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# Shifts in the life history of parasitic wasps correlate with pronounced alterations in early development

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**ABSTRACT** **Developmental processes have been traditionally viewed to be invariant within higher taxa. However, examples are known whereby closely related species exhibit alterations in early embryogenesis yet appear very similar as adults. Such developmental changes are thought to occur in response to shifts in life history. In insects, the regulation of embryonic development has been intensively studied in model species like *Drosophila melanogaster*. Previous comparative studies suggest that the developmental processes documented in *Drosophila* well describe embryogenesis of advanced, holometabolous, insects generally. There have been few attempts, however, to take into account how life history has influenced early development of insects or to characterize early development of species with life histories fundamentally different from flies. Here we compared early development of two species from the same family of parasitic wasps that exhibit very different life histories. *Bracon hebetor* is an ectoparasite that lays large, yolky eggs on the integument of its host that develop much like the free-living honeybee and *Drosophila*. In contrast, *Aphidius ervi* is an endoparasite that lays small and apparently yolk-free eggs that develop in the hemocoel of the host. This wasp exhibits a radically different mode of early development at both the cellular and molecular level from *B. hebetor*. The developmental changes in *A. ervi* reflect functional adaptations for its derived life history and argue that departures from the fly paradigm may occur commonly among insects whose eggs develop under conditions different from typical terrestrial species.**

Traditionally, changes in early development have been thought to occur rarely because such alterations are lethal or result in new adult phenotypes (1–3). A few examples among metazoans, however, describe pronounced differences in early development between closely related species without any concomitant changes in adult body form (4–9). These studies indicate not only that alterations in embryogenesis can occur without major consequence for the adult body plan but suggest that adaptations in early development may arise in response to changes in life history. How widespread punctuated modes of developmental evolution are among taxa and whether certain life history transformations lead to changes in early development more often than others are unclear (3).

In insects, regulation of embryogenesis has been intensively studied in the model species, *Drosophila melanogaster*, yet the role life history has played in shaping patterns of early development of this species-rich group of organisms is largely unknown. *Drosophila* and the honeybee, *Apis mellifera*, are members of two of the phylogenetically most advanced insect orders: the Diptera (flies) and Hymenoptera (bees, ants, and wasps) (Fig. 1). Both lay yolky eggs that undergo syncytial cleavage and long germband development whereby all segments of the body are established near simultaneously (10, 11). In *Drosophila*, the patterning process is initiated by maternal factors localized during oogenesis that trigger transcription of gap and pair-rule segmentation genes whose products diffuse within the syncytium to produce gradients of positional information (12, 13). By the time the blastoderm cellularizes, these factors have programmed the cells in different regions of the embryo to express segment polarity and homeotic genes that define segment-specific and regional identities.

If ancestry is the primary factor driving patterns of early development, we would expect that most insects in the monophyletic Hymenoptera (14, 15) would look much like the honeybee. However, if the environmental conditions in which insect eggs develop also influence embryogenesis, we would expect that changes in early development will occur in species with fundamentally different life histories from *Drosophila* and other free-living advanced insects like the honeybee. Analysis of the most advanced hymenopterans (the Apocrita) reveals that a sharp dichotomy in life history evolved within 50 million years: the aculeates, as represented by the honeybee, became free living pollinators or predators, whereas most other apocritans evolved into parasites of other arthropods (15–17). The parasitic wasps develop as either ectoparasites, which lay their eggs on hosts where the larvae feed through the host's exoskeleton, or as endoparasites, which inject their eggs into the body of hosts where the progeny feed directly in the host's hemocoel. The most primitive hymenopterans were either free-living, phytophagous insects (sawflies) or ectoparasites (Orussoidea) whose progeny fed on other insects. Phylogenetic analysis reveals that the monophyletic Apocrita likely evolved from an ectoparasitic ancestor, and that free-living and endoparasitic species arose within this group thereafter (15–17). Thus, replicate shifts in life history strategy have occurred in the Hymenoptera such that free-living, ectoparasitic, and endoparasitic lifestyles have arisen multiple times. At the superfamily level, the sister group to the Aculeata as represented by the honeybee, is the parasitic Ichneumonoidea whose species are divided into the families Braconidae and Ichneumonidae (16, 17) (Fig. 1). Both of these families are comprised of ecto- and endoparasitic species.

To examine how the transformation between free-living, ecto-, and endoparasitic life histories has influenced early development of insects, we examined embryogenesis of two parasitic wasps from the Ichneumonoidea in relation to the paradigms established through *Drosophila* and the honeybee. Our results show that the ectoparasite *Bracon hebetor* lays large, yolky eggs that develop very similarly to the honeybee and *Drosophila*. In contrast, the endoparasite *Aphidius ervi* lays small, apparently yolk-free eggs that develop in a manner radically different from most other insects described in the literature (10, 11).

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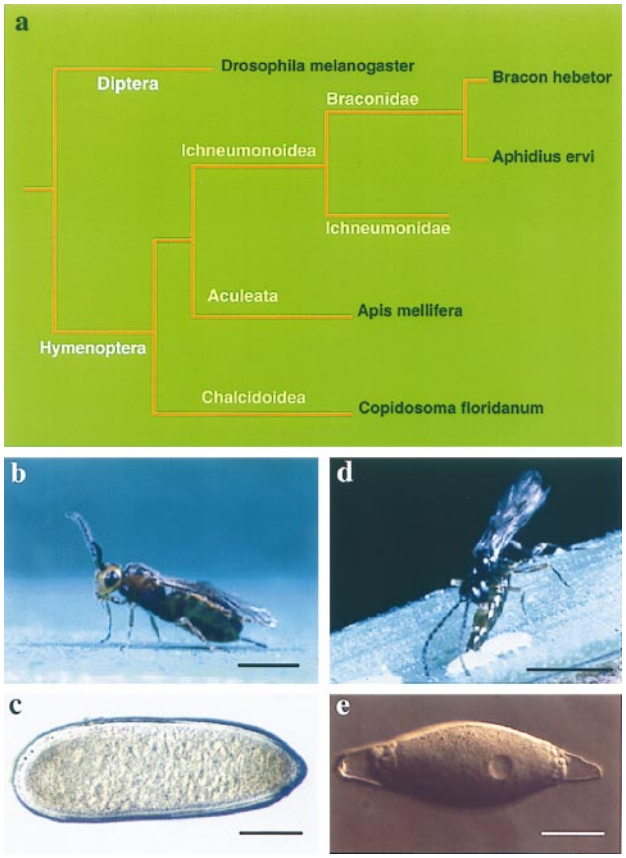


FIG. 1. Phylogeny and life history of *Bracon hebetor*, *Aphidius ervi*, and selected other hymenopterans (15–17). (*a*) Both *B. hebetor* and *A. ervi* are in the family Braconidae (superfamily Ichneumonoidea). Almost all ichneumonoids develop as either ecto- or endoparasites of other arthropods. The sister group to the Ichneumonoidea is the Aculeata that includes the honeybee, *Apis mellifera*. The other parasitic wasp in the phylogeny, *Copidosoma floridanum*, is the only other endoparasite whose embryonic development has been studied at the cellular and molecular level (20, 34). This wasp is in the family Encyrtidae (superfamily Chalcidoidea) and is more distantly related to *A. ervi* than *B. hebetor* and *A. mellifera*. Despite this, its eggs are devoid of yolk and early development proceeds in a cellularized environment like *A. ervi*. Although not completely resolved, the sister group to the Hymenoptera is thought to be the Mecopteroidea that includes the order Diptera and the model species *Drosophila melanogaster*. (*b*) *B. hebetor*: this wasp is a 3 mm long ectoparasite that lays its eggs on the integument of moth larvae such as *Plodia interpunctella*. (*c*) A close-up view of a *B. hebetor* egg. Like eggs of other terrestrial flies and wasps (10, 11), *B. hebetor* eggs are large (0.5 mm), yolk-rich, surrounded by a thick chorion, and elongated along their anterior-posterior axis. After hatching, *B. hebetor* larvae develop by rasping a hole through the host's integument and feeding on its tissues. (*d*) *A. ervi*: this wasp is a 1–2 mm long endoparasite that lays a single egg into the haemocoel of its aphid host, *Acyrthosiphon pisum*. (*e*) A close up view of an *A. ervi* egg. The egg is small (0.05 mm), yolkless, and is surrounded by a thin chorion. The *A. ervi* larva feeds inside the host and emerges as an adult by chewing a hole through the cuticle of the host. Scale bars = 1 mm (*b, d*); 100 μm (*c*); 15 μm (*e*).

## MATERIALS AND METHODS

**Insects.** *B. hebetor* was reared on the larval stage of its moth host, *Plodia interpunctella* (18). *A. ervi* was reared on its aphid host, *Acyrthosiphon pisum* (19). Fig. 1 summarizes the life history of these species and their phylogenetic relationships to selected other hymenopterans.

**Morphological Characterization.** *B. hebetor* eggs were collected from the surface of host larvae, whereas *A. ervi* eggs were dissected from parasitized aphids into physiological saline in a polyethyleneglycol-treated Petri dish. *B. hebetor* embryos at different stages of development were dechorionated in 50% bleach for 8 min, fixed for 30 min in 4% PEM buffer (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$) plus 0.5% Nonidet P-40 detergent. During fixation, embryos were constantly agitated on a vortex mixer. Embryonic development of *A. ervi* was monitored by placing newly laid eggs into *in vitro* cultures maintained in TC-100 medium (JRH Scientific, Lenexa, KS) supplemented with 10% fetal bovine serum (HyClone). At selected intervals *A. ervi* embryos were fixed for 25 min. in 4% PEM buffer. Embryos of both wasp species were stained with the nuclear dye 4,6-diamidino-1,2-diphenyl-3-methyl-2-butanol and fluorescein isothiocyanate-conjugated phalloidin that labels cortical actin (20). Embryos were examined by Nomarski, fluorescent, or confocal microscopy by using either a Nikon Diaphot inverted microscope or Bio-Rad 6000 laser scanning confocal microscope.

**Cell Injections.** *A. ervi* eggs were transferred into a nine-well glass dish in saline and treated with 100 μg/ml proteinase K for 4 min. After treatment, eggs were transferred to an injection well filled with TC-100 medium (JRH Scientific) and placed on an inverted Nikon Diaphot microscope with Hoffman modulation contrast optics. Ionotophoretic injections were performed by methods described previously (20) by using tetramethylrhodamine-conjugated lysine fixable dextran ($M_r$, $3 \times 10^3$; Molecular Probes). Embryos were fixed after injection as described above, counterstained with phalloidin, and examined by confocal microscopy.

**Antibody Staining.** To characterize antigen expression during embyrogenesis, *B. hebetor* eggs were dechorionated and fixed for 30 min in 4% formaldehyde, 0.5% Nonidet P-40 in PEM buffer as described above. *A. ervi* embryos were dissected from host aphids in physiological saline, fixed for 25 min in 4% formaldehyde in PEM buffer, and then dissected (with tungsten needles) from the extraembryonic membrane. Labeling reactions were conducted as described (20) with the following primary antibodies: even-skipped (Eve; mAb2B8; ref. 21), engrailed (En; mAbEN4F11; ref. 22), and Ultrabiothorax/Abdominal-A (Ubx/AbdA; mAbFP6.87; ref. 23). Secondary antibodies (biotinylated horse anti-mouse IgG; Jackson ImmunoResearch) and rhodamine-conjugated streptavidin (Jackson ImmunoResearch) were diluted 1:200 in PBST (phosphate-buffered saline plus 0.5% Triton X-100). Embryos were counterstained with phalloidin. For double labeling, antibody reactions were carried out sequentially with the first antibody detected with donkey anti-mouse IgG-conjugated to Cy5 (Jackson ImmunoResearch; 1:200 dilution) and the second antigen detected as described above. Embryos were examined by confocal microscopy. As controls, *A. ervi* embryos in the retracted germband stage (expressing Eve in neurons), and *Drosophila* embryos were processed together with early stage *A. ervi* embryos. These experiments were repeated on over 300 embryos by using different reagents and populations of *A. ervi* from the United States and the United Kingdom. We were always able to detect staining in the dorsal mesoderm and neurons of late stage *A. ervi* embryos as well as early and late stage *Drosophila* embryos. However, we never detected early Eve staining above background levels in early stage *A. ervi* embryos (see *Results*).

## RESULTS

**Embryogenesis of *B. hebetor* and *A. ervi*.** Morphological characterization revealed that *B. hebetor* lays yolky eggs surrounded by a rigid chorion. Early embryogenesis proceeded in a syncytium similar to other canonical long germband insects (Fig. 2 *a–f*). In contrast, *A. ervi* displayed a very different form of embryogenesis. Except for the first cleavage that proceeded without cytokinesis (Fig. 2 *f* and *h*), all other cleavages were cellular (Fig. 2 *i–o*). This resulted in formation of early blastomeres, and development of a morula stage embryo that

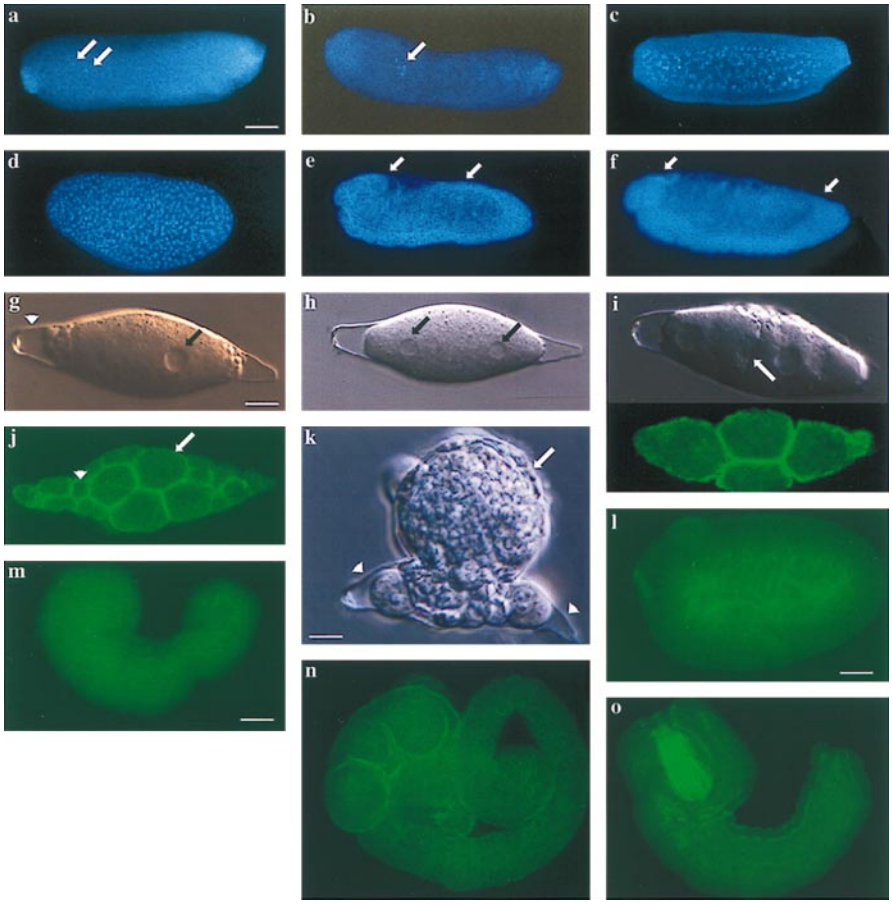


FIG. 2. Embryogenesis of *B. hebetor* and *A. ervi*. Confocal, fluorescent, and Nomarski images of embryonic development. (*a*) After oviposition the *B. hebetor* egg has a clear polarity corresponding to the dorsal-ventral and anterior-posterior embryonic axes. Embryonic nuclei (arrows) divide without cytokinesis. (*b, c*) During the first few syncytial cleavages nuclei remain in the yolk (arrow). (*d*) After the tenth cleavage nuclei migrate to the periphery of the egg where they undergo two additional division cycles in the syncytium before finally forming a cellular blastoderm (39). (*e*) The embryo then undergoes germband extension (anterior and posterior limits of the embryo marked by the arrows in *e* and *f*). (*f*) This is followed by germband retraction and segmentation. (*g*) After oviposition, the *A. ervi* egg is lemon-shaped and does not exhibit any axial polarity (nucleus marked by an arrow(s) in *g* and *h* and chorion by arrowhead). (*h*) The first nuclear division proceeds in a syncytium, without cytoplasmic cleavage. (*i*) The second cleavage results in formation of four nuclei that become separated by cell membranes (*Upper*, a single focal plane with two blastomeres, the cell membrane is marked by an arrow; *Lower*, the same stage embryo, phalloidin staining demarcates the cell cortex underlying the cell membranes in all four blastomeres). (*j*) The embryo undergoes cleavage to form large (which form the future extraembryonic membrane, arrow) and small blastomeres (which form the embryo proper, arrowhead). (*k*) The extraembryonic membrane surrounds the embryonic cells (arrow) and the embryo ruptures from the chorion (arrowhead). (*l–o*) The embryonic primordium remains surrounded by the extraembryonic membrane and initiates morphogenesis in the host's haemocoel. (*l*) Embryonic primordium. (*m*) The embryo undergoes germband extension by posterior growth. (*n*) Fully extended germband stage whereby the embryo assumes a coiled shape. (*o*) This is followed by condensation and segmentation of the embryo. (*l–o*) The extraembryonic membrane was removed. Bars = 80 μm (*a–f* and *m–o*); 7 μm (*g–k*); 50 μm (*l*).

ruptured from the chorion. These events were followed by morphogenesis that resembled short germband development (Fig. 2 *l–o*).

***A. ervi* Undergoes Holoblastic Cleavage.** To determine whether *A. ervi* embryos developed in a completely cellularized environment, we injected individual blastomeres with a fluorescently conjugated dextran tracer (Fig. 3). At the four- and eight-cell stage, our tracer diffused from each injected blastomere (Fig. 3*a*). However, when small and large blastomeres were injected at the 16-cell and later stages, the tracer remained only in the injected blastomere (Fig. 3 *b* and *c*). This indicated that early embryonic development of *A. ervi* proceeds in a cellularized environment, and that molecules larger than our tracer dye, such as transcription factors of the *Drosophila* patterning hierarchy, could not freely diffuse between embryonic cells.

**Eve, En, and Ubx/Abd-A Expression.** To compare patterning events at the molecular level, we stained *B. hebetor* and

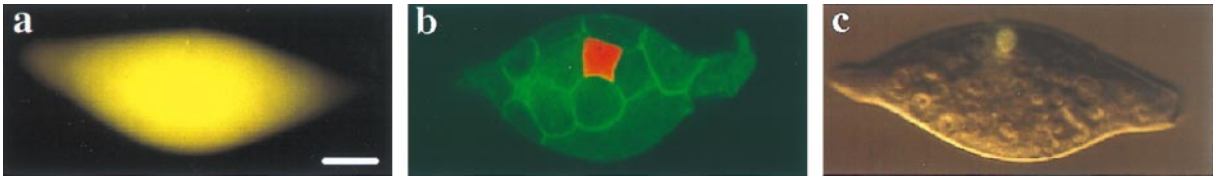


FIG. 3. Fluorescent and confocal images of *A. ervi* blastomere injections. (*a*) Fluorescent image of an embryo injected at the four-cell stage. The tracer dye spread throughout the embryo (yellow) ($n = 28$). (*b*) Confocal image of a blastomere injected at the 16-cell stage ($n = 36$). Injected cell (red), cortical actin (green). (*c*) Fluorescent image of an embryonic cell injected at the putative 64-cell stage ($n = 42$). The dye remains confined to the injected cell (yellow). Bar = 10 μm (*a–c*).

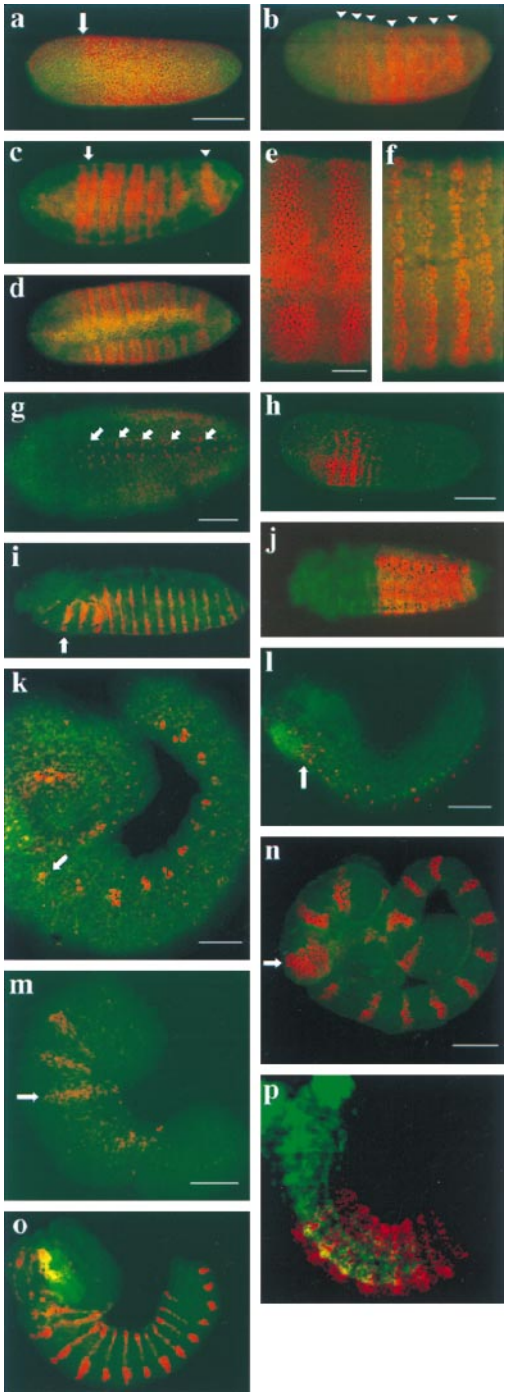


FIG. 4. Protein expression patterns of Eve, En, and Ubx/Abd-A in *B. hebetor* and *A. ervi* embryos. Confocal images of embryos stained with the anti-Eve antibody mAb2B813 (21), anti-En antibody EN4F11 (22), and anti-Ubx/Abd-A antibody mAbFP6.87 (23) (red) and counterstained with phalloidin (green). (*a–g*) Eve expression in *B. hebetor*. (*a*) Eve expression in the syncytial blastoderm (arrow demarcates anterior boundary). (*b*) Eve expression resolved into a pair-rule pattern in the syncytial blastoderm (arrowheads). (*c*) Individual pair-rule stripes begin to split into two stripes in a brief anterior to posterior progression (arrow marks split of the first pair-rule stripe and an arrowhead last, undivided, stripe). (*d*) Complete split of pair-rule stripes results in segmental iteration of the stripes (ventral up). (*e, f*) High magnification of pair-rule and segmental Eve expression. (*e*) Pair-rule stripes are eight nuclei wide. (*f*) Antigen fades in the central nuclei and continues to be expressed in two rows of peripheral nuclei. (*g*) Eve expression in neurons in a bilateral pattern along the ventral midline (arrows, ventral up). (*h, i*) En expression pattern in *B. hebetor*. (*h*) Formation of En stripes in a brief anteroposterior progression. (*i*) Mature En pattern (arrow marks mandibular stripe in the posterior segment compartment). (*j*) Ubx/Abd-A expression in the posterior thorax and abdomen of *B. hebetor*. (*k–l*) Eve expression in *A. ervi*. Eve protein is not expressed prior to germband condensation. (*k*) Expression of Eve in the dorsolateral mesoderm (arrow marks the most anterior group of dorsal cells expressing Eve). (*l*) Expression of Eve in neurons along the ventral midline (arrow marks anterior neuroblasts expressing Eve). (*m, n*) Formation of En stripes in *A. ervi*. (*m*) Initial expression of En in gnathal segments (arrow marks labial stripe). (*n*) En stripes formed sequentially as the germband extends (arrow marks labial stripe). (*o*) Mature En pattern demarcating the posterior segmental compartment. (*p*) Mature pattern of Ubx/Abd-A from the posterior thorax to the penultimate abdominal segment. In all panels, except *d* and *g*, anterior is on the left and dorsal is up. Scale bars: *a–d*, 110 μm; *e* and *f*, 16 μm; *g*, 60 μm; *h–j*, 80 μm; *k*, 60 μm; *l*, 100 μm; *m*, 80 μm; *n–p*, 100 μm.

*A. ervi* embryos with antibodies that recognize conserved epitopes of Eve, En, and Ubx/Abd-A in different insect species (21–23). *Eve*, a primary pair-rule gene is expressed in the *Drosophila* syncytium and forms a characteristic seven-stripe pattern with double segment periodicity (24). *En*, which is regulated by *Eve*, is a segment polarity gene that specifies the posterior segmental compartments (25). Ubx and Abd-A are *Drosophila* homeotic proteins that specify the posterior thorax and abdomen (26). In *B. hebetor*, Eve was expressed in a largely conserved fashion to *Drosophila* and other long germband insects. Initially, a broad domain of Eve expression (Fig. 4*a*) split into broad pair-rule stripes (Fig. 4*b*), followed by a split of the individual pair-rule stripes in rapid anteroposterior progression to form segmentally iterated stripes (Fig. 4 *c–f*). After germband retraction, Eve was localized in the cells of the dorsolateral mesoderm (not shown) and neurons (Fig. 4*g*); a pattern conserved in all examined insects (27). In *B. hebetor*, En was expressed in a rapid anteroposterior progression (Fig. 4*h*), forming a mature pattern of segmentally iterated stripes that localized to the posterior segmental compartments (Fig. 4*i*). Finally, the antibody against Ubx/Abd-A stained the region from the posterior thorax to the penultimate abdominal segment (Fig. 4*j*).

When we stained *A. ervi* embryos with anti-Eve, we were unable to detect either a pair-rule or segmental pattern. In the extended germband, however, an Eve antigen was detected in dorsolateral mesoderm and neurons (Fig. 4 *k* and *l*). En stripes appeared when embryos initiated germband extension (Fig. 4*m*). These stripes formed sequentially as the germband extended (Fig. 4*n*), resulting in a mature pattern of segmentally iterated stripes that localized to the posterior segmental compartments (Fig. 4*o*). Ubx/Abd-A was expressed in the posterior thorax and abdomen in the retracted germband stage (Fig. 4*p*).

## DISCUSSION

The contrasts between early development of *B. hebetor* and *A. ervi* are as large as any described for insects in the comparative developmental literature. However, unlike the differences reported between insects in phylogenetically distant orders such as the so called primitive (i.e., hemimetabolous) grasshopper, *Schistocerca gregaria* (Orthoptera), and advanced (holometabolous) fly, *Drosophila melanogaster* (Diptera) (14), our study compared development of wasps in a single monophyletic family of advanced insects. The implications of these results is that a change in life history strategy from a free-living or ectoparasitic existence to survival as an endoparasite is correlated with significant alterations in early development.

Prior to this study, segmental patterning had been examined in selected species of advanced insects from the orders Coleoptera (*Tribolium*, *Callosobruchus*), Lepidoptera (*Manduca*), Diptera (*Musca*), and Hymenoptera (*Apis*) (28–33). These studies reveal that each of these species utilizes homologs of *Drosophila* gap and pair-rule genes in patterning of the early embryo. Each of these

species also begins development by undergoing syncytial cleavage. Combined, these results suggest that patterning mechanisms in insects are broadly conserved, and have led to the conclusion that the paradigms established through *Drosophila* largely explain development of insects in phylogenetically advanced orders (27). Because *B. hebetor* exhibits a similar type of embryogenesis and patterning program, we suggest that the evolution of a parasitic life history *per se* also does not result in significant alterations in early development. Yet, when we compare other characteristics between *B. hebetor*, *A. ervi*, and the aforementioned free-living insect species described above, the following trend emerges. Most free-living insects develop in a terrestrial environment where adaptations for survival include a rigid chorion for protection from desiccation and a yolk source to supply nutrients for development. The eggs of ectoparasites, like *B. hebetor*, face the same ecological circumstances as the eggs of other terrestrial insects. They too develop in a terrestrial environment on the surface of the host where the risks of desiccation are significant and the need for a prepackaged yolk source is essential for completion of embryonic development. In contrast, the eggs of endoparasites, like *A. ervi*, develop in the nutrient-rich, aquatic environment of the host's hemocoel where protection from desiccation and a prepackaged source of nutrition are unnecessary.

We conceive the loss of yolk and a rigid chorion as underlying the change to holoblastic cleavage and concomitant loss of pair-rule patterning in *A. ervi*. In another endoparasitic wasp, *Copidosoma floridanum*, we also have documented the loss of yolk, major changes in the cellular aspect of embryogenesis, and the loss of an Eve pair-rule pattern (20). Because *C. floridanum* resides in a different superfamily (Chalcidoidea) of wasps from *A. ervi* strongly suggests that the changes in early development documented in these species have occurred in response to their shared endoparasitic life history. As noted previously, most apocritan superfamilies contain both ecto- and endoparasitic species; arguing that endoparasitism has evolved independently multiple times in the Hymenoptera (15–17). Inspection of the descriptive embryological literature reveals that total cleavage has arisen in association with endoparasitism in all of the major apocritan superfamilies, whereas syncytial cleavage occurs in ectoparasitic species in a manner similar to that of the honeybee and *B. hebetor* (34, 35). Despite the divergence of early patterning, late patterning in *B. hebetor* and *A. ervi* includes conserved expression of En and Ubx/Abd-A. This expression pattern in the germband, the phylotypic stage in insects (10), suggests conservation of this stage irrespective of how development begins. Based on these results, we would predict that changes in patterning mechanisms will occur in other advanced insect taxa that exhibit shifts in life history that favor the loss of yolk or early cellularization. By contrast, advanced insects with ectoparasitic or free-living life histories will exhibit patterning mechanisms that resemble those of *Drosophila*.

Finally, our findings raise questions about patterning mechanisms in short and long germband embryos more generally. Short, intermediate and long germband beetles display differences in the number of Eve pair-rule stripes at the time of gastrulation (32), whereas a more dramatic absence of pair-rule gene expression is found in the short germband grasshopper (21, 36). The common element shared during embryogenesis between the primitive (hemimetabolous) grasshopper and endoparasitic wasps like *A. ervi* is early cellularization (34, 37, 38, 40). This implicates the cellular environment as perhaps the key factor modifying patterning mechanisms. Because endoparasitic wasps arose from ectoparasitic long germband ancestors (16, 17), a switch to short germband development in *A. ervi* reveals the potential for short germband development from long germband ancestors in response to a switch in life history. Future studies on short germband species from primitive and advanced insect groups should clarify how this developmental transition occurs.

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