Morphogenetic Functions of Extraembryonic Membranes in Insects

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Summary

Morphogenetic functions of the amnioserosa, theserosa, the amnion, and the yolk sac are reviewed on the basis of recent studies in flies (*Drosophila*, *Megaselia*), beetles (*Tribolium*), and hemipteran bugs (*Oncopeltus*). Three hypotheses are presented. First, it is suggested that the amnioserosa of *Drosophila* and the dorsal amnion of other fly species function in a similar manner. Second, it is proposed that in many species with an amniotic cavity, the amnion determines the site of serosa rupture, which, through interactions between the serosa and the amnion, enables the embryo to break free from the amniotic cavity and to close its backside. Finally, it is concluded that the yolk sac is likely an important player in insect morphogenesis.

Introduction

The full complement of extraembryonic membranes in insects includes the serosa, the amnion, and the yolk sac [reviewed in 1,2-4]. They play important roles in embryo immunity, desiccation resistance, and morphogenesis, and may fulfill additional physiological roles [reviewed in 5,6,7]. This comparative review focuses on the functions of extraembryonic membranes in morphogenesis. Insect embryos undergo intertwined morphogenetic and blastokinetic functions. The former refer to cell and tissue movements within the embryo, whereas the latter refer to movements of the whole embryo within the egg but for the purpose of this review we will refer to them collectively as morphogenetic movements. Most research on the role of extraembryonic membranes in insect morphogenesis has been done with the genetic model organism *Drosophila melanogaster* because of its exceptional amenability to functional analysis, and this work has been reviewed extensively in the context of dorsal closure [for a concise summary of that literature, see 8]. However, in this species, the extraembryonic membranes are partly reduced, and it is not well understood how findings in *Drosophila* can be applied to insects with the full complement of extraembryonic membranes. In this brief “opinion paper”, we compare the morphologies and discuss specific functions of the diverse extraembryonic membranes in insects, drawing mostly from recent studies in flies (*Drosophila*, *Megaselia*), beetles (*Tribolium*), and bugs (*Oncopeltus*).

Diversity of Extraembryonic Membranes in Insects

The extraembryonic membranes of insects differ morphologically between species (Fig. 1) [reviewed in 1-4,5,9,10]. Nearly all insects develop a serosa after germ band formation. This epithelium lines the inner side of the eggshell and originates from the blastoderm cells that do not contribute to the germ rudiment (prospective embryo and amnion). The amnion develops from the rim of the germ rudiment but its topography varies between species. In most insects, it closes over the ventral side of the germ band and forms an “amniotic cavity”, a term that refers to the closed space between the amnion and germ band. In butterflies and some beetles (e.g. Chrysomelidae and Curculionidae), the amnion closes ventrally, like in most species, but then also dorsally, forming an outer circumferential amnion and an inner dorsal amnion that covers the yolk sac [2]. In wasps [11] and lower cyclorrhaphan flies such as phorids and syrphids [12], the amnion only grows dorsally over the yolk sac, forming a dorsal amnion. Higher cyclorrhaphan flies (Schizophora), such as *Drosophila* develop an “amnioserosa” instead of distinct serosa and amnion epithelia. The final topography of this tissue is comparable to the dorsal amnion in phorids and syrphids but the modes of dorsal amnion and amnioserosa formation differ. While the dorsal amnion of phorids and syrphids grows from the rim of the germ rudiment over the dorsal yolk sac and is completed after germ band elongation, the amnioserosa of *Drosophila* develops from blastoderm cells that form a narrow stripe along the dorsal midline and covers the yolk sac at all stages until dorsal closure occurs. The yolk sac of *Drosophila* is a membrane-lined multinucleate yolk cell that forms underneath the blastoderm. In other species, yolk nuclei underneath the yolk cell membrane cellularize and form a yolk cell lamella (primary entoderm), or the yolk cell cleaves into large membrane-lined mono- or multinucleate yolk cells.

Functions of the Serosa

The serosa can mount a strong innate immune response to counter bacterial infection [13-15] and secretes a cuticle underneath the eggshell that increases resistance to desiccation [16-23]. In many species, the serosa also controls dorsal closure by promoting the rupture of the amniotic cavity and pulling the amnion to the dorsal side [10]. Many non-holometabolous insects undergo longitudinal axis inversion and, in these species, the serosa also plays an important role in realigning the axes of the embryo with those of the egg (see Box 1) [5]. There is consensus among early and current investigators that, as the serosa ruptures and recedes to the anterior or dorsal egg, serosa cells actively contract. The contraction of serosa cells involves their apical constriction and transitioning from squamous to columnar cell shape. The mechanism for the directionality of this process, and the forces that finally drive the contracted serosa into the yolk sac, remain poorly understood [for a discussion of these events see 24,25]. The site of serosa rupture appears to be triggered by the amnion, and is discussed in the next section.

Functions of the Amnion

In this review, we treat the amnioserosa as a dorsal amnion. The rationale for this interpretation has been discussed elsewhere [12,26]. It is primarily based on the expression and function of the homeobox gene *zerknüllt* (*zen*) in different holometabolous species. In a range of holometabolous insects, *zen* specifies serosa cells in amnion-competent tissue and drives the expansion of the serosa; following knockdown of *zen*, serosa cells fail, or stop to expand and express amnion genes [12,27,28]. Amnioserosa specification in *Drosophila* is also dependent on *zen*, but the amnioserosa loses *zen* activity after gastrulation when other flies undergo serosa

expansion[29], suggesting that amnioserosa development might have evolved in response to a homeotic mutation that incorporated the serosa precursors into the amnion.

The amnioserosa of *Drosophila melanogaster* controls two vital morphogenetic movements: germ band retraction and dorsal closure[8,30-34]. Germ band retraction shortens the u-shaped germ band and aligns all body segments with the anteroposterior axis of the egg. The amnioserosa drives this process by crawling over the tail end and pulling on the crook of the folded germ band [35-38]. It may also induce cellular changes in adjacent embryonic tissue [34,39]. Dorsal closure seals the epidermis after germ band retraction. The amnioserosa controls this process in conjunction with the adjacent epidermis and the yolk sac[reviewed in 6,40,41-43] by regulating the volume and stiffness of amnioserosa cells [44], and, later in development, probably their apical constriction and invagination[45-47].

The morphogenetic roles of the amnion in other insects remain poorly understood. In the scuttle fly *Megaselia abdita* (Phoridae), the topography of the completed dorsal amnion is comparable to that of the amnioserosa in *Drosophila*. However, the dorsal amnion of *Megaselia* is still incomplete at the beginning of germ band retraction. After germ band retraction and amnion completion, the serosa adheres to the central portion of the dorsal amnion [supporting information in 12] and, following serosa rupture, both tissues move into the yolk as dorsal closure progresses [48].

In *Megaselia*, dorsal closure does not seem to be significantly altered when *zen* expression and serosa development are repressed [12]. Hence, serosa cells appear to be dispensable for dorsal closure in *Megaselia*. However, the amnion cells are probably essential for this process because *Megaselia* homologs of *Drosophila* genes that maintain the integrity of the amnioserosa (*u-shaped* group) are required for germ band retraction and dorsal closure in

Megaselia. If we assume that these genes exert similar roles in the maintenance of the amnioserosa of *Drosophila* and the dorsal amnion of *Megaselia* (which remains to be shown), these findings would suggest that the dorsal amnion and the amnioserosa of these species promote the same morphogenetic processes [28], even though the dorsal amnion of *Megaselia* is still incomplete at the beginning of germ band retraction. At the beginning of dorsal closure, the amnion of *Megaselia* has been completed. Therefore, it is conceivable that dorsal closure in *Megaselia* involves force-generating mechanisms in the amnion that are comparable to those in the amnioserosa of *Drosophila*. Given that the completed amnion of *Megaselia* adheres to the serosa cells prior to serosa rupture, it is conceivable that shrinking amnion cells not only would exert a pulling force of the lateral epidermis but would also increase tension in the serosa and, thereby, contribute to serosa rupture. It might be possible to test this hypothesis by genetically preventing amnion-serosa adhesion in *Megaselia*.

Research in *Oncopeltus* suggests that in species with a ventral amnion, serosa rupture occurs in three steps [25,27,49,50]. First, the serosa and amnion adhere locally. Second, the amnion begins to form a hole underneath the adhering portion of the serosa, which transiently seals this hole. Third, the serosa sealing the hole in the amnion ruptures and contracts, thereby ripping open the amniotic cavity and freeing the embryo. Knockdown of *zen* activity (*Of-zen*) suppresses the formation of the hole in the amnion and prevents the subsequent rupture of the overlying serosa [25]. Instead, the serosa in these embryos ruptures and contracts ectopically at a later stage, leaving the amniotic cavity intact and thereby causing ventral closure of the lateral epidermis around the legs. Likewise in *Tribolium*, knockdown of a *zen* homolog, *Tc-zen2*, leaves the amnion intact [27]. Additional data from the *Panfilio* group indicate that in this species, too, the amnion forms a hole at the site where the attached serosa will rupture [51].

Of-zen and *Tc-zen2* are expressed throughout the serosa and in the amnion at the site of serosa-amnion fusion [27, figure 6 and legend in 49]. How then does *zen* control the rupture of the serosa? It has been proposed that *zen* activity in the serosa controls its rupture by regulating the contractile behavior of the serosa, thereby creating a window of opportunity for site-specific rupture [7, 25, 50]. However, this hypothesis does not consider *zen* activity in the amnion of *Oncopeltus* and *Tribolium*, and the finding that knockdown of *zen* in these species leaves the amnion intact while exerting high strain on it (Fig. 2). Alternatively, the amnion might guide the site of serosa rupture. In this case, *zen* activity in the amnion would be required for the controlled release of a factor from the amniotic cavity that predisposes the exposed serosa to rupture under increased tension. Accordingly, the site of serosa adhesion and rupture should change when embryos rotate within the egg along their longitudinal axis, as observed by Panfilio's group [51, contra 52].

The cellular mechanism by which the amnion forms a hole underneath the attached serosa remains elusive. For *Oncopeltus*, it has been proposed that this process is driven by apoptosis [7, 25]. However, apoptosis of amnion cells in this region is also observed in *Of-zen* RNAi embryos in which the amniotic cavity remains intact [25], and apoptosis of amnion cells within the perimeter of the serosa-amnion fusion could simply reflect high mechanical stress [53].

Functions of the Yolk Sac

Potential morphogenetic roles of the yolk sac have been discussed extensively by early investigators [reviewed in 1-3, 4, 9]. However, the molecular basis for roles of the yolk sac in morphogenesis remains poorly understood. Only a few recent investigators have paid attention to

its role in morphogenesis [e.g. 45,54,55]. Along with these researchers, we believe this neglect is unjustified and seek to generate interest in this structure.

In *Drosophila melanogaster*, integrins in the yolk sac membrane are required for adhesion of the yolk sac to the amnioserosa via a laminin-containing extracellular matrix. The inhibition of this integrin function reveals autonomous yolk sac contraction and causes thinning and premature death of the overlying amnioserosa cell layer [45,55]. This phenotype led to the hypothesis that the contractile yolk sac may exert an apicobasal pulling force on overlying amnioserosa cells [55].

In *Tribolium*, the yolk sac and amnion fold over the tail end of the extending germ band[56]. A physical connection has been proposed between the yolk sac and the posterior end of the embryo-amnion rudiment to account for this fold [54]. When amnion involution stops, the yolk sac fold retracts from the space between the serosa and amnion, thereby allowing the close apposition of the serosa and amnion. A similar phenomenon might occur in *Oncopeltus*, where yolk removal from the cleft between the amnion and the serosa precedes serosa-amnion adhesion at the posterior pole of the egg [50]. Therefore, it is conceivable that in both species the yolk sac controls serosa-amnion apposition as a prerequisite for serosa-amnion fusion.

Early investigators provided strong evidence for an active role of the yolk sac in anatrepsis(longitudinal axis inversion;Box 1) [e.g. in leaf hoppers 57,58].In *Oncopeltus*, potential morphogenetic roles of the yolk sac have not yet been explored but a recent study shows that *Oncopeltus torso-like(Of-*tsl*)* is required for anatrepsis[59].The *tsl* protein (Tsl) is related to pore-forming MACPF proteins [60]. In *Drosophila*, Tsl is a localized eggshell component that mediates tyrosine-kinase signaling at the anterior and posterior poles of the syncytial embryo [61-64]. The receptor of this pathway, Torso (Tor), appears to be missing in *Oncopeltus*, but

Drosophila Tsl also fulfills Torso-independent functions in growth and developmental timing [65]. The transcripts of *Of-tsl* are expressed ubiquitously in freshly laid *Oncopeltus* eggs and degrade rapidly after egg laying, and could have a role in the reticular cytoplasm of the yolk system that would affect its contractility.

Conclusions and Outlook

Extraembryonic membranes are important players in insect morphogenesis, but their underlying molecular and cellular mechanisms remain poorly understood. One noteworthy exception is the role of the amnioserosa in the dorsal closure of *Drosophila* embryos. The model of how amnioserosa cells regulate dorsal closure might be applicable, in part, to the function of amnion cells in dorsal closure of other insects, given that the amnioserosa is functionally and evolutionarily comparable to a dorsal amnion. It should be kept in mind that the model for the role of the amnioserosa in dorsal closure depends on the developmental stage. In insects with ventral amnion closure, the amnion assumes a dorsal position only towards the end of dorsal closure. However, dorsal closure in these insects also proceeds when ventral amnion closure is genetically suppressed and a dorsal amnion is present throughout the process of dorsal closure [24,27]. This observation suggests, that aspects of the model for the role of amnioserosa cells in dorsal closure that are based on data from the early phase of dorsal closure, such as volume decrease of amnioserosa cells by controlled osmosis [e.g. 44], may also apply to the amnion cells of other species.

The mechanism that controls extraembryonic membrane rupture remains elusive, but the unexpected finding that serosa rupture is preceded and apparently triggered by the formation of a hole in the amnion marks a potential breakthrough [25,51]. Dissecting the cellular and molecular

events that generate the serosa-sealed hole in the amnion might be essential for understanding the process of membrane rupture. Furthermore, this discovery hints at the importance of the amniotic cavity, which may store factors that prepare for serosa rupture. It should be possible to identify such factors in genetic screens, unless they constitute a complex “cocktail” of partially redundant factors. Species that lack an amniotic cavity but rupture the serosa in a controlled manner, such as the scuttle flies [48,66], or that rupture the serosa and amnion independently, such as fleas [67], would require a different mechanism, which will be interesting to compare to those of *Tribolium* and *Oncopeltus*.

The molecular exploration of yolk sac functions in morphogenesis has hardly begun, though recent work in *Tribolium* has indicated a direction for study [54]. Examination of yolk sac functions in species with massive blastokinetic movements, such as crickets, leafhoppers, and damselflies, promise a further enhancement of our understanding.

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Figure Legends

Figure 1.

Sketches of embryos and extraembryonic membranes of four insect species [based on data reported in 12,27,48,50-52,56,68]. Consecutive developmental stages are depicted from top to bottom. *Oncopeltus* embryos are shown at the beginning of gastrulation (I), during anatrepsis (II), after serosa and amnion completion (III), after serosa-amnion adhesion (IV), after hole formation in the amnion (V), and after serosa rupture (VI). Developmental stages of other species are roughly matched. The serosa is shown in red, the amnion in blue, the embryo in grey, and the yolk sac in yellow. The eggshell is omitted. In *Drosophila*, amnioserosa cells are depicted in red or blue depending on whether *zen* is expressed (red) or repressed (blue). We propose that after the repression of *zen*, amnioserosa cells are comparable to amnion cells (see main text). Arrows indicate directed movement of extraembryonic epithelia. Anterior of the egg is left and dorsal up. H, head end. T, tail end.

Figure 2.

The amnion determines the site of serosa rupture in *Oncopeltus*. (a) A time sequence of *Of-zen* RNAi embryos. (b) Wild-type embryos during katatrepsis at three consecutive developmental stages. Note that knockdown of *zen* in *Oncopeltus* prevents the formation of a hole in the amnion and controlled rupture of the serosa. The asterisk marks the site of serosa-amnion adhesion. Curved arrows mark the site of amnion-serosa fusion during katatrepsis. The primary reference for these images [50] should be consulted for additional information. The eggs are

oriented with anterior left and dorsal up. Am, amnion; H, head; Ser, serosa; T, tail end of the germ band.

See Functions of the Amnion for a description of this phenotype.

Box 1 Anatrepsis and Katatrepsis

Many non-holometabolous insects, such as *Oncopeltus*, undergo prominent blastokinetic movements, called anatrepsis and katatrepsis. During anatrepsis, the embryo bends its tail end dorsally over the head and extends it towards the anterior pole. The attached amnion follows this movement and covers the ventral side of the extending germ band. As the embryo and amnion fold inwards, the head shifts posterior and the serosa expands. During this process, the orientation of the embryo is reversed. The serosa and the amnion close over the head and separate from each other. During katatrepsis, this process is reversed by the contracting serosa, which enforces a backflip movement of the embryo following its rupture and fusion to the opened amnion. Following anatrepsis, the embryo of certain species (e.g. the damselfly *Platycnemis pennipes* [9]) rotates 180° along the longitudinal axis, which is also corrected during katatrepsis.

Figure 1

Figure 2