Moreover, the tremendous diversity among insects provides a wide variety of species to sample the conserved and novel development opportunity to begin comparative studies between the well-studied Drosophila network and the genetic networks of other insect species. Moreover, the tremendous diversity among insects provides a wide variety of species to sample the conserved and novel developmental features that have evolved over time. The application of genetic screens, transgenic analysis and in particular, the development of RNAi in various insect model systems has also contributed significantly to the advancement of the field of evolution and development. The results presented in recent reports regarding Nasonia, Tribolium, Oncopeltus and Gryllus embryonic patterning have shown the power of comparative studies between different insects for studying evolution and development. This review will focus on the establishment of the wasp Nasonia vitripennis as a powerful model system for elucidating the various biological strategies employed during insect embryogenesis. Moreover, work presented throughout this review will highlight important results regarding comparative studies between the fruit fly and the wasp.

Keywords: bicoid, caudal, giant, nanos, orthodenticle, segmentation

INTRODUCTION

One of the first steps in the development of a single-celled embryo is the establishment of the anterior-posterior (AP) and dorsal-ventral (DV) axes. Subsequently, a complex genetic program patterns these axes to ultimately give rise to a basic adult body plan. The mechanisms employed by the embryo to achieve this initial polarization have long fascinated developmental biologists and various strategies have now been identified among different animal species for achieving this general polarization of the embryo. Moreover, it is clear that while some of these mechanisms are conserved throughout the animal kingdom, other patterning functions have significantly evolved.

Insects provide a fascinating group of organisms in which to study the diversity, as well as the conservation of mechanisms employed for both the polarization and early patterning of the embryo. The early patterning of the fruit fly Drosophila melanogaster is one of the best described developmental systems, providing an excellent comparison...
for work on the evolution of the patterning networks among insects (Davis and Patel 2002; Lynch and Desplan 2003a). It has become clear that the crux of the Drosophila patterning network, the gene bicaud, as well as the basic mode of development within the fruit fly are not well conserved developmental features among insects (Stauber et al. 2002; Lynch and Desplan 2003b). Many researchers have thus sought to discover more general, ancestral patterning network and mode of development within insects (Liu and Kaufman 2005b). To this end, a wealth of information has been reported regarding the development of the beetle Tribolium, the cricket Gryllus, as well as the milkweed bug Oncopeltus. In addition to descriptive reports regarding the temporal and spatial expression patterns of various genes during embryogenesis, elegant studies have made use of genetic mutant screens and RNA interference to uncover the functions of developmental genes and how their roles within their respective patterning network compare to their counterparts in the Drosophila network (Sulston and Anderson 1996; Schulz et al. 1998; Wolff et al. 1998; Schroder et al. 2000; Schroder 2003; Liu and Kaufman 2004; Cerny et al. 2005; Liu and Kaufman 2005a, 2005b; Mito et al. 2005; Shinmyo et al. 2005; van der Zee et al. 2005; Mito et al. 2006; Choe and Brown 2007). Among the various insects recently adapted as model organisms for use in comparative studies of embryogenesis with Drosophila, the wasp Nasonia vitripennis has been successfully used to identify mechanisms utilized for the initial polarization of the insect embryo and to identify features of the early patterning network. Studies presented throughout this review will highlight conserved aspects of insect embryogenesis including the use of gradients, cross repression between zygotic genes to refine expression boundaries, as well as the reliance on localization of maternal mRNAs. Additionally, unique characteristics of Nasonia embryogenesis have been uncovered which point to the plasticity of the genetic network responsible for insect embryogenesis throughout evolution (Pultz et al. 2005; Lynch et al. 2006a, 2006b; Olesnicky et al. 2006). In order to fully understand the impact of the Nasonia system and the recent data that have emerged regarding the development of the wasp in contrast to the embryonic network of Drosophila, it is first necessary to describe the well-known details of Drosophila embryogenesis.

DIFFERENT MODES OF EMBRYOGENESIS: SHORT VS. LONG GERM

A great deal of diversity is seen in mechanisms of embryogenesis among insects, ranging from polyembryonic embryogenesis, where thousands of embryos are derived from a single egg, to the highly derived system of long germ embryogenesis, characteristic of Drosophila. Despite this variety in insect embryogenesis, most insects can be categorized into 2 different developmental groups: short/intermediate germ and long germ insects (Grbic 2003; Liu and Kaufman 2005b). Short germ and intermediate germ insects are represented throughout basal insect orders. This is in contrast to long germ insects, which are confined to some holometabolous insect species. This suggests that short or intermediate germ embryogenesis is the ancestral mode of development (Liu and Kaufman 2005b) while long is a derived state.

In short germ embryogenesis, the majority of the embryo is occupied by extraembryonic membranes, while the embryo develops in the posterior of the egg and only anterior embryonic structures are patterned in the early syncytial environment of the germ anlage. Later, after gastrulation, abdominal and posterior structures are formed in a cellularized environment through a region in the posterior of the germ rudiment termed the “growth zone”. The growth zone undergoes extensive elongation during which abdominal segments become specified in an anterior to posterior progression. Within short and intermediate germ insects, there is a difference in the number of segments patterned syncytially, prior to gastrulation. Short germ embryos pattern typically only head segments prior to gastrulation, while intermediate germ insects will also pattern thoracic segments within the syncytial germ anlage (Davis and Patel 2002). Additionally, the proportion of the egg devoted to either the embryonic anlage or the extraembryonic membranes, varies among short and intermediate germ insects (Davis and Patel 2002; Liu and Kaufman 2005b).

Short germ embryogenesis is in sharp contrast to the derived long germ mode of patterning found in both Drosophila and Nasonia, where the embryo occupies the entire egg and the entire adult fate map is specified completely within a syncytial environment prior to gastrulation. As all segments are patterned in the syncytial blastoderm embryo, the long germ embryo lacks a posterior growth zone. The honeybee was the first organism in which long germ embryogenesis was described. In the honeybee, the entire length of the egg is occupied by the embryo and it undergoes almost no germ band elongation during gastrulation (Davis and Patel 2002). This is in contrast to Drosophila, the most studied long germ embryo, which does undergo germ band elongation, during which the embryo is lengthened though a dramatic series of cellular rearrangements. At the conclusion of germ band elongation, the embryo returns to almost its original size as it continues to pattern the insect body plan. Interestingly, however, unlike Drosophila, where all segments are patterned simultaneously, the honeybee undergoes development in an anterior-posterior progression, which is reminiscent of short germ embryogenesis, underlining an obvious diversity among insects within the mechanism of long germ embryogenesis (Davis and Patel 2002). The diversity among these groups of insects that undergo drastically different modes of embryogenesis highlights the need for sampling a broader range of species within both the short and long germ categories to obtain a greater understanding and dissection of the genetic networks involved in embryogenesis. Such information will be important for identifying conserved features among insect embryogenesis and for the general understanding of the evolution of genetic networks.

THE PATTERNING NETWORK OF DROSOPHILA MELANOGASTER

Patterning along the AP axis in the Drosophila embryo involves the coordinate inputs of an intricate genetic hierarchy. The initial coordinating information in this patterning cascade is communicated by the maternal system, i.e. genes whose function is required in the mother, during oogenesis, for the proper patterning of the embryo. Opposing gradients of maternal signaling molecules are loaded into the oocyte during oogenesis. Upon fertilization, an anterior to posterior gradient of the homeodomain transcription factor Bicoid (Bcd) is created (Driever and Nusslein-Volhard 1988a). An opposing posterior to anterior gradient is formed when Bcd represses translation at the anterior of the embryo of the homogeneously loaded maternal caudal (cad) mRNA, which encodes the homeodomain transcription factor (Macdonald and Struhl 1986; Mlodzik and Gehring 1987). Similarly, maternal hunchback (hb) mRNA is homogenously present in the early embryo. However, the translation of this zinc finger transcription factor is prevented in the posterior half of the embryo by nanos (nos), a factor required for abdome formation (Irish et al. 1989; Lehmann and Nusslein-Volhard 1991; Rivera-Pomar and Jackle 1996).

The restricted expression of maternal factors serves to divide the embryo in domains, with Bcd and Hb positioned at the anterior and Cad and Nos occupying the posterior half of the embryo. The information encoded by these factors is then interpreted by downstream targets, termed gap genes. These are the first zygotically expressed segmentation genes that serve to subdivide the embryo into large subdomains. In the anterior, gap genes such as the homeodomain transcription factor Orthodenticle (Od) become expressed in response to the Bcd gradient (Driever et
Thoracic and central gap genes such as Krüppel (Kr) are activated in response to the coordinate functions of Hb and Bcd (Hoch et al. 1991). In the posterior abdomen, the embryo is divided into the expression domains of knirps (kni) and giant (gt). Activation of the kni posterior expression domain is achieved by the functions of both Bcd and Cad (Rivera-Pomar et al. 1995; Schulz and Tautz 1995; Schroeder et al. 2004).

THE BCD MORPHOGEN GRADIENT

Morphogenetic gradients represent a powerful strategy employed by cells to supply both positional and instructional information to surrounding cells or tissues. The use of this mechanism has been uncovered as a common theme in developmental programs ranging from early embryogenesis to neurogenesis. The establishment of a graded signal allows for cells to interpret their location relative to the signal source. Moreover, a signaling gradient, in addition to supplying positional information, instructs a variety of cell fate decisions that depend on the concentration of signal present (Ashe and Briscoe 2006).

One well-studied example of a morphogen is the Bicoid gradient, which is established in the early Drosophila embryo. The Bicoid gradient provides biologists with an exceptional system to study morphogens, as the Drosophila embryo develops in a syncytium, allowing simple diffusion to generate a protein gradient (Driever and Nusslein-Volhard 1988a; Struhl et al. 1989; Houchmandzadeh et al. 2002; Crauk and Dostatni 2005; Gregor et al. 2005). Thus instead of initiating a signaling cascade that must penetrate the cell membrane to impart an effect, Bicoid is a transcription factor that can easily diffuse within the syncytial environment of the early embryo. This feature allows the signal to function quickly in controlling cell fate decisions during development.

bcd is expressed maternally and its mRNA is localized to the anterior-most region of the oocyte. Upon fertilization of the embryo, bcd mRNA is translated and the protein product forms an anterior to posterior gradient (Driever and Nusslein-Volhard 1988a). bcd− mutant mothers produce embryos that lack all anterior structures including the head and thorax, consistent with Bcd’s role in anterior patterning, and instead develop posterior structures at the anterior of the embryo (Frohnhoefer et al. 1986). As a result of the morphogenetic properties of the Bcd gradient, adjusting levels of the protein results in shifts in the fate map of the embryo. For example, reducing bcd gene dosage results in an anterior shift of the embryonic head fold. Moreover, an increase in gene dosage results in the posterior shift of the head fold, as well as posterior shift of expression of all stripes of the pair rule gene eve (Driever and Nusslein-Volhard 1988b).

Once a gradient has been established, cells along the AP axis must interpret the positional and functional information encoded within the Bcd morphogenetic gradient. The strategy employed by the early Drosophila embryo involves the differential affinities of Bcd for binding sites present within the enhancers that regulate expression of target genes. For example, the gene hunchback (hb) contains high affinity Bcd binding sites that allow hb expression in more posterior regions of the embryo (Fig. 1) (Driever et al. 1989; Struhl et al. 1989). The target gene orthodenticle (otd), on the other hand, is less sensitive to the Bcd gradient and contains low affinity binding sites that drive expression in a narrow region of the anterior of the embryo, which contains the highest levels of Bcd protein (Fig. 1) (Driever et al. 1989). It has also become apparent that although the positional information supplied by morphogenetic gradients is indeed indispensable, often combinatorial inputs are necessary to impart precise positional information and sufficient information for cells to make appropriate cell fate decisions (Ochoa-Espinosa et al. 2005).
BCD FUNCTIONS AS A TRANSLATIONAL REPRESSOR

In addition to Bcd’s role in directing anterior patterning through the activation of downstream targets, Bcd also functions by inhibiting posterior patterning at the anterior by acting as a translational repressor. The maternal gene otd-1, which encodes a posterior patterning protein, is present homogeneously in the early embryo. Cad translation is repressed at the anterior of the embryo by Bcd binding to a 120 base pair region in the cad 3'UTR, termed the Bicoid Response Element (BRE). This ensures that Cad protein is excluded from the anterior of the embryo, in turn creating a posterior to anterior gradient of the Cad protein (Dubnau and Struhl 1996; Rivera-Pomar et al. 1996; Niesing et al. 1999, 2000, 2002).

THE EVOLUTION OF BCD

Although bcd is the anterior patterning center with multiple indispensable functions during Drosophila embryogenesis, it has become clear that bcd is not a conserved feature of insect embryogenesis and is not found outside members of a highly derived group within the dipteran lineage (Lynch and Desplan 2003a, 2003b). bcd is located in a rapidly evolving region of the genome within the homeotic gene complex (Hox-C), adjacent to the gene zerknullt (zen), the insect Hox3 homolog. It is thought that despite a great deal of sequence divergence between the two genes, bcd arose as a duplication of zen (Dearden and Akam 1999; Stauber et al. 1999). In Drosophila, zen is expressed in the dorsal region of the embryo corresponding to the extraembryonic membranes. Interestingly, a study revealed that in certain non-Cyclorrhaphan flies, such as Empis livida, Haematopota pluvialis and Clognia albipunctata, the Hox3 genes show a greater sequence similarity to zen. The expression patterns of these genes however, show characteristics of both the anterior bcd expression, as well as the dorsal zen expression. These findings support the idea that bcd evolved as a duplication of zen and took on some of the maternal aspects of zen expression, whereas zen evolved to be restricted to zygotic patterning of the dorsal extraembryonic membranes (Stauber et al. 2002). The Bcd homeodomain then acquired the same DNA binding specificity as that of Otd, imparted by the presence of a lysine residue at position 50 of their homeodomains (Schroder 2003). The hypothesis that Bb and Otd might function together ancestrally to achieve anterior patterning in the absence of Bcd has been thoroughly tested in both Tribolium and Nasonia (Schroder 2003; Lynch et al. 2006a). In contrast to the solely zygotic otd expression in Drosophila, Tribolium otd-1 is expressed maternally in the anterior of the developing embryo. Using parental RNA interference (pRNAi) to knock down the function of otd-1 in Tribolium results in loss of head structures. Conversely, knock down of Tribolium hb via pRNAi results in loss of thoracic and gnathal structures. In the absence of both hb and otd-1, however, the head, thorax and anterior abdomen of the beetle do not form. This phenotype is strikingly similar to that reported for strong bcd mutant embryos (Schroder 2003).

NASONIA AS A MODEL SYSTEM

The realization that the well understood genetic network and mechanism of embryogenesis of Drosophila is in fact highly derived, spurred many researchers within the field of evolution and development to decipher the true ancestral network and mechanisms driving insect embryogenesis. As work in a number of short germ insects has highlighted both common and divergent features within embryogenesis, it has become increasingly apparent that a greater understanding of long germ development is crucial in understanding the evolution of insect embryogenesis from short to long modes. As a result, developmental biologists have sought out other long germ insects to study the common features of this mode of embryogenesis in relation to what is known in Drosophila. Nasonia vitripennis was recently established as a model system to explore these questions (Pultz et al. 1999, 2000, 2005; Lynch 2005; Lynch et al. 2006a; Olesnicky et al. 2006b; Olesnicky et al. 2006). Nasonia is a long germ embryo that lacks the anterior patterning gene bcd (Lynch et al. 2006a). Moreover, recent evidence suggests that long germ development among Hymenopterans (Nasonia) and Dipterans was derived independently (Savard et al. 2006). This system, therefore gives us an opportunity to study the result of convergent evolution within the developing insect embryo.

Nasonia is a member of the Family Chalcidae and the genus is comprised of three species of wasps that are-
sitoids of large fly’s pupae: *Nasonia vitripennis*, *Nasonia girault* and *Nasonia longicornis* (Campbell et al. 1993). *N. vitripennis* is found throughout the world and has been used both commercially in pest control of larger flies and academically for genetic and developmental research (Werren et al. 1986; Pultz and Leaf 2003; Beukeboom et al. 2007). The genome size of *Nasonia* is approximately 335 Megabases in five chromosomes, as opposed to *Drosophila* whose genome is 180 Megabases in four chromosomes. Despite the fact that *Nasonia*, a Hymenopteran, and Diptera are separated by over 200 million years of evolution, *Nasonia* and *Drosophila* share many similar characteristics in their modes of embryogenesis, making these species ideal for studying the (convergent) characteristics of long germ development (Pultz and Leaf 2003).

*Nasonia* is easily maintained in the laboratory and has a life span comparable to *Drosophila*. This wasp provides a unique system for the study of zygotic lethal mutations because of the ability to apply haplo-diploid genetics to the system (Pultz et al. 1999, 2000, 2005; Olesnicky et al. 2006). Females deposit eggs inside the pupae of large flies and fertilized eggs develop into females, while unfertilized eggs will develop as males, facilitating screening for zygotic lethal mutations in the all male progeny of unmated females. Under favorable conditions, a female will lay a clutch of between 20 and 35 embryos per host pupa (Davies and King 1972; Parker and Orzack 1985).

**EMBRYOGENESIS**

The *Nasonia* embryo is covered by a smooth and flexible chorion and a vitelline membrane, both of which contain no visible structures. Both layers are clear and allow for observation of embryogenesis through a dissecting microscope (Fig. 2A). At egg deposition, the embryo is arrested in meiosis I. As meiosis completes, the cytoplasm at both anterior and posterior poles clears in contrast to the more densely stained cytoplasm at the center of the embryo (Pultz and Leaf 2003). Additionally, the oosome, a structure containing the germplasm, becomes evident at the posterior of the embryo (Fig. 2B, 2C). One hour after the completion of meiosis I and the fusion of pronuclei in fertilized embryos, the nuclei enter into the first mitosis and undergo a series of synchronous divisions, resulting in the formation of a syncytial embryo (Tram and Sullivan 2000; Pultz and Leaf 2003). After the eighth nuclear division (~3 hours after egg deposition at 25°C), pole cells begin to bud off from the posterior pole of the embryo (Fig. 2D). The formation of pole cells coincides with the migration of embryonic nuclei to the periphery of the embryo. Here the nuclei will continue to divide until cellularization, when membranes are laid down around the nuclei to form individual cells. As cellularization proceeds (~7 hours after egg deposition at 25°C), the embryo becomes narrower, elongating at the posterior pole, with an obvious thickening along the periphery of the embryo, making a physical separation from the yolky center of the embryo. Gastrulation occurs at approximately 10 hours after egg deposition at 25°C and is easily identified.
by the formation of folds at the anterior of the embryo, corresponding to the gnathal head region (Fig. 2F). The germ band begins to extend and segments become apparent in an anterior to posterior progression (Fig. 2G, 2H). After 30 hours at 25°C, the Nasonia larva hatches (reviewed by Pultz and Leaf 2003).

A SCREEN FOR ZYGOTIC EMBRYONIC PATTERNING MUTANTS

In order to appropriately compare the developmental programs of Drosophila and Nasonia, a genetic screen for the identification of zygotic patterning mutants was conducted by Pultz et al. (1999). The screen was designed to identify the major players involved in AP axis patterning, similar to the saturation screens carried out by Nusslein-Volhard and Wieschaus (1980) in Drosophila. In the Nasonia screen, approximately 100 embryonic patterning mutants were identified including mutants that resemble the phenotypes associated with mutations in Drosophila genes of the axial-patterning, gap, pair-rule and Polycomb groups. A number of novel mutant phenotypes that do not resemble any known Drosophila mutants were also isolated. Mutations were characterized based on cuticular patterns, as well as altered expression of engrailed and the thoracic HOX genes Ultrabithorax and Abdominal-A. In wild type cuticles of Nasonia first instar larvae, there are distinct denticle belts for the three thoracic and ten abdominal segments. Furthermore, there are large spiracles on the second thoracic segment, as well as the first three abdominal segments making their identification straightforward (Fig. 3A) (Pultz et al. 1999, 2000). Overall, the screen showed that zygotic mutations in Nasonia often create much more severe patterns than those observed for any Drosophila zygotic mutants (Fig. 3B, 3C). This suggested that Nasonia relies more on zygotic patterning input for axis patterning than on maternal function (Pultz et al. 2000), as is the case for Drosophila (Pultz et al. 1999; Pultz and Leaf 2003). Alternatively, the severity of the zygotic phenotypes may be due to an inability of maternal genes to rescue loss of their zygotic counterparts, as their expression patterns do not overlap temporally. This may be due to developmental timing as pre-gastrulation events in Nasonia take approximately 10 hours compared to the 3 hours in Drosophila (Olesnicky et al. 2006).

The success of the Nasonia genetic screen and its impact within the field of evolution and development became apparent with the isolation of the zygotic Nasonia hunchback null allele, headless. Pultz et al. (2005) identified a deletion spanning 1.5 kb in the hunchback locus responsible for the severe headless mutant phenotype (Fig. 3B). Loss of zygotic Nv hb results in loss of most of the head and the entire thorax, whereas the loss of zygotic hb in Drosophila is much less severe due to the ability of maternal Dm hb to rescue patterning of anterior structures. These results suggest that the wasp cannot compensate for loss of zygotic function with maternal contributions as early development is much longer than in flies and the maternal supply is exhausted before zygotic gene expression is turned on (Pultz et al. 2005).

The development of parental RNA interference (pRNAi) in Nasonia has facilitated the study of both maternal and zygotic factors involved in early embryonic development (Lynch 2005; Lynch et al. 2006a, 2006b; Brent AE 2007). Lynch et al. (2006) provide striking evidence in support of the long-standing theory that otd and hb function ancestrally to replace the role of bcd in anterior patterning. Additionally, work by Olesnicky et al. (2006) using pRNAi and genetic mutant analysis shows that the posterior patterning gene caudal (cad) acts as a posterior patterning center, and in conjunction with Otd-1 patterns the entire abdomen of the wasp embryo (Fig. 4).

OTD-1 FUNCTIONS AS A MORPHOGEN IN THE NASONIA EMBRYO

In the long germ insect Nasonia, otd-1 mRNA is maternally expressed and localized to both poles of the oocyte during oogenesis (Fig. 5A, 5B) (Lynch et al. 2006). During early embryogenesis, Otd-1 protein is first made at the anterior

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**Fig. 3** A genetic screen for patterning mutants in *Nasonia*. Wild type cuticle composed of head segments, 3 thoracic segments and 10 abdominal segments (A). headless (hunchback) cuticle shows thoracic and head defects (B). head only cuticle shows loss of abdomen and posterior structures (C).
pole and translationally repressed at the posterior of the embryo. The presence of nanos response elements in the otd-1 3’UTR suggests that Nos protein functions to prevent Otd-1 (and Hb) translation at the posterior of the embryo, similar to its role as a translational repressor of Hb in the posterior of the Drosophila embryo (Hulskamp et al. 1989; Irish et al. 1989; Sonoda and Wharton 1999; Lynch et al. 2006a). Eventually, Otd-1 protein gradients form at both poles. Knockdown of otd-1 expression via pRNAi results in a shift of target gene expression towards the poles of the embryo, consistent with otd-1 functioning as a morphogen at both poles. Specifically, the anterior patterning gap gene empty spiracles (ems) is shifted farther towards the anterior pole in response to increased strength of otd-1 pRNAi. Both the anterior and posterior expression domains of the gap genes giant (gt) and zygotic hb are also shifted towards the poles in response to otd-1 knockdown. The molecular phenotypes resulting from otd-1 pRNAi show that Otd-1 functions as a morphogen in the wasp, similar to the way Bcd functions in the fly (Lynch et al. 2006a).

The cuticular phenotype resulting from otd-1 knockdown results in loss of head segments, as well as loss of posteriormost abdominal segments (Fig. 4). This phenotype is much more severe than that of loss of the zygotic gap gene Dm otd. Although the otd-1 knockdown phenotype is not identical to the bcd\textsuperscript{-} mutant phenotype, which also lacks thoracic segments (Frohnhofer et al. 1986; Cohen and Jurgens 1990), these results do further confirm the hypothesis that Otd, at least in part, functions ancestrally as a major anterior determinant in the absence of Bcd. Moreover, as both Bcd and Otd have the same binding specificity, they likely share similar downstream targets (Treisman et al. 1989; Lynch et al. 2006a).

**hb is a conserved anterior patterning gene in Nasonia**

The zinc finger transcription factor hb is expressed maternally and zygotically in the Nasonia embryo. Maternal hb mRNA is first transcribed during oogenesis in the nurse cells and loaded throughout the oocyte. In the early embryo, hb mRNA is homogenously present throughout the entire length of the embryo. Upon translation of the maternal hb mRNA, Hb protein creates an anterior to posterior gradient in the early embryo (Pultz et al. 2005). The presence of multiple sequences resembling nanos response elements (NREs) in the Nvit hb 3’UTR suggests that the hb mRNA is translationally repressed in the posterior of the embryo by nanos, as is Dm hb in Drosophila (Hulskamp et al. 1989; Irish et al. 1989; Struhl 1989; Murata and Wharton 1995; Pultz et al. 2005).

At the anterior of the embryo, zygotic hb forms a large cap of expression that eventually fades to form a stripe, similar to the zygotic anterior Dm hb expression (Fig. 1G, 1H). Zygotic hb is also expressed in a stripe at the posterior of the embryo and as a dorsal strip corresponding to the extraembryonic membranes (Pultz et al. 2005). Using the hb\textsuperscript{headless} mutant wasp strain, a null allele of zygotic hb, Pultz et al. (1999, 2005) examined the phenotype resulting from loss of zygotic hb in the wasp embryo (Pultz et al. 1999, 2000, 2005). Cuticles show both posterior and thoracic defects as expected from the zygotic hb expression pattern. Specifically, the posterior abdominal segments A8-A10 are missing and thoracic, gnathal and antennal segments in the head region are defective or missing. Labral structures, however, are present, suggesting that zygotic hb does not function alone in anterior patterning (Fig. 4). It is also clear that Nasonia relies more heavily on zygotic hb than the fly, whose loss leads to less severe cuticular phenotypes (Pultz et al. 1999). This may be due to redundant functions of Bcd and Hb in flies. Conversely, the greater reliance on zygotic hb in the wasp embryo may be a reflection of the lack of bcd during wasp embryogenesis.

**otd-1 and hb function synergistically to pattern the anterior of the wasp embryo**

otd-1 knockdown in Nasonia results in loss of head segments, as well as loss of posterior segments. Knocking down both hb and otd-1, however, results in a complete loss of head and thorax in addition to loss of posterior structures. The anterior segmentation phenotype is more severe than the combination of the individual pRNAi knockdowns of either hb or otd-1, suggesting that these genes work synergistically in the anterior of the embryo (Fig. 4). There is no evidence for a synergistic relationship between hb and otd-1, however, in patterning the posterior of the wasp embryo (Lynch et al. 2006a). This study, in conjunction with the investigation of anterior patterning in the beetle (Schröder 2003) provide strong support for the hypothesis that Hb and Otd play the ancestral role of Bcd in patterning the anterior of the embryo.

**Maternal giant is a key repressor in the anterior of the wasp embryo**

Although many basic mechanisms in embryogenesis are conserved throughout the insect world, a common theme that has emerged as a result of many recently reported com-
parative studies is that many members within the genetic network of early embryogenesis seem to take on new roles throughout evolution. This is exemplified by the recent report regarding the expression and function of giant (gt) in the wasp embryo (Brent et al. 2007). In Drosophila, gt is a zygotic gap gene that is expressed in a bcd activated anterior stripe, as well as in a posterior stripe (Kraut and Levine 1991b). In Nasonia, the expression of this zygotic gap gene is very similar to its Drosophila counterpart (Fig. 1I, 1J) (Olesencky 2006; Brent et al. 2007). Strikingly, however, freshly laid wasp embryos show an anterior to posterior gt mRNA gradient, indicating that gt has taken on a new role as a maternal determinant during wasp embryogenesis (Brent et al. 2007). In fact, follicles harvested from female wasps show that gt mRNA is tightly localized to the oocyte nucleus in the anterior of the developing oocyte (Fig. 5E, 5F). This again highlights the use among insects of conserved mechanisms, such as mRNA localization, and the adaptation of these strategies to create novel roles for existing genes within the genetic network.

The evolution of gt as a maternally expressed factor in Nasonia suggests a new function for gt during development. gt pRNAi embryos show loss of all head and thoracic segments, as well as fusion or deletion of abdominal segments 6 and 7, likely due to loss of the posterior zygotic gt domain (Fig. 4). Therefore, Nasonia gt has gained a new essential role in head and thoracic patterning resulting in a phenotype much more severe than the mild head phenotype of Dm gt mutants, which only show loss of labral and labial structures (Kraut and Levine 1991b). An elegant molecular analysis by Brent et al. (2007) on the effect on the genetic network resulting from loss of gt ascribed a major role for gt as a potent repressor of Kr in the anterior of the wasp embryo. Thus, the loss of gt results in an expansion of Kr to the anterior tip of the developing embryo, preventing the formation of any head or thoracic segments. This study highlights the ever-changing roles of developmental genes throughout evolution and the ability of these plastic genetic networks to pattern a basic segmented body plan that is conserved among insects.

COMBINATORIAL INPUT ALONG THE AP AXIS: BICOID AND CAUDAL

One study in Drosophila showed that the combined activities of Bcd and Cad are involved in driving expression of knirps (kni) in the posterior of the embryo via two response elements in the kni regulatory region. This study is of particular interest as bcd is an anterior determinant, yet here it plays a role in driving expression of a target gene in a more posterior region of the embryo. Moreover, neither Bcd nor Cad alone is sufficient to drive wild type expression of kni in the embryo (Rivera-Pomar et al. 1995). Another example of a target gene responding to the reciprocal gradients of the transcription factors Bcd and Cad, is hairy (h), a pair-rule gene expressed in 7 stripes in the embryo. Similar to even skipped (eve), the elements driving h stripes can be divided into separable modules. In the case of h stripes 7, the most posteriorly expressed h stripe, a number of Bcd and Cad sites are necessary to drive wild type expression. Furthermore, removal of both cad and bcd results in a severe reduction in h stripe 7 expression (La Rosee et al. 1997).

Using the computer algorithm Ahab, a recent study utilized known binding sites of various maternal and gap genes, as well as equilibrium thermodynamics to recover novel transcription elements in 7 of segmentation genes (Schroeder et al. 2004). The expression patterns of these elements revealed that genes expressed in the anterior of the embryo showed a great number of Bcd binding sites, whereas Cad binding sites were few. Conversely, elements expressed in the posterior half of the embryo tended to have a greater number of Cad sites and few Bcd binding sites. Furthermore, the elements expressed early in the embryo contained binding sites for a number of maternal factors including Bcd and Cad, strongly suggesting that these maternal factors directly control most of early zygotic patterning (Schroeder et al. 2004).

CAD MAY BE A POSTERIOR PATTERNING CENTER IN SHORT GERM EMBRYOS

It has been proposed that an anterior patterning center, such as Bcd in the long germ Drosophila, would not function well to pattern the anterior of the embryo in short germ insects (Stauber et al. 1999; Stauber et al. 2002). Anteriorly localized factors would not be able to reach the germ rudiment at the posterior of the oocyte and would instead pattern the extraembryonic membranes, which lie at the anterior (Lall and Patel 2001; van der Zee et al. 2005). Instead, the ancestral system may have exclusively utilized a posterior patterning center, allowing for posteriorly localized factors to easily reach the developing embryo. One candidate for an ancestral posterior patterning function is cad. In both Tribolium and Gryllus, cad is expressed early in a broad posterior to anterior gradient and it appears to regulate expression of anterior hb and Kr placing it atop the genetic patterning hierarchy (Copf et al. 2003; Shimmyo et al. 2005).

CAD IS A POSTERIOR PATTERNING CENTER IN THE NASONIA EMBRYO

Among the various mutant phenotypes isolated in the Pultz et al screen, head only (ho) was shown to have dramatic posterior patterning defects that partly overlap with the posterior cuticular phenotypes derived from knockdown of otd. ho is a mutant that develops only head and thorax, lacking all abdominal and posterior structures (Fig. 3C). Comparison of the ho phenotype to known Drosophila mutant phenotypes strongly suggested that ho is due to a lesion in the Nasonia cad locus. Moreover, cad pRNAi phenocopies the ho mutant phenotype and genetic mapping experiments place the ho genetic lesion very close to the Nv cad locus (Fig. 4) (Olesencky et al. 2006). Similar to the phenotype resulting from loss of zygotic Nv hb, however, ho results in a much more dramatic phenotype than loss of zygotic cad alone in Drosophila and instead resembles loss of both maternal and zygotic Dm cad. This strongly suggests that the wasp needs both genetic components during embryogenesis (Pultz et al. 1999).

In Nasonia, cad is expressed both maternally and zygotically. Expression analysis of ovarian follicles and early embryos uncovered a surprising mechanism for the generation of the maternal Cad gradient in the wasp embryo (Olesencky et al. 2006). In Drosophila, maternal Nv cad, however, is expressed both maternally and zygotically. Formation of the Cad gradient is achieved through translational repression of cad mRNA in the anterior of the embryo (Macdonald and Struhl 1986; Mlodzik and Gehring 1987; Hunter and Kenyon 1996; Schulz et al. 1998). In the fly, Bcd functions as the Cad translational repressor by binding to the cad 3’UTR (Dubnau and Struhl 1996; Rivera-Pomar et al. 1996; Niessing et al. 1999, 2000, 2002). While the factors involved in establishing the Cad gradient in Tribolium are not known, in C. elegans, formation of the Cad gradient is achieved through translational repression of cad mRNA in the anterior of the embryo (Macdonald and Struhl 1986; Mlodzik and Gehring 1987; Hunter and Kenyon 1996). In Nasonia, cad mRNA is instead localized to the posterior pole of the oocyte during oogenesis and forms a posterior to anterior mRNA gradient by simple diffusion from the posterior pole (Fig. 5G, 5H). This result is particularly interesting because the use of mRNA localization and diffusion obviates the need for translational repression at the anterior of the embryo to efficiently establish a Cad gradient (Olesencky et al. 2006).

Zygotic cad is expressed in the posterior two thirds of the early embryo and begins to recede from the anterior as embryogenesis progresses. Later, cad is expressed as two stripes in the posterior, and finally in a single stripe at gastrulation. Functional analysis of cad using pRNAi showed that maternal Nv Cad is a major transcriptional regulator of
genes expressed in the thoracic and abdominal regions of the embryo, whereas Dm Cad does not function in thoracic patterning. For example, in Nasonia, Cad is a transcriptional activator of the gene Kr, which forms a broad stripe at the center of the embryo and is essential for normal abdominal patterning. Similarly, anterior and posterior kni expression is absent in embryos lacking Nv cad. Moreover, in the wasp embryo, cad plays a role as an activator of gt and tailless (tl). In contrast, removal of both maternal and zygotic cad components has no effect on Kr expression in Drosophila and only functions as a weak activator of posterior kni expression. Additionally, by comparing results obtained from cad pRNai mutant embryos and the zygotic Nv cad mutant embryos, it becomes clear that maternal cad functions in Nasonia specifically as an activator of anterior gap and pair rule gene expression, whereas zygotic cad functions as an activator in more posterior regions of the wasp embryo (Olesnicky et al. 2006).

Thus, Drosophila relies much less on cad function than Nasonia or other insects such as Tribolium and Gryllus (Copf et al. 2004; Shinnyo et al. 2005; Olesnicky et al. 2006): As the genetic network evolved, Cad progressively lost in flies its importance as an activator of the gap genes. The fact that Bed, in conjunction with Cad, is also important for the activation of posterior genes in the fly suggests that Bed may have usurped much of Cad’s role as the major posterior patterning gene. Cad does, however retain an important role as a regulator of pair rule gene expression in the posterior half of the fly embryo, maintaining some of its role as a major posterior patterning factor (Fig. 6A-D) (Olesnicky et al. 2006).

mRNA LOCALIZATION: A COMMON FEATURE OF LONG GERM EMBRYOGENESIS

mRNA localization is commonly used for targeting factors to particular regions of a cell or tissue. In Drosophila, mRNA localization of maternal factors is used extensively to set up the antero-posterior axis of the early embryo, prior to the onset of zygotic gap gene expression (St Johnston 2005; Zimyanin et al. 2007). In fact, the use of localized mRNAs, such as the anterior patterning factor bed, allows for the generation of steep concentration gradients of proteins through translation and simple diffusion. Thus mRNA localization in a syncytial embryo is a superb mechanism for setting up an environment that utilizes morphogens for patterning.

In Drosophila, bed assumed the role of the anterior patterning center and its mRNA is localized maternally to the anteriormost pole of the embryo. Similarly, in the long germ wasp Nasonia, otd-1 is maternally localized to the anterior pole of the early embryo and functions similarly to bed in patterning the anterior segments through a morphogenetic protein gradient. Surprisingly, otd-1 mRNA is also localized to the posterior pole of the Nasonia embryo to help direct posterior patterning (Lynch et al. 2006a). In addition to otd-1, a number of maternal mRNAs are also localized maternally and localized to the anterior pole of the developing oocyte (Brent et al. 2007). Moreover, nos and cad mRNAs are localized to the posterior pole of the embryo (Fig. 5A-H) (Lynch et al. 2006a; Lynch and Desplan submitted). As Nasonia undergoes long germ development similar to Drosophila, it becomes evident that in addition to a heavy reliance on maternal patterning factors, one common feature of long germ embryogenesis is the importance of proper localization of these maternal determinants.

Recent work by Olesnicky and Desplan (2007) shows that the major mechanisms employed for maternal mRNA localization are common to both Nasonia and Drosophila. Specifically, transport of mRNAs along the microtubule cytoskeleton is utilized for the localization of most mRNAs within the oocyte. Most posteriorly localized mRNAs, however, exploit the germ plasm to achieve a tight posterior localization that persists much longer than micro-tubule mediated mRNA localization. By using drug treatments to inhibit the polymerization of microtubules, Olesnicky and Desplan also find that Nasonia relies heavily on the microtubule cytoskeleton, not only for transport of factors from the nurse cells to the oocyte, but also during oogenesis for oocyte specification. A similar role in oocyte specification has been ascribed to microtubules in Drosophila (Pokrywka 1995; Riparbelli et al. 2007).

COMMON STRATEGIES USED DURING LONG GERM INSECT EMBRYOGENESIS

The Drosophila embryo utilizes many strategies including mRNA localization, gradient formation and mutual repression to create a very fast and efficient developmental program. Other insects undergo development in a much slower manner and utilize very different mechanisms to achieve their adult body plan. One important aspect of Drosophila embryogenesis is the evolution of a syncytial embryo that persists even during the beginning stages of zygotic transcription, allowing the specification of the entire antero-posterior axis in the early embryo. This strategy is also employed by many short germ insects, but only for patterning the anterior embryonic structures. In short germ embryos, the abdominal and posterior structures form later within a cellularized environment at the posterior of the embryo in a region termed the growth zone. Thus, the use of a syncytium to pattern the entire length of the embryo is a feature specific to long germ development (Lall and Patel 2001; Davis and Patel 2002; Liu and Kaufman 2005b).

In a syncytial environment, there are no cellular membranes to create a barrier between nuclei, allowing for formation of gradients of transcription factors via simple diffusion and the simultaneous patterning of the whole length of the antero-posterior axis. For example, Drosophila establishes gradients of Hb and Bcd at the anterior embryonic pole, while Cad and Nos gradients are formed at the posterior pole, thus establishing the basic asymmetry along the antero-posterior axis in the earliest phase of development (Liu and Kaufman 2005b; St Johnston 2005). Although gradients of extracellular growth factors are also observed in cellularized environments, they must rely on additional factors to transduce the signal within the cell.
Results presented in this review show that a similar strategy of opposing gradients is used in the Nasonia embryo, where Hb and Otd create gradients at the anterior of the embryo and Cad and Otd are graded at the posterior of the embryo. In short germ embryos, although gradients are formed in the early embryo, they are incapable of patterning the entire length of the embryo, as abdominal structures develop much later and within a cellularized environment. Thus, in addition to major differences in the embryonic environment between long and short germ insects, there is also a delay in posterior patterning within short germ embryos. Therefore, with the advent of long germ embryogenesis, a temporal shift must have taken place to accommodate simultaneous expression of the anterior and posterior patterning centers, thus allowing patterning along the entire antero-posterior axis (Lynch and Desplan 2003a; Lynch et al. 2006b; Olesenick et al. 2006).

The Bcd gradient is formed in the anterior of the embryo and functions in a morphogenetic manner in Drosophila. The Bcd protein serves many purposes in development including activation and translational repression. Strikingly, despite its concentration at the anterior tip of the embryo, Bcd is also important for the activation of genes in posterior regions of the embryo. The combinatorial input of Bcd and Cad has been reported to regulate expression of both gap and pair-rule genes in the posterior or the embryo, whereas Hb and Bcd function together at the anterior of the embryo to activate gap and pair rule genes (La Rosee et al. 1997; Hader et al. 1998).

In Nasonia, the use of a morphogen is also extremely important for normal embryonic development. The wasp, however, lacks Bcd and instead establishes an Otd morphogenetic gradient at both poles of the early embryo. Thus, Otd is able to function throughout the length of the embryo in a morphogenetic manner, similar to Bcd in Drosophila, yet is positioned very differently from Bcd. Since Otd is expressed at both poles, it is important for cofactors to create asymmetry in the wasp embryo. Hb serves as a cofactor for Otd in patterning the anterior of the embryo, similar to its role with Bcd in Drosophila (Lynch et al. 2006a). Recent work shows that Cad is an extremely important posterior patterning gene that serves as a cofactor for Otd in posterior embryonic patterning (Olesenick et al. 2006). Thus both Otd in the wasp and Bcd in the fly provide essential patterning information throughout the entire length of the embryo that serves to initiate transcription of zygotic target genes. Both morphogens, however, require cofactors to initiate the proper developmental program in the different regions of the embryo. Additionally, although a similar combinatorial effect is seen between Cad and Otd in Nasonia, as compared to that of Cad and Bcd in Drosophila, Cad functions more extensively in directing posterior or patterning in the Nasonia embryo than it does in the fly.

**CONCLUSIONS**

In conclusion, research in Nasonia and Drosophila highlights a number of conserved features employed by long germ insects to establish the antero-posterior axis and initiate patterning along this axis. Initially, maternal mRNAs are transcribed by the nurse cells and transported into the developing oocyte. Next, groups of polarized microtubules within the dynamic cytoskeleton are used as tracks for motors to shuttle mRNAs to various regions of the oocyte. Additionally, the germ plasm is used for the tight localization of mRNAs and other factors to the posterior cortex of the egg. Upon the completion of oogenesis and subsequent egg deposition, these localized mRNAs begin to diffuse and generate opposing gradients within the syncytial embryo. These gradients provide the positional information along the axis that is necessary to initiate the complex genetic program that will establish the adult body plan. Thus the increasing reliance on maternal factors, the utilization of mRNA localization, gradient formation and a syncytial embryo are hallmarks of long germ embryogenesis. Yet these genes that make up the developmental network are plastic and show a remarkable ability to evolve new functions and expression patterns to adapt to the changing mechanisms and environments of the quickly evolving insect world.

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