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Polyembryony in parasitic wasps: evolution of a novel mode of development

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ABSTRACT Major developmental innovations have been associated with adaptive radiations that have allowed particular groups of organisms to occupy empty ecospace. Well-known developmental novelties associated with the conquest of new habitats include the evolution of the tetrapode limb, allowing the radiation of vertebrates into a terrestrial habitat, and formation of insect wings that permitted their dispersal into the air. However, an understanding of the evolutionary forces and molecular mechanisms behind developmental novelties still remains tenuous. A little-studied adaptive radiation in insects from the developmental perspective is the evolution of parasitism. The parasitic lifestyle has allowed parasitic insects to occupy a novel ecological niche where they have evolved a plethora of life history strategies and modes of embryogenesis, developing on or within the body of the host. One of the most striking adaptations to development within the body of the host includes polyembryonic development, where certain wasps form clonally up to 2000 embryos from a single egg. Taking advantage of well-established insect phylogeny, techniques developed in a model insect, the fruit fly, and a wealth of knowledge in comparative insect embryology, we are starting to tease apart the evolutionary events that have led to this novel mode of development in insects.

KEY WORDS: *polyembryony, developmental novelty, evolution of development, maternal specification, pattern formation*

Introduction

Polyembryonic development represents the formation of multiple embryos from a single zygote. The accidental form of polyembryonic development, where an individual egg occasionally forms multiple embryos, has been described in almost all animal groups studied to date (Olsen 1962; Stansfie, 1968; Kaufman, 1982; Laale, 1984; Ashwort *et al.,* 1998). This accidental form of polyembryony suggests that eggs of otherwise monoembryonic species have the regulative capacity to generate multiple embryos. On the other hand, obligatory polyembryonic development, where a single zygote of certain species invariably produces multiple embryos, is a relatively rare event in metazoans, but quite frequent in plants (Shaanker and Ganeshaiah, 1996; Carman, 1997). In metazoans, obligatory forms of polyembryonic development are present in both vertebrates and invertebrates. Species exhibiting polyembryonic development are scattered in multiple phyla including Cnidaria, Platyhelmintes, Arthropoda, Bryozoa, Echinodermata and Chordata (reviewed in Craig *et al.,* 1997). It should be noted that in certain groups, the source of clones is not the embryo but the larva, as in all described cases of polyembryony in the phila Cnidaria and Echinodermata, and in Cestodea and Trematoda (Platyhelmintes) and Crustacea (Arthropoda) (Noble *et al.,* 1989; Shostak, 1993; Glenner and Hoeg, 1995; Jaeckle, 1994).

The focus of this review is obligatory polyembryony in insects that arises by embryonic cloning. The term *polyembryony* denotes both the developmental process, and the form of reproduction. Developmental processes include complex cellular and molecular events whereby multiple embryos form clonally from a single zygote (acquired by parasitic wasps at least 100 MYR before Dolly, the sheep). In addition, polyembryony refers to a unique form of reproduction in which a single egg results in multiple progeny, maximizing the reproductive capacity of the species and increasing its fitness. Along with its ecological and reproductive ramifications, study of the phenomenon of polyembryony in insects has the potential for addressing one of crucial questions in the evolution of development: How do developmental novelties arise? Polyembryony in insects represents a developmental novelty whereby both precursor structure and evolutionary processes are basically unknown (type A novelty *sensu* Wilkins 2001). In general, true developmental novelties are rare and often their evolution is not easily tractable. However, the combination of a relatively well-established insect phylogeny, embryological studies of insect polyembryony that span more than a century (Marchal, 1898), and

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techniques and concepts established in a closely-related model Arthropod, *Drosophila melanogaster*, demonstrate a promising system that could provide clues as to how complex developmental novelties are formed.

In this review, I will present the current model for the evolution of embryogenesis in insects, and develop the phylogenetic context of the evolution of polyembryony in parasitic wasps. The phylogenetic perspective will allow us to understand the polarity of embryological evolution and will help to clarify how mechanisms utilized in polyembryonic embryogenesis, that are challenging current paradigms of *Drosophila* development, evolved. Finally, I will propose a sequence of evolutionary events and testable scenarios that could have led to this novel form of development.

Evolution of embryonic development in insects

In order to be able to map changes in the embryonic development of polyembryonic insects it is necessary to have a typical "road map" of the evolutionary trajectory of insect embryogenesis. The insect egg is formed in the ovary where the initial coordinates of embryo axial polarity are established (Buning, 1994). After oviposition, the oocyte nucleus undergoes a variable number of nuclear divisions in syncytium (without division of cytoplasm) to form the critical number of nuclei necessary for formation of the cellular blastoderm. At this point embryogenesis bifurcates in two groups of insects. Primitive insects (hemimetabolous, whose larvae resemble adults and which do not undergo metamorphosis) initially form small embryonic primordium consisting of anterior structures (short germband insects) or anterior structures plus some portion of the trunk (intermediate germband insects). The posterior structures are formed by the growth of the posterior growth zone (Sander, 1976). In contrast, advanced, holometabolous insects (that evolved a pupal stage) such as *Drosophila* and the honeybee, form long germband embryos, which contain all future body regions at the blastoderm stage (Sander, 1976). Thus, the future structures of long germband embryos are formed *in situ*, without further differential growth. Even though embryonic developmental programs differ in these early stages of development at fully extended germband stage both embryos are virtually the same. Data from different phylogenetic groups of insects supports an evolutionary trajectory in which some sort of short or intermediate form of embryogenesis was ancestral in the insect lineage, subsequently evolving into long germband development in advanced insects (reviewed in Tautz *et al.,* 1994).

It appears that type of ovarial structure which is involved in the process of egg formation is associated with a certain type of embryogenesis (Sander, 1976; Patel *et al.,* 1994). It has been suggested that short germband development is associated with oogenesis in panoistic ovaries (that lack nurse cells, thus all factors for embryo development have to be transcribed from the oocyte nucleus) and long germband with meroistic polytrophic ovaries (where nurse cells synthesize maternal factors crucial for embryo development, and the oocyte nucleus is largely transcriptionally silent). However, this difference is not clear-cut, illustrated by specific cases of association of panoistic ovaries with long germband development and meroistic ovaries with short germband development (Sander 1976).

These two patterns of embryogenesis attracted the attention of embryologists due to not only their morphological differences, but also the different developmental potentials of short and long germband embryos. Accidental cases of insect embryo twinning are abundant in the embryological literature (Cappe de Baillon, 1927; Slifer and Shulow, 1947; Prevost and McFarlane, 1979; Cabrero *et al.,* 1996). Interestingly, described cases of embryo twinning are exclusive to short germband insects. In contrast to spontaneous twinning in primitive insects, there is no report of accidental twinning in long germband insects (Sander, 1984). In addition, embryological manipulative data suggest a unique regulative potential of primitive insects, where simple cauterization or chilling can generate multiple embryos or embryo duplication in the single insect egg (Sander, 1976; Sander, 1984). Collectively, this suggests that primitive, short germband insect embryos have a regulative capacity that is absent from modern, long germband insect embryos.

Multiple events of independent evolution of polyembryonic development in wasps

Hymenoptera (wasps) represents a holometabolous insect order that consists of two suborders. Suborder Symphita includes basal plant-eating groups, and Apocrita, an advanced group of parasitic species (Fig. 1). Hymenoptera posses polytrophic meroistic ovaries (Buning, 1994) and basal groups produce yolky eggs which undergo long germband embryogenesis (Speicher, 1936; Fleig

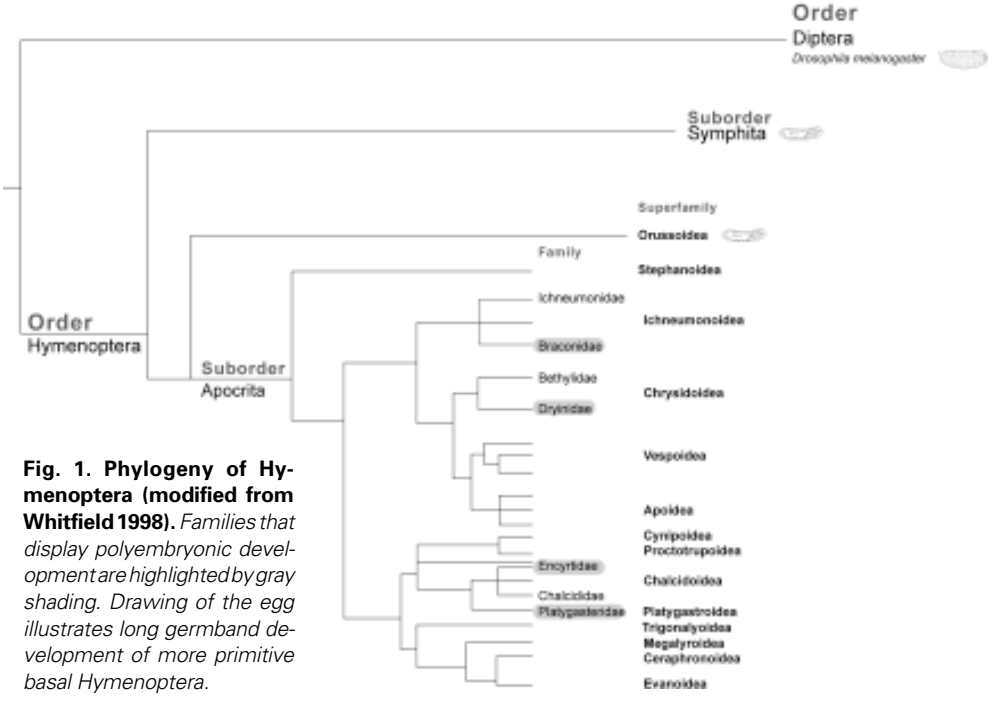

Fig. 1. Phylogeny of Hymenoptera (modified from Whitfield 1998). *Families that display polyembryonic development are highlighted by gray shading. Drawing of the egg illustrates long germband development of more primitive basal Hymenoptera.*

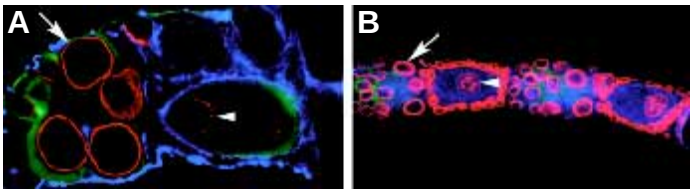

Fig. 2. Confocal images of ovaries of *Copidosoma* and *Macrocentrus*. (A) Copidosoma *ovaries.* **(B)** Macrocentrus *ovaries. Red staining with anti-nuclear pore protein highlights nuclei. Blue represents phalloidin staining of filamentous actin. Anterior is to the left. Arrowhead marks oocyte nucleus and arrow points to nurse cell nucleus.*

and Sander, 1986). Apocrita (parasitic wasps plus ants and bees) represents a monophyletic assemblage which includes ectoparasitic species (laying the egg on the surface of the host), endoparasitic species (ovipositing within the body of the host), and free-living pollinators including eusocial species (Whitfield, 1998). Basal species in all parasitic groups whose life histories are known appear to be ectoparasitic. They lay large yolky eggs, and undergoing long germband development, such as described in the honeybee (Fleig, 1990; Binner and Sander, 1997) and the endoparasitic basal braconid *Bracon hebetor* (Grbic and Strand, 1998). This suggests that the basal state of embryonic development in parasitic wasps includes canonical long germband development associated with meroistic polytrophic oogenesis, where critical determinants are transcribed in nurse cells and transported to the oocyte in a manner described in *Drosophila*. However, many parasitic lineages contain parasitic species that have evolved a derived form of development within the body of the host (endoparisites). This switch in life history strategy subjects them to a different selection regime compared to other terrestrial insects. The evolution of endoparasitism appears to be crucial for further evolutionary innovations, such as polyembryony. Polyembryony evolved independently four times in wasps: in Braconidae, Encyrtidae, Dryinidae and Platygasteridae (Ivanova-Kazas, 1972). The association of endoparasitc lifestyle with evolution of polyembryony is strengthened by the fact that the only other case of polyembryony in insects is displayed by endoparasitic Strepsiptera (Noskiewicz and Poluszynski, 1935).

Polyembryonic embryogenesis: embryological innovations

Independent evolution of polyembryony evokes several important questions. First, what is qualitatively novel in polyembryonic development relative to canonical insect embryogenesis? Second, which elements of the regulatory mechanisms were modified to result in a novel, obligatory form of embryo cloning? Finally, understanding such independently evolved, but similar novelties could inform us about evolutionary constraints and plasticity. For example, are there multiple pathways in the evolution of certain features, or are similar evolutionary innovations based on a common program?

Thus far, our model insect for polyembryonic development has been the polyembryonic encyrtid *Copidosoma floridanum* (Silvestri, 1906; Grbic *et al.,* 1996a; Grbic *et al.,* 1996b; Grbic *et al.,* 1998). This wasp parasitizes noctuid moths and produces up to 2000 embryos from a single egg. However, a poor understanding of encyrtid phylogeny and a lack of knowledge of closest monoembryonic ancestors led us to initiate studies on another independently-evolved polyembryonic wasp, the braconid *Macrocentrus grandii*. A better understanding of the phylogeny of braconids could help us to determine the closest monoembryonic relatives, and to generate a hypothesis about transitory forms that may have led to polyembryonic development. In addition, studies of multiple forms of polyembryony could uncover common features and possible variations in polyembryonic development.

Polyembryonic development is relatively well described in *Copidosoma.* It consists of three phases: early cleavages, which leads to formation of a single proliferative morula; the novel proliferative phase, that generates thousands of embryos; and the morphogenetic phase, where the patterning of individual embryos takes place (Grbic *et al.,* 1998; reviewed in Grbic, 2000). Association of ovary type and form of embryogenesis including developmental capacity of the embryo highlights the importance of ovaries in embryogenesis. Thus far, it has been assumed that all wasps have polytrophic meroistic ovaries. However, if organization of the ovaries is important for type of regulative capacity, as the examples of short germband development in primitive insects may imply, it is possible that polyembryonic wasps required a redesigning of ovarian structure as a prerequisite to evolving polyembryony. Staining of *Copidosoma* and *Macrocentrus* ovaries reveals that both wasps have polytrophic meroistic ovaries, where nurse cells are associated with the egg chamber (Fig. 2). However, adult *Copidosoma* females store almost all eggs in the terminal stages of oogenesis. Nurse cells are visible only during the pupal stage (Fig. 2A), but are degenerated in adult females. In contrast, *Macrocentrus* ovaries in adult females contain all stages of egg maturation closely resembling the pattern seen in *Drosophila* (Fig. 2B). This heterochronic change in egg maturation strategy probably reflects a difference in the adult life span and foraging behavior between the two wasps.

As a result of oogenesis both wasps form small and transparent eggs which they oviposit within the hemocoel of the host. Eggs of both species are transparent, surrounded by tiny chorion and in contrast to those of their basal, ectoparasitic relatives, contain almost no yolk (Fig. 3). Initial cleavage events in these tiny eggs differ from the canonical type of insect syncytial cleavage. Both wasps undergo total (holoblastic) cleavage in which nuclear division is immediately followed by cytoplasmic division, forming individual cells (blastomeres). This novel type of early cleavage appears to be common also in polyembryonic platygasterids (Ivanova-Kazas, 1972), and its general presence in all polyembry-

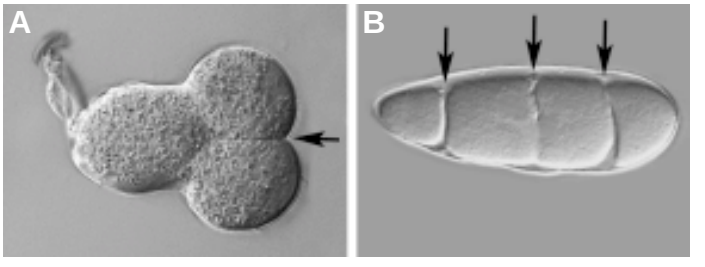

Fig. 3. Differential interference contrast images of *Copidosoma* and *Macrocentrus* eggs. (A) *First cleavage of* Copidosoma *egg.* **(B)** *Second cleavage of* Macrocentrus *egg. Arrow points to cleavage furrows. Anterior is to the left.*

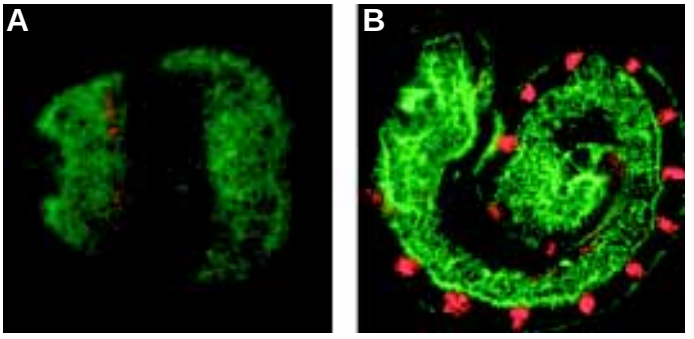

Fig. 4. Confocal images of *Macrocentrus* embryos stained with anti-Engrailed antibody. (A) Macrocentrus *heart-shaped embryonic primordium expressing two Engrailed stripes (ventral view).* **(B)** *Germband extension of* Macrocentrus *embryo expressing 15 Engrailed stripes. Anterior is to the left (A,B) and dorsal is up in (B).*

onic species suggests that it represents a prerequisite for the evolution of polyembryonic development.

Following early cleavages, *Copidosoma* embryos emerge from the tiny chorion into the host hemocoel and enter the proliferative phase of development (Grbic *et al.,* 1998; reviewed in Grbic, 2000). In this phase, the number of cells increases and thousands of cells become subdivided by the extraembryonic membrane into numerous spatial domains. The proliferative stage is also apparent in *Macrocentrus*, albeit resulting in a smaller rate of proliferation to form ultimately up to 20 embryos. Anti-histone staining in *Copidosoma* during the proliferative phase suggests that cells are not undergoing synchronous rounds of division as described in *Drosophila* syncytium (Grbic, unpublished). Instead, these divisions appear to be random and scattered in cells of the proliferative morula, without any discernable pattern.

Finally, initiation of the morphogenetic phase results in the formation of the embryonic primordium. In both species embryonic primordia are formed from the very beginning as cellularized structures. However, in *Copidosoma* the embryonic primordium is solid, without the blastocoel (Grbic *et al.,* 1996a), while the *Macrocentrus* primordium consists of single layer of cells that surrounds the hollow space of the blastocoel. These species also differ in the type of germband. *Copidosoma* embryogenesis was hard to classify. It more resembled long germband development by its proportional growth and expression of molecular markers (Grbic *et al.,* 1996a). On the other hand, *Macrocentrus* embryogenesis is clearly of a short germband type. The initial primordium consists of anterior structures and the remaining trunk is generated by posterior growth (Fig. 4 A,B).

The comparison of development in two independently evolved polyembryonic species suggests that evolution of polyembryony is compatible with meroistic ovarial apparatus present in basal monoembryonic wasps. On the other hand, innovations that are conserved in both polyembryonic species include a novel type of cleavage, and the proliferative phase responsible for creation of multiple embryos. It appears that in both polyembryonic wasps the proliferative phase has been simply "inserted" into the monoembryonic developmental program without any consequences for the later phases of development. Even though the proliferative phase seems to be similar in specific embryological events but different in the amount of proliferation, the late morphogenetic phase displays two completely different trajectories. In *Copidosoma* three-dimensional tissue specification proceeds from the morphogenesis of a solid ball of cells, resembling the long germband type of embryogenesis (Grbic *et al.,* 1996a). In contrast, the *Macrocentrus* primordium forms a single cell layer, and extension of the embryo trunk represents a form of short germband development, as described in primitive insects. Even though short germband development is considered to be a primitive remnant of insect ancestors, its secondarly-derived development in *Macrocentrus* indicates that the evolutionary trajectory can be inverted: short germband development can evolve from a long germband ancestor.

Collectively, descriptions of embryogenesis in two wasps illustrate the surprising level of plasticity and modularity of developmental programs. First, meroistic polytrophic ovaries that synthesize determinants for syncytial cleavage and long germband development are compatible with specification of determinants for polyembryonic development. Second, innovations in the cleavage type and proliferative phase which should theoretically scramble *Drosophila* localized maternal determinants and diffusion-based action of the transcription factors (as will be discussed in next section) are perfectly compatible with *de novo* formation of thousands of embryonic axes many days after oviposition. On the other hand, these multiple independent evolutionary events of polyembryony suggest that evolution of such a complex developmental program could have a relatively simple genetic basis that includes changes in very few genes.

Mechanistic changes in polyembryony

The innovations in polyembryonic development have important ramifications for the developmental mechanism and potentials of insect embryos. However, in order to understand the mechanical problems that polyembryonic development poses to paradigms of early insect development, *Drosophila* early development will be briefly summarized.

Specification of *Drosophila* oocyte axial polarity is a complex, multi-step process that is initiated in the ovaries (reviewed in van Eeden and St Johnston, 1999). This process includes a sequence of cell signaling events that results in differential polarization of the cytoskeleton, that in turn provides transport structures for the localization of cytoplasmic determinants. The source of early polarizing signals is the oocyte nucleus (Gonzalez-Reyes and St Johnston, 1994). At the early stages of oogenesis, the oocyte nucleus migrates toward the posterior and signals via *gurken* protein (TGFα signaling molecule) to induce the posterior fate of surrounding somatic follicular cells (Gonzalez-Reyes *et al.,* 1995; Roth *et al.,* 1995). The posterior follicle cells signal back, resulting in cytoskeletal polarization that facilitates migration of the oocyte nucleus to the anterior. When the nucleus reaches an anterior dorsal position, a second event of *gurken* signaling from the nucleus induces dorsal follicular cell fate, and initiates specification of the dorsal-ventral axis of the oocyte. Posterior polarization of microtubules results in an organization with the minus ends of microtubules oriented toward the anterior of the oocyte and plus ends pointing toward the posterior pole (van Eeden and St Johnston, 1999).

Such polarization of microtubules sets the stage for the transport of the maternal determinants to the posterior and anterior poles. An essential component for the organization of the embryonic posterior is the localization of *oskar* RNA (Ephrussi and

Lehman 1992). Translated Oskar protein has a key role in two different, but in *Drosophila* spatially-coupled, processes: organization of posterior patterning and specification of the germ line. Oskar protein is important for localization of the pole plasm components including *vasa* and *nanos* (Ephrussi *et al.,* 1991; reviewed in Saffman and Lasko, 1999). Nanos in *Drosophila* has a dual role. The posterior morphogenetic gradient of Nanos protein determines the posterior embryonic fate, reflected by the lack of abdominal structures in *nanos* mutants. In addition, its expression in the germ line is required in the process of germ cell formation (Deshpande *et al.,* 1999). At the anterior pole, *bicoid* RNA is also localized in a microtubule-dependant manner (St Johnston and Nüsslein-Volhard, 1992). Following oviposition, due to unique syncytial cleavage of *Drosophila,* Bicoid maternal protein is translated to form a transcription factor morphogen gradient in a manner similar to Nanos but with opposite orientation. Different concentrations of Bicoid protein regulate downstream gap genes, including *hunchback* and *Kruppel*, which also form diffusion gradients of transcription factors to subdivide the *Drosophila* embryo into smaller domains (reviewed in Rivera-Pomar and Jäckle, 1996). Mutual interaction within overlaping gap gene domains initiates the remainder of the patterning cascade including pair-rule and segment-polarity genes.

Paradoxically, despite considerable understanding of *Drosophila* axial patterning, one of the main obstacles in extending this paradigm to other insects is its peculiarity and a still incomplete understanding of the genetic interactions that polarize embryonic axis. Recent isolation of 23 novel mutants that disrupt anteroposterior axial development in *Drosophila* (Martin *et al.,* 2003) may provide novel insights into the axial patterning mechanism of the fly. In the light of the current *Drosophila* paradigm it is hard to imagine how a polyembryonic developmental program can evolve to keep "old" ovaries, invent a new type of cleavage, insert lengthy proliferative stage and subsequently form thousands of *de novo* specified embryonic axes.

The main evolutionary questions that would help guide an examination of how polyembryonic patterning evolved include the following. Is there any polarization of the oocyte during oogenesis that is involved in specification of early developmental asymmetries? How much do maternal determinants contribute to the specification of early cell fats in polyembryonic development? This constitutes the main conceptual unknown, given their significant role in *Drosophila* development. Finally, if conserved patterning genes are used in *Copidosoma* development (i.e. maternal coordinate and gap genes) how do they operate in cellularized embryos to specify the pattern? Alternatively, if early determinants of axial polarity are not conserved, what is establishing the axial polarity in *de novo* formed embryonic axes?

Some of the early markers of *Drosophila* axial polarity include the localization of the posterior gene products and germ plasm components at the posterior of the egg. In order to detect early signs of oocyte polarity we have isolated *vasa* mRNA from the *Copidosoma* cDNA library (Terzin and Grbic, unpublished), and have examined the expression of Vasa protein in both *Copidosoma* and *Macrocentrus* using cross-reactive anti-Vasa antibodies. In a fixed *Copidosoma* egg, a prominent circular structure at the posterior of the egg becomes visible with differential interference contrast (DIC) microscopy (Fig. 5A'', DIC panel). This structure was named the "oosome" by Silvestri (1906) who proposed that it represents the germ cell determinant. Staining with anti-vasa antibody co-localizes with the "oosome" (Fig. 5A''', merged panel), suggesting that this structure represents a highly condensed equivalent of pole plasm. In *Macrocentus,* even though a specific structure is not visible, anti-Vasa staining localizes to the posterior of the oocyte at early stages of oogenesis, marking putative germ plasm (Fig. 5B). This suggests that common mechanisms of protein localization operate in the ovaries of both polyembryonic species and that maternal factors participate in polyembryonic embryogenesis.

How are these determinants maintained in the proliferative phase and do they contribute to the specification of the embryo? Both determinants become segregated in individual cells and are maintained during the proliferative phase (Zhurov, Terzin and Grbic, unpublished) to become segregated in individual embryos and mark the germ line (Fig. 5 C,D). Even though a lack of other markers does not allow us to examine other components of the posterior group and germ plasm, it becomes clear that in both wasps evolution of the proliferative phase required uncoupling of the pattern formation function from germ line specification. Thus, to evolve polyembryony, it is necessary to separate the germ cell specification from posterior patterning in order to "insert" the proliferative stage that serves to generate the cellular mass for formation and patterning of multiple embryos.

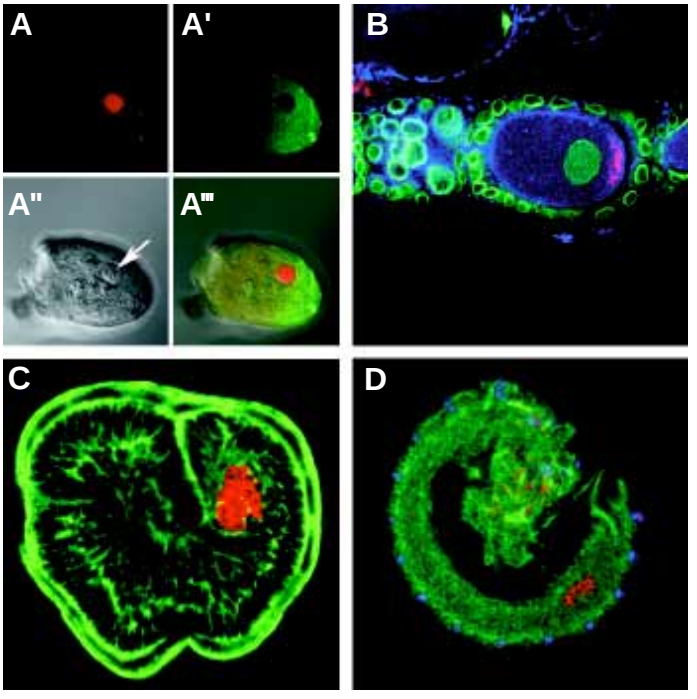

Fig. 5. Confocal images of *Copidosoma* and *Macrocentrus* eggs and embryos stained with anti-Vasa antibody. (A) *Ovarial* Copidosoma *egg: (A) panel Vasa, red; (A') phalloidin, green; (A'') transmitted DIC image; (A''') merged three previous panels.* **(B)** *Ovarial* Macrocentrus *egg (Vasa, red; anti-nuclear pore protein, green; phalloidin, blue).* **(C)** Copidosoma *late germband embryo (phalloidin, green; Vasa, red).* **(D)** Macrocentrus *late germband embryo (phalloidin, green; engrailed, blue; Vasa, red). Anterior is to the left (A,B,C,D) and Dorsal is up in (C,D). Paul Lasko's anti-Vasa antibody cross-reacts in* Copidosoma *(Lasko and Ashburner 1991) and Chun-Che Chang's grasshopper anti-vasa cross-reacts in* Macrocentrus *(Chang* et al., *2002).*

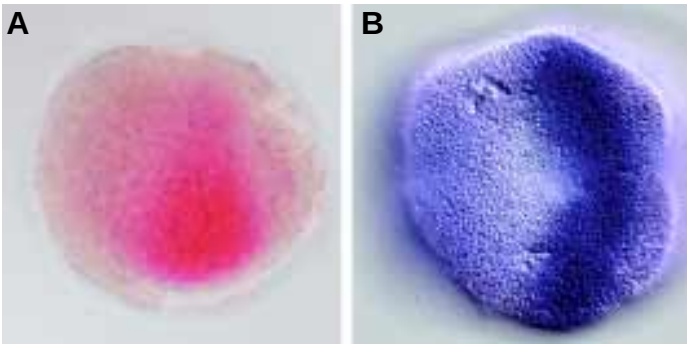

Fig. 6. Differential interference contrast images of *Copidosoma* and *Macrocentrus kruppel* mRNA expression. (A) Copidosoma kruppel *mRNA pattern (red).* **(B)** Macrocentrus kruppel *mRNA pattern (blue, ventral view). Anterior is to the left (A,B) and dorsal is up in (A).*

Finally, it was important to determine how the embryo is being patterned. It was determined earlier that *Copidosoma* uses the pair-rule protein Eve for embryo patterning, albeit in a pattern resembling segment polarity expression (Grbic *et al.,* 1996a). However, it remains unclear if these cellularised embryos are using maternal coordinate and gap transcription factors that are postulated to form diffusion gradients. Analysis of expression of gap gene *kruppel*/mRNA expression showed that this gene operates in embryo patterning (Terzin and Grbic, unpublished; Sucena and Grbic, unpublished). The *kruppel* expression domain was restricted to the central region of *Copidosoma* and *Macrocentrus* early morphogenetic embryos (Fig. 6 A,B), roughly corresponding to the domain described in *Drosophila*. *kruppel* expression was never observed during the proliferative phase. Thus utilization of a gap gene in embryo patterning indicates that elements of a conserved patterning program are activated once embryonic primordia have been assembled, following the proliferative phase.

Thus far it appears that maternal specification takes place in polyembryonic insects and that uncoupling of patterning from germ cell specification is required for the evolution of polyembryony. We know that following the "inserted" proliferative phase, the conserved patterning program is initiated during individual embryo formation, utilizing at least gap and pair-rule genes. What is less clear is its relationship, if any, to *de novo* establishment of axial polarity in polyembryonic wasps. Recent analysis of early axial specification in insects indicates that both anterior and posterior systems appear to represent a "retrograde" construction, where conserved elements remain at the bottom of the cascade, while new elements are recruited at the top of the regulatory pyramid (Wilkins, 2001). First, *bicoid,* the gene crucial for anterior patterning in *Drosophila*, does not appear to function outside of higher Diptera (Schröder and Sander, 1993). Second, in *Tribolium, bicoid* could not be found in the putative location in the Hox complex (Brown *et al.,* 1999). Finally, it appears that *bicoid* anterior patterning function evolved late in Diptera. In primitive *Tribolium orthodenticle* and *hunchback* genes appear to specify anterior fates ancestrally, in absence of *bicoid* (Schröder, 2003). The posterior group genes illustrate a similar trend. *oskar*, a critical element of posterior gene group and germ plasm localization is not found outside of drosophilids (Lehmann, personal communication) and does not exist in the recently sequenced *Anopheles* genome. On the other hand, the role of the posterior determinant *nanos* turns out to be crucial for germ cell specification in animals (Tsuda *et al.,* 2003). This suggests that the posterior morphogen role in *Drosophila* represents a relatively recently-derived function. This is a tempting speculation given that the only role of *nanos* in posterior patterning is to prevent translation of the maternal *hunchback* gene, illustrated by a completely normal phenotype of *nanos* and maternal *hunchback* double mutants (St Jonston and Nüsslein-Volhard, 1992). Bearing in mind the highly dynamic evolution of genes at the top of the axial patterning cascade, we should not expect conserved participation of maternal coordinate genes in polyembryonic *de novo* axis formation. In fact, in order for polyembryony to evolve it is necessary for *nanos* to lose its role in posterior patterning and remain involved in germ cell specification.

Transitory steps preceding polyembryonic development

Analysis of development of two polyembryonic wasps as a model to understand the evolution of developmental novelties yielded a testable hypothesis as to how the proliferative phase could be inserted in the developmental program. However, we remain ignorant of the factor(s) that could be involved in the *de novo* establishment of multiple embryonic axes. In order to address this question it is necessary to turn to the system that preceded the evolution of polyembryonic development and to look at the axial patterning in the closest monoembryonic ancestor. In an analysis of the developmental features of both polyembryonic wasps, it is possible to make predictions about the putative ancestor. First, it has to be an endoparasitic wasp. Second, it should undergo total egg cleavage. Finally, it should emerge from the chorion into the host hemocoel, and should utilize the polar body-derived cell to form the extraembryonic membrane surrounding the embryo.

During the course of its development, the braconid endoparasite *Aphidius ervi* exhibits the predicted features of the hypothesized ancestor of polyembryonic wasps. *Aphidius* has meroistic polytrophic ovaries, in which the oocyte is associated with a nurse cell complex (Fig. 7A). This wasp lays tiny transparent eggs that undergoe total cleavage (Fig. 7B). Its embryo emerges from the egg shell into the host hemocoel and remains enveloped by the polar body-derived extraembryonic membrane (Fig. 7 C,D). Following the emergence from the chorion, morphogenesis is initiated by the formation of an embryonic primordium that consists of a solid ball of cells, similar to the *Copidosoma* embryonic primordium. The embryo of *Aphidius* initially forms just the anterior structures of the embryo (Fig. 7E). The rest of the trunk is formed by sequential

TABLE 1

ESTIMATED GENOME SIZE OF PARASITIC WASPS (FEULGENE DENSITOMETRY METHOD)

Species	Estimated genome size (1C) (Mega base pairs)	% *Drosophila* genome
Macrocentrus grandii (Braconidae)	103	61
Praon sp (Braconidae)	122	72
Aphidius ervi (Braconidae)	103	61
Peristenis stygicus (Braconidae)	94	56
Peristenis digoneutis (Braconidae)	122	72
Copidosoma floridanum (Chalcidoidae)	534	316

proliferation (Fig. 7F), exhibiting characteristic short germband development. Since basal braconids display long germband development, *Aphidius* development represents secondarily derived short germband embryogenesis.

Finding a group with a well-established phylogeny, such as the braconids, allowed us to make other comparisons including relating genome size to evolution of polyembryony. On examination, all braconids were found to have a smaller genome size than *Drosophila* (Table 1). Additionally, no difference exists in the genome size of polyembryonic versus monoembryonic species (Gregory and Grbic, unpublished). The genome size of only *Copidosoma* appears to be significantly larger than *Drosophila* and other wasps. However, since the genome size of other encyrtids is unknown, *Copidosoma* genome size could not be attributed solely to the polyembryony.

Thus far in developmental processes it has been easier to find components of developmental circuitry that are conserved. Based on the flexibility of axial polarity genes discussed earlier, we should not expect their conservation in the establishment of polyembryonic axial polarity. However, we should at least be able to make predictions about the types of molecules that might be involved in axial patterning in the predecessor, as exemplified in other cellularized monoembryonic metazoan species. First, we expect that the posterior determinant in *Drosophila*, *nanos*, will lose its role in posterior patterning and retain its function in germ cell specification. Recent isolation of an *Aphidius nanos* homologue will allow us to test this hypothesis (Zhurov and Grbic, unpublished). Second, we expect that signaling genes are prime candidates for initiating the axial polarity as is indicated in other metazoan embryos undergoing early cellularization. The candidate genes should include Wnt, TGFβ, BMP and hedgehog gene families (Wikramanayake *et al.,* 1998; Thisse *et al.,* 2000; Schier and Shen, 2000). Our screen for signaling genes that show an axial pattern pinpointed a novel domain of the *Aphidius wingless* homologue that is expressed early at the anterior of the embryonic primordium (Fig. 7G). This early polar domain precedes the conserved, late, segmentally repeated *wingless* stripes. Such novel domains of signaling genes may indicate their role in axiogenesis. Even though techniques of gene manipulation are not currently available in *Aphidius,* simple drug experiments could be informative about the function of signaling genes in axial patterning. For example, culturing the embryo in a medium supplemented with lithium should affect *wingless* signaling (Wikramanayake *et al.,* 1998) and provide clues about its early function.

Scenarios for evolution of polyembryony

An analysis of multiple independent events of polyembryony in wasps within the phylogenetic framework suggests that it consists of a complex and stepwise processes. The ancestral type of development in all polyembryonic lineages included an ectoparasitic life history strategy and a large yolky egg, exhibiting long germband embryogenesis. With the evolution of endoparasitism, wasp embryos gained the advantage of exploiting the nutritive environment of the host not only for larval feeding, but also for embryo development. This shift resulted in several changes in egg architecture. First, the chorion which consists of elaborate structures in ectoparasites and other terrestrial insects protecting them from dessication, decreased in its complexity once the embryo evolved emergence from the chorion into the host nutritive hemolymph. In addition, because host nutrients were utilized for embryo development it was not necessary to stockpile a large amount of yolk in the eggs. Consequently, endoparasitic egg size decreased. In smaller eggs evolution favoured a new type of cleavage: total cleavage, immediately forming individual cells.

It is unique that in many endoparasitic wasps polar nuclei do not degenerate as in other terrestrial insects (Tremblay and Calvert, 1972). Instead, they participate in the formation of extraembryonic membranes that completely surround the embryo. It appears that this structure evolved many new functions in contrast to the extraembryonic membranes in terrestrial insects. In many endoparasitic wasps, at the completion of morphogenesis the extraembryonic membrane fragments into individual polyploid cells called teratocytes. In some endoparasitic wasps teratocytes circulate in the host hemolymph and synthesize proteins which are

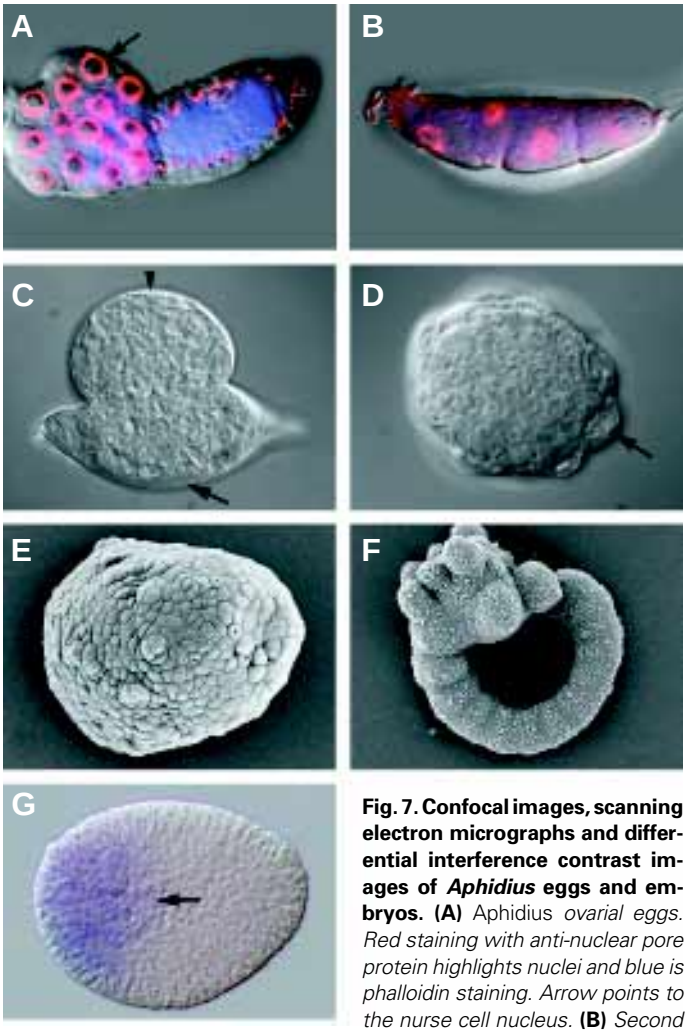

Fig. 7. Confocal images, scanning electron micrographs and differential interference contrast images of *Aphidius* eggs and embryos. (A) Aphidius *ovarial eggs. Red staining with anti-nuclear pore protein highlights nuclei and blue is phalloidin staining. Arrow points to the nurse cell nucleus.* **(B)** *Second cleavage of Aphidius; red, anti-nuclear pore protein.* **(C)** *Emergence of* Aphidius *egg from the chorion. Arrowhead points to the emerged embryo and arrow marks chorion.* **(D)** *Round embryonic primordium surrounded by the extraembryonic membrane (arrow).* **(E)** *Scanning electron micrograph of* Aphidius *embryonic primordium.* **(F)** *Posterior growth of the* Aphidius *embryo.* **(G)** *Early anterior domain of* wingless *mRNA in* Aphidius *embryonic primordium (arrow). Anterior is to the left and dorsal is up in E-G.*

secreted, altering host physiology in support of endoparasitic development (Rana *et al.,* 2002). However, in the polyembryonic embryogenesis of *Copidosoma,* the extraembryonic membrane is involved in the proliferative phase of development, separating proliferative cells into spatial domains. It never fragments to form the teratocytes and continues to surround both embryos and larvae. Even though endoparasitic embryos can take advantage of the host nutritive environment, they must first evolve a defense against the host immune system. Recent findings by Corley and Strand (2003) that the extraembryonic membrane in *Copidosoma* protects the larvae from the host immune system may provide a clue as to the primary reason for the evolution of this structure. In addition, it has been proposed that the polar cell-derived extraembryonic membrane plays a role in the uptake of nutrients from the host hemolymph (Koscielski and Koscielska, 1985). Analyzing the expression pattern of genes in the proliferative phase of development, it was determined that all cells of the extraembryonic membrane in *Copidosoma* express alkaline phosphatase mRNA (Terzin and Grbic, unpublished). This enzyme is involved in nutrient absorption and transport mechanisms in insects and vertebrates (Eguchi, 1995), suggesting that the extraembryonic membrane actively absorbs nutrients from the host hemolymph. Thus, the primary role of the extraembryonic membrane initially was probably to protect the emerged embryo of monoembryonic endoparasites against the host immune system, and to absorb nutrients. Later on, the existing structure was likely co-opted to the proliferative phase of embryogenesis in polyembryonic insects to organize proliferative growth.

Evolution of small egg size, total cleavage, and novel, multifunctional extraembryonic membranes were the prerequisites for the evolution of the novel proliferative stage. This stage represents the true developmental innovation (Type A) because it was derived from novel structures (the extraembryonic membrane) and a cleavage type that does not have a known precursor in ancestral, ectoparasitic, insects. It is hard to conceptualize the evolution of a novel stage that disrupts one of the crucial paradigms of *Drosophila* development, maternal specification of the embryonic axis, while at the same time creating *de novo* 2000 independent embryonic axes! If the syncytial environment of the *Drosophila* pre-blastoderm embryo has created complications in understanding how pattern formation proceeds in the cellular milleu of short and intermediate germband insects (Wilkins, 2001), then polyembryonic development represents a real challenge for the *Drosophila* paradigm. One of first prerequisites for such an event appears to be the uncoupling of posterior patterning and germ cell specification. The second step should include the initiation of the proliferation mechanisms to generate at least 40,000 cells necessary for initiation of 2000 embryonic primordia (Grbic at al. 1998). There are several relatively simple possible means how to initiate proliferation. In the monoembryonic ancestor cleavages must generate enough cells for the formation of the single embryonic primordium. At this point proliferation has to stop and become coupled with axial patterning. Thus, a simple change in the regulatory region of the mitogenic signal could extend the period of proliferation necessary for polyembryonic development. Another avenue generating the same effect would be to produce a mutation in the putative suppressor of proliferation that terminates early proliferation and regulates entry into the blastoderm stage of the monoembryonic ancestor. Both of these changes are relatively simple and could involve existing genes without requiring new gene recruitment (Wilkins, 2001). In a likewise manner, removal of the mitogenic signal by a similar mechanism at the completion of proliferation could regulate the exit from the proliferative stage.

It is hard to conceptualize how is the proliferative stage integrated with *de novo* establishment of embryonic axes. All 2000 embryo axes appear to form independently with random axial orientation relative to each other (Grbic *et al.,* 1996b). This favours an independent specification of the axial polarity within each embryo rather than a global mechanism specifying simultaneous polarity in 2000 embryos. However, recent genetic analysis of the basal long germband wasp reveals differences relative to fly development that could be utilized to develop the model of evolution of polyembryony. Genetic analysis of the long germ ectoparasitic wasp *Nasonia virtripennis* revealed mutations in embryo pattern that correspond to putative gap and pair-rule mutant phenotypes in *Drosophila*, as well as zygotic phenotypes that have no fly mutant counterparts (Pultz *et al.,* 1999). Most importantly, it appears that in *Nasonia* zygotic control has a more prominent effect on embryo patterning, contrasting predominantly maternal early control as determined in the fly (Pultz *et al.,* 1999). It is hard to conceive that at the stage of embryonic primordium (and during its formation) a *Drosophila*-like transcription gradient operates in the cellular environment of *Copidosoma* and *Macrocentrus* embryos. However, gap genes appear to be involved in embryo patterning in both wasps. It is possible that the predominance of zygotic control of embryo patterning in the ancestral long germband wasps such as *Nasonia* could be used as a stepping stone to shift embryo patterning to the zygotic genes at the late stages of embryogenesis (following the proliferation) and thus allow “insertion” of the proliferative stage. However, this still does not explain how *de novo* axial polarity is initiated at the polyembryonic blastoderm. Emerging evolutionary flexibility of early genes involved in polarization of the embryonic axis in insects suggests that it is impossible to use the candidate gene approach based on the *Drosophila* paradigm to isolate the earliest axial organizers in polyembryonic wasps. The cellular environment in endoparasitic wasps narrows the choice of genes to a group of signaling genes that are used in other systems to establish embryonic axis. Current knowledge of the patterning of polyembryonic and monoembryonic wasps suggests two approaches to isolate putative genes involved in *de novo* establishment of axial polarity. One approach would be to utilize genomic EST expression screens in both monoembryonic holoblastic cleaving and polyembryonic wasps to isolate those that are expressed at the future embryo poles. In addition, isolation of the regulatory regions of *Kruppel*could serve as a tool in determining the gene products binding to its regulatory regions in *Copidosoma* and *Macrocentrus*. This could provide clues as to how the conserved phase of the gap patterning cascade is integrated with the regulatory elements directing *de novo* establishment of axial polarity.

Concluding remarks

Evolution of developmental novelties is a complex phenomenon that requires understanding of both the ecological processes and developmental mechanisms responsible for its creation. Analysis of the evolution of polyembryonic development within the phylogenetic context, and studies of multiple independent events of poly-

embryony have been important stepping stones toward beginning to understand the processes and mechanisms shaping the evolution of this novel form of development. As stated by Wilkins (2001), there is no general analytical method that can be applied to all developmental novelties. However, clues derived from a broader phylogenetic context suggesting the polarity of change and an examination of possible ancestral states are essential in constructing testable hypotheses. In addition, this approach could narrow down the choice of candidate gene groups and suggest the most promising experimental approaches for isolation of critical genes.

One of the unknown elements in the evolution of polyembryony is the time scale of evolutionary events. Even though Hymenoptera as a group appeared 220 MYA, the first parasitic wasps fossil records date from 160 MYA (Whitfield,1998). Polyembryonic wasps represent the most derived groups and it is plausible that they emerged relatively late. New advances in the estimation of age of parasitic wasps (Whitfield, 2002) are promising breakthroughs in understanding the evolutionary timing of developmental events leading to polyembryony. In addition, variations in early developmental programs that can be correlated with the switch in the life history strategies, coupled with a small genome size and transparent embryos make these wasps an amenable system for dissecting evolutionary forces and mechanisms that are shaping the evolution of early development. Finally, embryo culturing techniques (Grbic *et al.,* 1997) and uptake of nutrients by the embryo may facilitate implementation of manipulative techniques such as RNAi that will be crucial for understanding the evolution of gene function in these systems.

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