 1973). Yolky embryos gastrulate in a pattern that appears somewhat similar to the chelicerates; they form a blastoderm and undergo gastrulation by means of a linear blastoporal region on the ventral side (Anderson, 1966, 1973). An important difference is that the majority of the gastrulating cells (thought to be prospective midgut) are organized into two bilaterally symmetric groups that appear to internalize by means of proliferative mitosis or involution or both. Also, the position of the prospective mesoderm is thought to be posterior to the prospective midgut. From these comparisons, it seems that the chelicerate gastrulation pattern, namely internalization of spatially continuous me-

soderm and endoderm through a ventral groove, is not ancestral to the arthropods, because such spatial continuity of mesoderm and endoderm is not typical of yolky onychophorans or most arthropods. It should be noted, however, that the precise position of the germ layers relative to the ventral groove is uncertain in the Onychophora, and in virtually all arthropods, because most maps are interpretations of fixed and sectioned specimens. Even Holm's (1952) map for the spider *Agelena labyrinthica* does not positively distinguish between mesoderm and endoderm.

It has become increasingly clear that early embryonic development is flexible, with considerable variation observed even within a single genus in yolk content, mechanism of embryonic axis determination, and mode of gastrulation. For example, direct development has evolved multiple times in the echinoderms and the amphibians (Wilkins, 2002). In some cases, the cell movements of gastrulation have been radically altered to accommodate the derived developmental pattern. In the sea urchin *Heliocidaris tuberculata*, which has a planktotrophic larva, gastrulation follows the pattern typical for indirect-developing sea urchins: the archenteron forms initially by invagination and limited involution, and then elongates by way of convergent–extension cell rearrangement (Wray and Raff, 1991). *Heliocidaris erythrogramma* has a much larger egg that develops into a lecithotrophic larva. In this species, archenteron elongation results primarily from involution of sheets of cells (Wray and Raff, 1991). Another example comes from eggs of the frog *Gastrotheca riobambae*, which develop into feeding tadpoles, but do so in an unusual manner. The embryos form the blastopore near the vegetal pole, and involution of mesoderm and endoderm proceeds locally and symmetrically. This process results in formation of an embryonic disc that will form the body and that is attached to a yolk sac that develops from the remainder of the embryo (del Pino and Elinson, 1983). Other frogs with similar-sized eggs typically do not form an embryonic disc (Keller and Shook, 2004). Early spider development also appears to be rather flexible; a variety of patterns is described in the classic literature, and gastrulation in *A. tepidariorum* (Yamazaki et al., 2005; Oda et al., 2007) and *Zygiella x-notata* appears to differ in fundamental ways. Exploring the variations of spider gastrulation at both the cellular and molecular levels will contribute to an understanding of the evolution of these different strategies.

EXPERIMENTAL PROCEDURES

Collection of Embryos

Adults of *Zygiella x-notata* Clerk (Aranaeidae) and their egg sacs were collected locally. Egg sacs contained 20–80 embryos that developed synchronously. Embryos varied in color from golden-brown (collected in the laboratory) to lavender to dark purple (collected in the field). After collection, silk was removed and embryos were visualized by immersing a few in mineral oil (Sigma). Embryos were then kept in Petri dishes at room temperature (~23°C). The rate of development could be slowed by keeping embryos at cooler temperatures, although early stages did not tolerate long periods at <14°C.

Time-Lapse Imaging

Embryos were placed in mineral oil or dechorionated and embedded in 3% gelatin (300 Bloom, Sigma) in 2 mM HEPES with 50 μg/ml Kanamycin, pH 7.2. Embryos were imaged with Optronics 750-line video cameras and digitized by means of Canopus capture boards. Typically, one frame was captured every 5 min. Movies were made of MPEG-4–compressed files by BTV Pro software running on Macintosh computers. Cells were traced by following their position frame-by-frame in videos, then exporting image files to Photoshop and false-coloring their position at standard time intervals. Movies were made of the false-colored files with BTV Pro software.

Fixation

A fixative was developed that provides excellent results for histology (the heptane-formaldehyde technique gave inferior results in *Z. x-notata*). Embryos were dechorionated in 50% household bleach for 15 min. After rinsing in DI water, embryos were fixed in Ilsa (58% methanol, 17% chloroform, 17% DMSO, 8% acetic acid made fresh each time) for 20–30 min or until embryos were a very pale orange or white. Embryos were then stepped into 100% methanol for storage at −20°C or immediately post-fixed. Fixed embryos were manually devitellined with sharpened Dumont #5 forceps and rehydrated into phosphate buffered saline (PBS) through a stepwise methanol series (10 min/step). Embryos were post-fixed 10 min in 2% paraformaldehyde, 5% DMSO, and then washed 3 × 10 min in PBS with 5% DMSO. Embryos were then stepped into methanol for histology or storage at −20°C.

Embedding and Sectioning

Post-fixed, dehydrated embryos were prestained to facilitate embedding. They were stained with dilute Eosin–Phloxine (3 drops 0.1% stock in 10 ml of methanol) until they were hot pink with distinct cell boundaries (45–120 min, depending on stage). They were then cleared in toluene and passed through three changes of molten paraffin. After embedding, blocks were sectioned at 10 μm until tissue was encountered, and then soaked overnight in 5% glycerol, tissue side down. After soaking, blocks were wiped dry and sectioned at 6–7 μm. Sections were mounted on a degassed solution of Mayer's albumen (Carolina), stained with Delafield's Hematoxylin and Eosin B–Phloxine B, and coverslipped with Permount.

ACKNOWLEDGMENTS

We thank David Rasmussen for sharing unpublished results and for interesting discussions. We also thank Drs. Greta Binford, Paula Cushing, and Susan Masta not only for identifying spiders and clarifying phylogenetic relationships, but also for their enthusiasm, comments, and support. This research was supported by a Mellon grant to Reed College, by a Betty C. Liu Memorial Biology Research Fellowship (to E.V.), and HHMI student research grants.

REFERENCES

Abd-el Wahhab A. 1954. The formation of the germ layers and their embryonic coverings in the scorpion, *Buthus quinquestriatus* (H.E.). Proc Egypt Acad Sci 10: 110–118.

Akiyama-Oda Y, Oda H. 2003. Early patterning of the spider embryo: a cluster of mesenchymal cells at the cumulus produces dpp signals by germ disc epithelial cells. Development 104:391–402.

Akiyama-Oda Y, Oda H. 2006. Axis specification in the spider embryo: *dpp* is required for radial-to-axial symmetry transformation and *sog* for ventral patterning. Development 133:2347–2352.

Anderson D. 1966. The comparative early embryology of the Oligochaeta, Hirudinea and Onychophora. Proc Linn Soc N S W 91:10–43.

Anderson D. 1973. Embryology and phylogeny in annelids and arthropods. New York: Pergamon. 495 p.

Ayoub N, Garb J, Hedin M, Hayashi C. 2007. Utility of the nuclear protein-coding gene, elongation factor-1 gamma (*EF-1*γ), for spider systematics, emphasizing family level relationships of tarantulas and their kin (Araneae: Mygalomorphae). Mol Phylogen Evol 42:394–409.

Brusca R, Brusca G. 2002. Invertebrates. 2nd ed. Sunderland, MA: Sinauer. 936 p.

Crome W. 1963. Embryonalentwicklung ohne "Umrollung" (=Reversion) bei Vogelspinnen. Dtsch Entomol Zeitschrift 10: 83–95.

Davis G, Patel N. 2002. Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu Rev Entomol 47:669–699.

de Rosa R, Prudhomme B, Balavoine G. 2005. *caudal* and *even-skipped* in the annelid *Platynereis dumerili* and the ancestry of posterior growth. Evol Dev 7:574–587.

del Pino E, Elinson R. 1983. A novel development pattern for frogs: gastrulation produces an embryonic disk. Nature 306: 589–591.

Ehn A. 1963. The embryonic development of the spider *Torania variata* Poc. (Sparassidae). Zool Bidr Uppsala 36:37–48.

Foelix R. 1996. Biology of spiders. 2nd ed. New York: Oxford Thieme. 330 p.

Gerberding M, Patel N. 2004. Gastrulation in crustaceans. In: Stern C, editor. Gastrulation. New York: Cold Spring Harbor Laboratory Press. p 79–89.

Giribet G, Edgecombe G, Wheeler W. 2001. Arthropod phylogeny based on eight molecular loci and morphology. Nature 413: 157–161.

Holm A. 1940. Studien uber die Entwicklung und Entwicklungsbiologie der Spinnen. Zool Bidr Uppsala 19:1–215.

Holm A. 1947. On the development of Opilio parietinus Deg. Zool Bidr Uppsala 29:409–422.

Holm A. 1952. Experimentelle Untersuchungen uber die Entwicklung und Entwicklungsphysiologie des Spinnenembryos. Zool Bidrag Uppsala 25:293–424.

Holm A. 1954. Notes on the development of an orthognath spider, *Ischnothele karschi* Bos. & Lenz. Zool Bidr Uppsala 30: 199–221.

Keller R, Shook D. 2004. Gastrulation in amphibians. In: Stern C, editor. Gastrulation. New York: Cold Spring Harbor Laboratory Press. p 171–203.

Kiehart D, Edwards K, Rickoll W, Montague R. 2000. Multiple forces contribute to cell sheet morphogenesis for dorsal closure in *Drosophila*. J Cell Biol 149: 471–490.

Montgomery T. 1909. The development of *Theridium*, an araneid, up to the stage of reversion. J Morphol 20:297–352.

Oda H, Nishimura O, Hirao Y, Tarui H, Agata K, Akiyama-Oda Y. 2007. Progressive activation of Delta-Notch signaling from around the blastopore is required to set up a functional caudal lobe in the spider *Achaearanea tepidariorum*. Development 134:2195–2205.

Rempel J. 1957. The embryology of the black widow spider, *Latrodectus mactans*. Can J Zool 35:35–74.

Roth S. 2004. Gastrulation in other insects. In: Stern C, editor. Gastrulation. New York: Cold Spring Harbor Laboratory Press. p 105–121.

Saaristo M. 2006. Theridiid or cobweb spiders of the granitic Seychelles islands (Araneae, Theridiidae). Phelsuma 14:49–89.

Schimkewitsch L, Schimkewitsch W. 1911. Ein Betrag zur Entwicklungsgeschichte der Tetrapneumones. Bull Acad Imp Sci St. Petersbourg 8:637–654.

Schoppmeier M, Damen W. 2005. Expression of Pax group III genes suggests a single-segmental periodicity for opisthosomal segment patterning in the spider *Cupiennius salei*. Evol Dev 7: 160–169.

Seitz K-A. 1966. Normale Entwicklung des Arachniden-Embryos *Cupiennius salei* Keyserling und seine Regulationsbefähigung nach Röntgenbestrahlungen. Zool Jahrb Anat Ontogenie 83:327–447.

Sekiguchi K, Yamamichi Y, Seshimo H, Sugita H. 1988. Normal development. In: Sekiguchi K, editor. Biology of horseshoe crabs. Tokyo: Science House. p 133–224.

Stollewerk A, Schoppmeier M, Damen W. 2003. Involvement of *Notch* and *Delta* genes in spider segmentation. Nature 423: 863–865.

Wilkins A. 2002. The evolution of developmental pathways. Sunderland, MA: Sinauer. 603 p.

Wray G, Raff R. 1991. Rapid evolution of gastrulation mechanisms in a sea urchin with lecithotrophic larvae. Evolution 45: 1741–1750.

Yamakazi K, Akiyama-Oda Y, Oda H. 2005. Expression Patterns of a *twist*-related gene in embryos of the spider *Achaearanea tepidariorum* reveal divergent aspects of mesoderm development in the fly and spider. Zool Sci 22:177–185.

Yoshikura M. 1955. Embryological studies on the liphistiid spider *Heptathela kimurai*. Kumamoto J Sci Ser B 2:1–86.

Yoshikura M. 1958. On the development of the purse-web spider, *Atypus karschi* Donitz. Kumamoto J Sci Ser B 3: 73–85.