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Neofunctionalization of Toll Signaling in Insects: From Immunity to Dorsoventral Patterning

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Abstract

Toll signaling plays a crucial role in pathogen defense throughout the animal kingdom. It was discovered, however, for its function in dorsoventral (DV) axis formation in *Drosophila*. In all other insects studied so far, but not outside the insects, Toll is also required for DV patterning. However, in insects more distantly related to *Drosophila*, Toll's patterning role is frequently reduced and substituted by an expanded influence of BMP signaling, the pathway implicated in DV axis formation in all major metazoan lineages. This suggests that Toll was integrated into an ancestral BMP-based patterning system at the base of the insects or during insect evolution. The observation that Toll signaling has an immune function in the extraembryonic serosa, an early differentiating tissue of most insect embryos, suggests a scenario of how Toll was co-opted from an ancestral immune function for its new role in axis formation.

Contents

INTRODUCTION

Toll signaling was discovered in screens for maternal-effect mutations that disrupt the formation of the body axes of *Drosophila* larvae (Anderson & Nüsslein-Volhard 1984; Anderson et al. 1985a,b; Nüsslein-Volhard 2022). Toll codes for a transmembrane receptor and occupies a central position within a relay mechanism that transmits spatial information of the eggshell to the embryo and thereby establishes the embryonic dorsoventral (DV) axis (Stein & Stevens 2014) (**Figure 1**). The work by many labs on Toll signaling and its target genes as well as on the establishment of DV

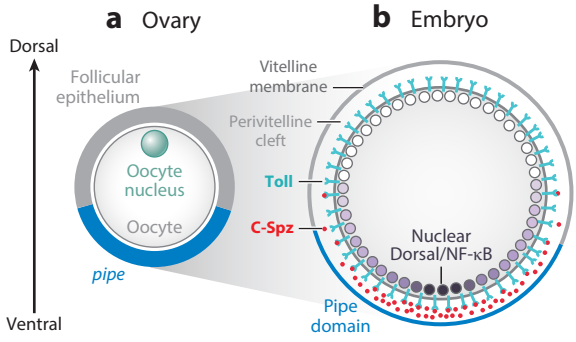

Figure 1

Dorsoventral axis formation in *Drosophila*: From ovary to embryo. (*a*) Cross-section through a *Drosophila* egg chamber. The oocyte nucleus (*green*) is asymmetrically localized. The follicular epithelium surrounds the oocyte. Signaling from the asymmetrically localized oocyte nucleus to the follicle cells leads to the repression of *pipe* coding for a sulfotransferase (*blue*). The follicle cells secrete the inner (vitelline membrane) and outer (chorion) eggshell. (*b*) Cross-section through a *Drosophila* blastoderm embryo surrounded by the vitelline membrane. The ventral vitelline membrane harbors proteins with sulfated polysaccharide side chains (Pipe domain, *blue*). Here, a proteolytic cascade is activated, which leads to the production of the Toll ligand C-Spätzle (C-Spz, *red*) within the perivitelline cleft. C-Spz binds to and activates the Toll receptor (*light blue*), a transmembrane protein evenly distributed in the plasma membrane of the embryo. Toll signaling leads to the nuclear import of Dorsal/NF-κB (*purple*).

polarity during oogenesis has produced one of the best understood patterning processes in all developmental biology. In particular, the gradient of the transcription factor (TF) Dorsal (Dl)/NF-κB, which is established in the early embryo in response to Toll signaling, became a paradigm for a morphogen controlling cell fates in a concentration-dependent manner (Roth et al. 1989, Rushlow et al. 1989, Schloop et al. 2020a, Steward 1989). This alone would provide an excellent justification for studying the evolution of DV patterning within insects. How did this complex multilayered network involving oogenesis, the eggshell, and the embryo evolve in the face of major changes in ovary types, eggshell ecology, and modes of embryonic development found in different insect lineages? However, there is an additional point of interest. More than a decade after the discovery of Toll's developmental function, Toll was shown to be involved in pathogen defense in flies (Lemaitre et al. 1996), humans (Medzhitov et al. 1997), and mice (Poltorak et al. 1998). These findings had far-reaching consequences for a molecular understanding of innate immunity in insects (Ferrandon et al. 2007) and mammals, including humans (Fitzgerald & Kagan 2020), and were awarded with the Nobel Prize in Physiology or Medicine in 2011 to Jules Hoffmann and Bruce Beutler (O'Neill et al. 2013). Subsequent work revealed that Toll's role in innate immunity is broadly conserved in metazoan phyla ranging from polyps to sea urchins and vertebrates (Brennan & Gilmore 2018, Nie et al. 2018). The DV function of Toll, however, is so far known only from insects.

In this review, I present data suggesting that the situation found in *Drosophila* is the result of an evolutionary process in which Toll became the dominant source of patterning information, mostly supplanting the role of the BMP system that ancestrally had a dominant function. This probably happened independently in different insect lineages and may have started from an ancestral immune function Toll provided to the insect egg and early embryo. Thus, Toll signaling offers an excellent case for analyzing the emergence of an evolutionary novelty. At the same time, Toll signaling is integrated into a patterning system that produces a constant phenotypic output and thus offers the opportunity to study the molecular details of developmental system drift, the divergence of developmental mechanisms that give rise to homologous characters (True & Haag 2001). Indeed, the output of DV patterning, the sequence of cell fates along the DV axis, is remarkably stable throughout insect evolution (Benton 2018, Roth 2004) (**Figure 2*a***). This is reflected in certain molecular features of the DV gene regulatory network (GRN): (*a*) Key target genes of Toll signaling in *Drosophila*, like the TFs Twist (Twi) and Single minded (Sim), have conserved roles in morphogenesis and cell type specification in all insects studied so far. (*b*) BMP signaling is conserved for patterning the dorsal side of insect embryos (Lynch & Roth 2011). In contrast to Toll signaling, BMP's role in DV axis formation is highly conserved even outside the insects and has been well characterized in spiders, planarians, echinoderms, and vertebrates (Bier & De Robertis 2015, Genikhovich et al. 2015). The conserved nature of BMP signaling outside insects suggests that Toll signaling was integrated into a BMP-based patterning system at the base of the hexapods.

Our current knowledge on the evolution of DV patterning in insects is focused on a small number of species for which sufficient functional data have been obtained to allow a mechanistic comparison with *Drosophila* (**Figure 2*b***). I start by reviewing some aspects of *Drosophila* DV patterning in light of what we have learned from the other insects.

DROSOPHILA: UNIPOLAR DV AXIS FORMATION WITH STRONG MATERNAL PREPATTERNING

The cell fates along the DV axis of blastoderm *Drosophila* embryos comprise the ventral mesoderm, which will invaginate during gastrulation, the ventrolateral neuroectoderm giving rise to both ventral epidermis and central nervous system, the dorsal non-neurogenic ectoderm, and the extraembryonic amnioserosa (**Figure 2*a***) (Hartenstein 1993).

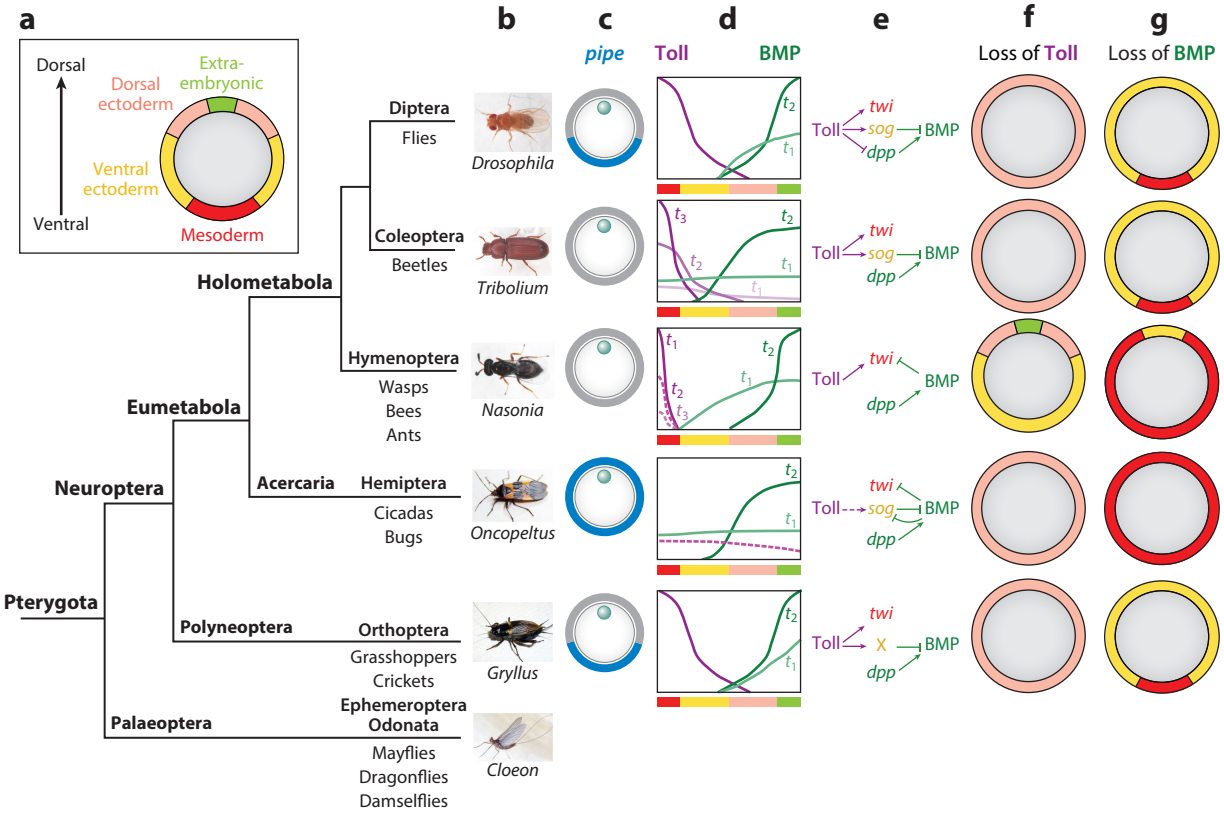

Figure 2

The evolution of dorsoventral (DV) patterning in insects. (*a*) Fate map of the dorsoventral axis. Cross-section through a blastoderm embryo. (*b*) The phylogenetic relationship for the species subject to detailed studies of DV patterning. Species icon for *Cloeon* is from **https://commons.wikimedia.org/w/index.php?curid=32720159**. (*c*) Cross-sections through egg chambers with the asymmetrically localized oocyte nucleus and *pipe* (*blue*) expression. (*d*) Diagrams showing the approximate distribution of Toll and BMP signaling along the DV axis at different time points indicated by t_1, t_2, and t_3. The data for Toll signaling in *Nasonia*, *Oncopeltus*, and *Gryllus* are based on indirect evidence. (*e*) Basic structure of gene regulatory networks. Arrows indicate positive and T-bars negative interactions. (*f*) The cross-sections through blastoderm embryos depicting fate map shifts upon loss of Toll signaling. (*g*) The cross-sections through blastoderm embryos depicting fate map shifts upon loss of BMP signaling. Panels *a*,*c*,*g* adapted from Pechmann et al. (2021). Panels *b*,*d*,*e* adapted from Sachs et al. (2015).

The establishment of this cell fate pattern can be traced back to the origin of DV polarity during mid-oogenesis when the oocyte nucleus migrates to an asymmetric position with regard to the anteroposterior (AP) axis of the egg chamber (**Figures 1** and **2*c*,*d***). Nuclear migration is apparently a symmetry breaking event as no spatial cues have been detected that influence its outcome (Roth & Lynch 2009). The asymmetric position of the nucleus determines the dorsal side of the egg and, later, embryo. The mRNA of *gurken* (*grk*) coding for TGFα, an EGF-type ligand, accumulates at the nucleus. From there, Grk protein is secreted and activates the EGF receptor in overlying follicle cells, which results in DV patterning of the follicular epithelium. The most important outcome of EGF signaling with regard to embryonic DV patterning is the repression of the gene *pipe*, whose transcription becomes restricted to the ventral 40% of the follicular epithelium (**Figures 1** and **2*c***) (Stein & Stevens 2014).

Pipe is a sulfotransferase that transfers sulfate groups to polysaccharide side chains present on several vitelline membrane (inner eggshell) proteins. The follicle cells secrete these proteins

during vitelline membrane formation (Stein & Stevens 2014). As a result, the ventral 40% of the vitelline membrane carries sulfated polysaccharide side chains. I refer to this eggshell region as the Pipe domain (**Figures 1** and **2*c***). The sulfated polysaccharides in this region recruit a complex of three serine proteases, which are secreted as inactive zymogens from the oocyte or embryo into the perivitelline cleft, a fluid-filled extraembryonic space bounded by the vitelline membrane and plasma membrane of the oocyte/embryo (**Figure 1**). The proteases engage in an activation cascade which leads to the cleavage of Spätzle protein releasing the active ligand of the Toll receptor, a C-terminal fragment of Spätzle (C-Spz) (**Figure 1**). This cascade requires the activity of Nudel (Ndl), which is a structural component of the vitelline membrane and carries a central serine protease domain required for self-cleavage. Ndl has been suggested to provide the appropriate environment for activating the proteolytic cascade (Stein & Stevens 2014).

The production of C-Spz presumably is confined to the Pipe domain and leads to a bell-shaped C-Spz profile with peak levels along the ventral midline and, in turn, to a corresponding profile of Toll receptor activation in the ventral half of the embryo (Stein & Stevens 2014) (**Figure 1**). Gradient formation of C-Spz has properties of a self-organizing process. If the Pipe domain is enlarged or if the amount of Spz is increased, DV pattern duplications are observed (Morisato 2001, Roth & Schupbach 1994). To account for these phenotypes, a shuttling mechanism for ventral C-Spz accumulation has been proposed (Haskel-Ittah et al. 2012).

Graded activation of Toll triggers a cascade of cytoplasmic signal transducers including the kinase Pelle, which phosphorylates Cactus (Cact)/I-κB and leads to its degradation (Daigneault et al. 2013). In absence of Toll signaling, Cact/I-κB binds to the TF Dl/NF-κB, preventing its transport into the nucleus. The resulting nuclear gradient of Dl/NF-κB appears to be a largely linear response to Toll activation (Stathopoulos & Levine 2002). By extension, the Dl/NF-κB nuclear gradient should broadly correspond to the extracellular gradient of C-Spz (**Figure 1**). Its final shape, however, is influenced by cytoplasmic diffusion of Dl/NF-κB, which is facilitated when Dl/NF-κB binds Cact/I-κB (Schloop et al. 2020b).

Dl/NF-κB acts as a concentration-dependent activator and repressor of zygotic target genes, which can be split into two groups (Reeves & Stathopoulos 2009) (**Figures 3*a*** and **2*d*,*e***). The first group comprises TFs and signaling components directly involved in specifying cell fates and controlling cell behavior within the ventral half of the embryonic circumference. Typical examples are the TFs *twist* (*twi*) and *snail* (*sna*) and components of Fog signaling (*fog*, *mist*) (Manning & Rogers 2014), which are involved in mesoderm specification and morphogenesis. The second group consists of components of the BMP signaling network, such as *decapentaplegic* (*dpp*) coding for a ligand of the BMP2/4 family and *short gastrulation* (*sog*), a homolog of vertebrate chordin acting as a secreted BMP inhibitor (gene names are framed in green in **Figure 3*a***). By regulating this second group of genes, Dl/NF-κB initiates the dynamic formation of a BMP signaling gradient with opposite polarity to that of Toll signaling, i.e., with peak levels along the dorsal midline. In turn, graded BMP signaling specifies the cell fates within the dorsal half the embryonic circumference (**Figure 3*a*** and **2*d*,*e***) (O'Connor et al. 2006).

Collectively, more than 50 Dl/NF-κB target genes have been identified, and the enhancer elements controlling their expression have been thoroughly analyzed, providing one of the most complete sets of regulatory elements for any morphogen system (Schloop et al. 2020a). The readout of the Dl/NF-κB gradient employs several distinct concentration thresholds for target gene control (**Figure 3*a***) that depend on the affinity of Dl/NF-κB binding sites present in the respective enhancers and on combinatorial binding of co-activators or co-repressors. At least six different regulatory codes have been described, accounting for the diverse expression domains of Dl/NF-κB target genes (Hong et al. 2008, Reeves & Stathopoulos 2009).

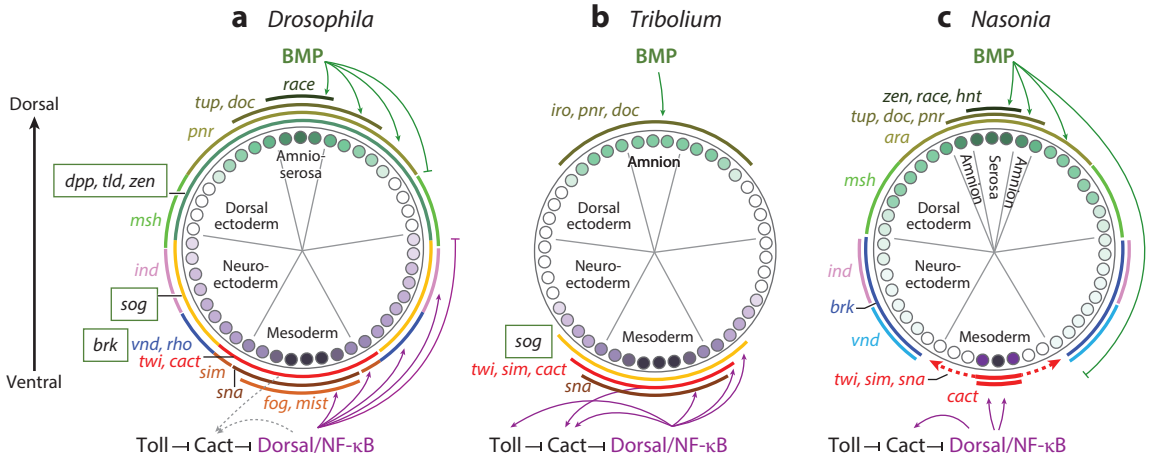

Figure 3

Target genes of Toll and BMP signaling in *Drosophila*, *Tribolium*, and *Nasonia*. Cross-sections through blastoderm embryos. Nuclear Dorsal/NF-κB with peak levels in ventral nuclei (*shades of purple*) reflects the strength of Toll signaling. Nuclear pMAD with peak levels in dorsal nuclei (*shades of green*) reflects the strength of BMP signaling (O'Connor et al. 2006). Gene expression domains are depicted by different colors. Arrows indicate activation and T-bars repression. (*a*,*b*) *Drosophila* and *Tribolium*: Toll target genes, which are components of the BMP signaling network, are framed (*green*). (*c*) *Nasonia:* Red arrows with dotted lines indicate that *twi*, *sim*, and *sna* expression expand laterally, starting from a narrow domain at the ventral midline.

Dl/NF-κB and its target genes form a GRN with extensive mutual interactions. For example, Twi binds to many ventrally active enhancers and acts synergistically with Dl/NF-κB, establishing a coherent feedforward loop (Sandmann et al. 2007). Through binding to its own enhancer, Twi initiates positive feedback, which widens the expression domain resulting from Dl/NF-κB inputs alone to establish the final mesodermal domain. Since Twi binds to the *sna* enhancer, Dl/NF-κB is only required to initiate early *sna* expression, which later can be maintained independently from Dl/NF-κB by Twi inputs alone (Irizarry et al. 2020).

The formation of the opposing BMP signaling gradient occurs via several parallel inputs of Dl/NF-κB (**Figure 3*a***). Besides *dpp*, *tolloid* (*tld*) coding for the protease cleaving Sog is ventrally repressed by Dl/NF-κB. Besides *sog*, *brinker* (*brk*) coding for a repressor of BMP target genes is ventrally activated by Dl/NF-κB. Brk acts in concert with secreted Sog to antagonize BMP signaling in the ventral neurogenic ectoderm (Jazwinska et al. 1999). In addition, Sog transports BMP ligands to the dorsal side of the embryo, where its cleavage by Tld releases the ligands and allows receptor binding (O'Connor et al. 2006, Wang & Ferguson 2005). This process generates a flat gradient, which initiates local autoactivation of BMP signaling at the dorsal side (**Figure 2*d***) (Gavin-Smyth et al. 2013, Wang & Ferguson 2005). Enhanced BMP signaling activates EGF signaling, providing negative feedback control (Deignan et al. 2016). Thus, the final profile of BMP signaling results from a subtle balance between positive and negative effects. High signaling levels are restricted to a narrow domain straddling the dorsal midline, with intermediate and low levels present in adjacent dorsolateral regions (**Figure 3*a***). High activity is required for specification of the amnioserosa, whereas low and intermediate activity suppress neuroectoderm specification and promote the development of dorsal epidermis.

Taken together, Toll signaling with peak levels along the ventral midline initiates a cascade of patterning processes that reliably produce peak levels of BMP signaling in a narrow stripe along the dorsal midline. This is a remarkable process of relaying spatial information from one pole of the body axis to the opposite pole. Despite some evidence that maternal BMP signaling influences

embryonic DV patterning in a Toll-independent manner (Araujo & Bier 2000), all available data suggest that in *Drosophila*, Toll signaling is the only source for DV patterning information in the embryo (**Figure 2*d–f***).

VARIATIONS OF OOGENESIS AND EMBRYOGENESIS IN INSECTS

Ovary types in insects are distinguished with regard to the presence (meroistic ovary: *Drosophila*, *Tribolium*, *Nasonia*, *Oncopeltus*) or absence (panoistic ovary: *Gryllus*) of nurse cells, which are connected with the oocyte by cytoplasmic bridges and provide the oocyte with cytoplasmic components including mRNAs and proteins. The meroistic ovaries are further subdivided into telotrophic (*Tribolium*, *Oncopeltus*) and polytrophic (*Drosophila*, *Nasonia*) types based on the arrangement of nurse cells relative to the oocyte (Büning 1994). These distinctions, however, have no major impact on the origin of DV polarity as asymmetric oocyte nucleus movement and EGF signaling to the overlaying follicle cells are conserved for all ovary types (Lynch et al. 2010) (**Figure 2*c***). Likewise, in all insects, the follicle cells surrounding the oocyte secrete an eggshell composed of an inner vitelline membrane and an outer chorion. The vitelline membrane closely abuts the embryo surface (Rezende et al. 2016) (**Figures 1** and **4**). As insects deposit their eggs into highly diverse environments, the eggshells show great morphological and biochemical variety allowing adaptation to different ecological niches (Church et al. 2019, Hilker & Meiners 2002, Hinton 1981, Rezende et al. 2016). In the face of this variety, it is remarkable that one of the proteins required for vitelline membrane integrity and production of the Toll ligand, the serine protease Ndl, is deeply conserved within insects (Pechmann et al. 2021).

Major steps of DV patterning occur during the uniform blastoderm stage. The fate map of different DV territories at this stage is broadly similar for all insects and corresponds to what has been previously mentioned for *Drosophila* (**Figure 2*a***), with the exception of the amnioserosa, a derived extraembryonic tissue, which is replaced by the amnion (**Figure 3*b***) or a combination of amnion and serosa (**Figure 3*c***) in most insects (Benton 2018, Roth 2004, Schmidt-Ott & Kwan 2022).

The uniform blastoderm gives rise to the differentiated blastoderm in many insects (**Figure 4**), a stage absent in *Drosophila*, which develops according to the long-germ type of embryogenesis (Roth 2004). During long-germ development, all segments along the AP axis are represented in the blastoderm fate map, and segmentation occurs simultaneously or in a progressive manner (Cheatle Jarvela et al. 2023, Clark et al. 2019). This embryogenesis type is a derived state found only in insects with complete metamorphosis (holometabola). Some holometabolous insects and all basally branching lineages with incomplete metamorphosis (hemimetabola) show short-germ development. At the blastoderm stage, only anterior segments (most frequently those from head and thorax) are specified while the posterior segments emerge from a posterior segment addition zone (Clark et al. 2019). Consequently, DV patterning also has two phases: blastoderm patterning, which can employ maternally provided spatial cues like in *Drosophila*, and DV patterning within the segment addition zone, which occurs in absence of such cues.

As short-germ insects specify only anterior segments at the blastoderm stage, they usually do not use the entire blastoderm surface for embryonic development. Variably sized regions of the anterior blastoderm may give rise to extraembryonic serosa (Roth 2004). The distinction between serosa and germ (or embryonic) rudiment, the region giving rise to the amnion and embryo proper, is frequently visible prior to gastrulation. The germ rudiment condenses to form a columnar epithelium (**Figure 4**). Concomitantly, the future serosa cells stop dividing, increase their DNA content by endoreduplication, and flatten to produce a squamous epithelium (Benton et al. 2019, Handel et al. 2000). This stage is referred to as differentiated blastoderm. The serosal cells are the earliest cells to become terminally specified in many insect embryos (Roth 2004).

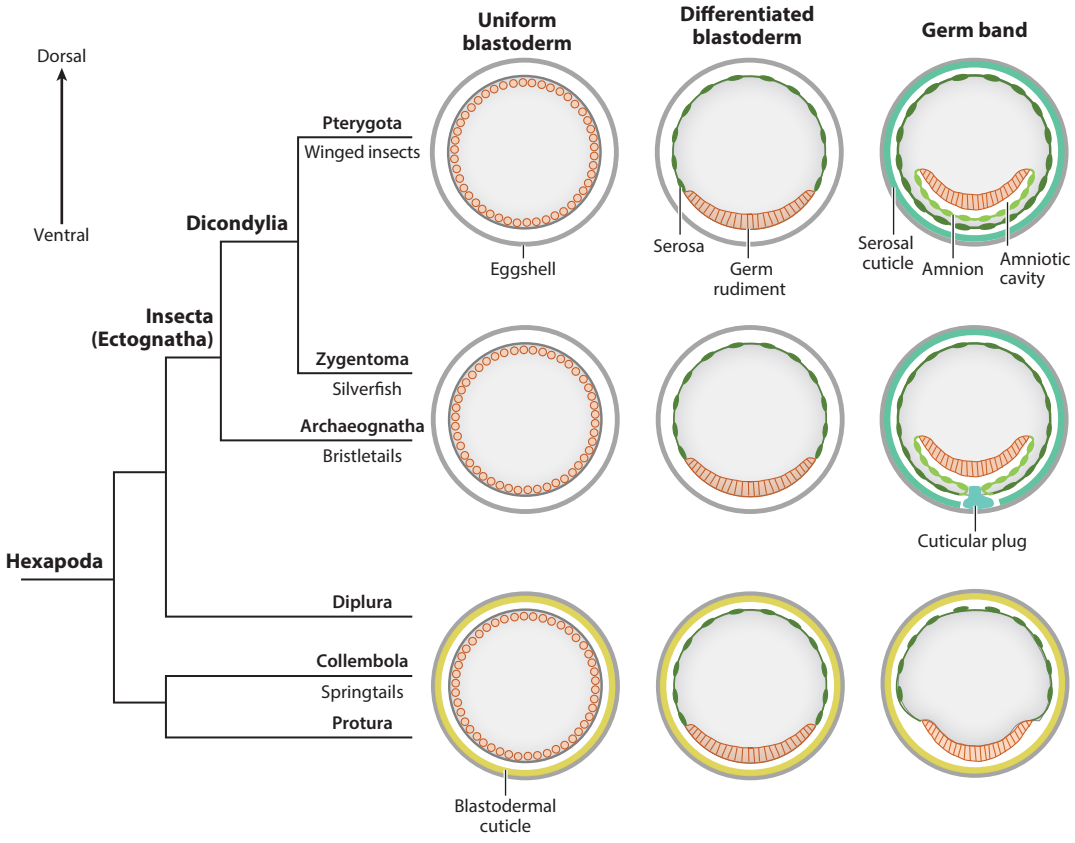

Figure 4

Embryonic cuticle formation and the evolutionary history of extraembryonic membranes in hexapods. While non-insect hexapods form a blastodermal cuticle, insects produce a serosal cuticle. The insect serosa has gained the capacity to fold over the embryo and, in winged insects (Pterygota), gives rise to a continuous serosal epithelium enveloping the entire egg. The sister groups of the winged insects (Zygentoma and Archaeognatha) show variability in ventral closure of the serosa. Pterygota secrete a continuous serosal cuticle, while Zygentoma and Archaeognatha often have a gap in the serosal cuticle that is closed by a cuticular plug.

During gastrulation in most insects, serosa and amnion cells fold over the embryo at the ventral side to create an amniotic cavity (**Figure 4**). When the cavity closes, serosa and amnion become separate epithelia, with the serosa covering the entire egg and embryo (Panfilio 2008).

TRIBOLIUM: DYNAMIC TOLL SIGNALING WITH WEAK MATERNAL PREPATTERNING

With a wide range of functional tools, the red flour beetle *Tribolium castaneum* has become the second-best model for studying insect development (Klingler & Bucher 2022). Apart from *Drosophila*, *Tribolium* is the only insect model for which unbiased genome-wide RNAi screens have been performed (Schmitt-Engel et al. 2015). Although the Coleoptera are typical holometabolous insects, *Tribolium* shares many features with basal hemimetabolous lineages, like a serosa derived from a large anterior region of the blastoderm and short germ development (Handel et al. 2000). Despite these more ancestral features, functional studies of Toll and BMP signaling show that the basic regulatory logic of DV axis formation in *Tribolium* is similar to *Drosophila* (**Figure 2*e–g***). Like in *Drosophila*, Toll is required along the entire DV axis of the *Tribolium* embryo, where it

also polarizes BMP signaling (da Fonseca et al. 2008). Likewise, in both insects, lack of BMP signaling leads to an expansion of the neuroectoderm, while the mesoderm is largely unaffected. Its specification relies predominantly on Toll signaling (**Figure 2*f*,*g***) (van der Zee et al. 2006).

Yet the way Toll signaling is polarized and how it affects embryonic patterning in *Tribolium* appear to be very different from *Drosophila*. Major components of the Toll signaling cascade are not provided as uniformly distributed maternal mRNAs like in *Drosophila*. They are zygotically expressed in the embryo and subject to feedback control by Toll signaling (Chen et al. 2000, da Fonseca et al. 2008, Maxton-Küchenmeister et al. 1999). This applies to the Toll receptor itself, which initially shows uniform zygotic expression but later becomes ventrally up-regulated due to positive feedback by Toll signaling (**Figure 3*b***). Negative feedback results from *Toll*- and *twi*-dependent activation of *cact/I-κB*. The resulting combination of positive and negative feedback control is likely to contribute to the spatiotemporal dynamics of Toll signaling in *Tribolium* as reflected by the nuclear accumulation Dl/NF-κB (Chen et al. 2000) (**Figure 2*d***). During early blastoderm stages Dl/NF-κB accumulates at low levels in nuclei around the entire embryonic circumference. Subsequently, the nuclear Dl/NF-κB levels increase ventrally, and a dynamic gradient forms whose ventral peak levels rise while its lateral range declines.

DV target genes of Toll signaling, like *twi*, *sim*, and *sog*, are conserved between *Drosophila* and *Tribolium* (**Figure 3*b***) (Stappert et al. 2016). In contrast to *Drosophila*, *Tribolium sna* activation is entirely dependent on *twi*, and the homologs of the *Drosophila* Toll target genes *rho*, *brk*, *vnd*, and *ind* lack early expression in *Tribolium* and thus cannot be subject to direct control by Toll (Rousso et al. 2010, van der Zee et al. 2006, Wheeler et al. 2005). Furthermore, Dl/NF-κB apparently does not act as a repressor like in *Drosophila*. For instance, *dpp* and *tld* are not repressed by Dl/NF-κB like in *Drosophila* (da Fonseca et al. 2010). Although a global transcriptome analysis revealed potential Toll target genes in *Tribolium* with no counterpart in *Drosophila*, the available data suggest that the number of genes directly controlled by Toll is considerably smaller in *Tribolium* than in *Drosophila* (Stappert et al. 2016). In addition, there is no clear evidence that the *Tribolium* Dl/NF-κB gradient controls its target genes by multiple thresholds like the *Drosophila* gradient. As *twi* expression is nested within the *sog* domain in *Tribolium* (da Fonseca et al. 2008), it is possible that *twi* activation requires higher levels of Dl/NF-κB than does *sog*. However, given the dynamics of the *Tribolium* Dl/NF-κB gradient, it could be that Toll signaling employs only one spatially shifting concentration threshold and hence should not be classified as a typical morphogen.

As Dl/NF-κB nuclear accumulation starts uniformly or with very weak DV asymmetry (**Figure 2*d***), we assume only weakly polarized spatial cues are needed to initiate Toll signaling. Toll activation depends on Spz and, as mentioned earlier, on Ndl (Muhammad 2018). However, *pipe* is not expressed in the follicular epithelium, suggesting that the proteolytic cascade leading to Spz processing uses eggshell cues different from those in *Drosophila* (Pechmann et al. 2021) (**Figure 2*c***). As locally activated EGF signaling can be detected in the follicle cells overlying the asymmetrically localized oocyte nucleus, asymmetric eggshell cues are likely present also in *Tribolium* (Lynch et al. 2010). The mRNA of TGFα is evenly distributed in the oocyte without accumulation at the oocyte nucleus, and cellular mechanisms of localized signaling are unknown. Although EGF signaling provides apparently only a weak asymmetry, knockdown (KD) experiments show that this asymmetry is crucial for axis formation. Strikingly, DV cell fates are duplicated along the DV axis, or even arranged along the AP axis in absence of EGF signaling, supporting the idea of self-organization at the level of Toll signaling (Lynch et al. 2010).

In contrast to *Drosophila*, BMP signaling in *Tribolium* does not refine to a narrow peak along the dorsal midline during blastoderm stages (**Figure 3*b***). Rather, BMP activity is found in a broad dorsal domain (da Fonseca et al. 2010, van der Zee et al. 2006). How does Toll polarize BMP signaling in *Tribolium*? Both *dpp* and *tld* are uniformly expressed along the DV axis, and *brk* is not

present in early embryos. Sog is the only known component of the BMP signaling system with localized Toll-dependent expression (**Figure 3*b***). Indeed, the loss of *sog* results in a complete lack of ectodermal DV polarity, associated with a loss of the entire ventral nervous system like in *brk sog* double mutants of *Drosophila* (Jazwinska et al. 1999, van der Zee et al. 2006). Thus, in *Tribolium*, Toll-dependent activation of *sog* is the major, if not the only, way that BMP polarity is generated.

The fact that the columnar genes *vnd*, *ind*, and *msh* begin to be expressed after the Dl/NF-κB gradient has already disappeared suggests that the interplay of EGF signaling and BMP signaling has a greater role for neural ectodermal patterning in *Tribolium* compared with *Drosophila* (Chen et al. 2000, Rousso et al. 2010, Wheeler et al. 2005). The main role of Toll signaling in *Tribolium* appears to be the ventral activation of *twi*, *sim*, and *sog* (**Figure 3*b***). Toll's direct patterning range is therefore more restricted to the ventral side while that of BMP is expanded relative to *Drosophila*. Moreover, the network of BMP signaling components is not dependent on multiple inputs from Toll signaling compared with *Drosophila*.

NASONIA: BIPOLAR DV AXIS FORMATION WITH DRASTICALLY REDUCED ROLE OF TOLL SIGNALING

The jewel wasp *Nasonia vitripennis*, representing the taxon Hymenoptera, belongs to a lineage that split from the remainder of the holometabolous insects more than 300 million years ago (Misof et al. 2014) (**Figure 2*b***). Analyzing development of *Nasonia* has the promise to uncover features of GRNs present in the last common ancestor of the Holometabola. *Nasonia* and other members of the Apocrita (ants, bees and wasps) like the honey bee *Apis mellifera* are also interesting since they possess a mode of long germ embryogenesis similar to, but independently derived from, that of *Drosophila* (El-Sherif et al. 2012, Schmidt-Ott & Lynch 2016). This convergence offers the possibility to study how different evolutionary and developmental trajectories lead to similar phenotypic outputs.

A comprehensive comparative analysis of the expression of DV patterning genes from *Nasonia* and *Drosophila* reveals striking similarities at the cellular blastoderm stage shortly before gastrulation (**Figure 3*c***). However, the way the expression patterns emerge and the subsequent events during gastrulation are very different (Buchta et al. 2013). The expression of ventral genes like *twi*, *sna*, and *sim* is initiated in a discrete narrow stripe straddling the ventral midline (5% of the embryonic circumference) and only later expands to form the final domain spanning 20% of the embryonic circumference (**Figure 3*c***). This is clearly different from both *Drosophila* and *Tribolium*, where the final pattern is not the result of expanding a narrow, well-defined stripe. Simultaneously with the narrow expression domains along the ventral midline, a number of genes are expressed in narrow domains along the dorsal midline (**Figure 3*c***). While the former are typical Toll targets in *Tribolium* and *Drosophila*, the latter genes like *zen*, *race*, *hindsight* (*hnt*), *dorsocross* (*doc*), and *tailup* (*tup*) are typical BMP targets in *Drosophila*. These observations pose the question of how refined spatial information can arise at the same time at both poles of the DV axis in the *Nasonia* embryo.

The KD of Toll and BMP signaling components provides some answers. In contrast to *Drosophila* and *Tribolium*, KD of Toll in *Nasonia* does not lead to a complete loss of DV polarity (Özüak et al. 2014b). Indeed, the embryos lacking Toll signaling are highly polarized (**Figure 2*f***). Only ventral genes are affected while patterning at the dorsal side of the embryo remains intact, implying the existence of a dorsally localized Toll-independent patterning system.

As expected, dorsal patterning requires BMP. Surprisingly, BMP is also essential for delimiting the expression of ventral genes (**Figures 2*g*** and **3*c***). The loss of dorsally expressed genes upon BMP KD is accompanied by a massive expansion of *twi* expression, resulting in largely mesodermalized embryos (Özüak et al. 2014a,b). Thus, in *Nasonia*, mesoderm specification is not solely dependent on Toll signaling but requires repressive inputs from BMP. However, embryos lacking

both Toll and BMP signaling are devoid of mesoderm, demonstrating that *Toll* is necessary for mesoderm specification even in the absence of BMP.

Gene expression dynamics of the Toll target gene *cact* suggest that Toll signaling in *Nasonia* is restricted to a narrow region along the ventral midline (**Figures 2*d*** and **3*c***). Here, it is required to initiate the expression of *twi* and other early target genes like *sna* and *sim*. A positive feedback loop of one or more of these early Toll target genes drives the expansion of the expression domains. BMP signaling emanating from the dorsal side counteracts this positive feedback loop and thereby determines the expression boundaries (**Figures 2*d*,*e*** and **3*c***). Thus, in *Nasonia*, Toll signaling apparently does not act as a morphogen but rather acts as a local inducer while BMP provides long-range positional information for all target genes along the DV axis. Indeed, BMP signaling initially forms a broad, shallow gradient, which expands to the ventral side. Subsequently, the gradient narrows, becomes steeper, and finally reaches peak levels in a narrow domain along the dorsal midline (**Figures 2*d*** and **3*c***).

The BMP signaling dynamics thus has similarity to that in *Drosophila* (**Figure 2*d***). However, since Toll is not required for BMP polarity in *Nasonia*, the mechanism initiating these dynamics cannot be the same. This idea is in line with the observation that *Nasonia* lacks a Sog/chordin homolog, the BMP inhibitor highly conserved throughout Eumetazoa and the most important Toll target gene for polarizing BMP signaling in *Tribolium* and *Drosophila* (Özüak et al. 2014a,b). What, then, could be the source for BMP polarity? One possibility is to postulate maternal cues provided during oogenesis in a Toll-independent manner.

Like in all insects studied so far, the asymmetrically localized oocyte nucleus defines the dorsal side of the egg chamber in *Nasonia* and polarizes EGF signaling from the oocyte to the follicle cells (Lynch et al. 2010, Özüak et al. 2014b) (**Figure 2*c***). Asymmetric EGF signaling, in turn, is required for normal DV patterning in *Nasonia*. Like in *Drosophila*, but unlike *Tribolium*, polarization of EGF signaling occurs via localization of the mRNA of the EGF ligand. However, *Nasonia* and another hymenopteran species (*Apis mellifera*) have evolved the capacity of localizing mRNA not just to the oocyte nucleus, but rather in a highly refined pattern: a narrow strip along the dorsal midline of the oocyte (Lynch et al. 2010, Wilson et al. 2011). Consequently, EGF signaling occurs in a corresponding dorsal stripe of overlying follicle cells. Dorsally localized EGF signaling is required to confine Toll signaling to the ventral side of the embryo (Özüak et al. 2014b). How this happens at the molecular level is not known since, like in *Tribolium*, *pipe* is not expressed in the follicular epithelium in *Nasonia* (and *Apis*) (Pechmann et al. 2021, Pers et al. 2022, Wilson et al. 2014) (**Figure 2*c***).

Interestingly, BMP signaling is not affected upon loss of ovarian EGF signaling, suggesting that EGF only controls Toll signaling (Özüak et al. 2014b). What, then, could provide the polarity information for BMP signaling? In both *Apis* and *Nasonia*, the mRNAs of BMP components are localized in a dorsal stripe in the oocyte like TGFα mRNA. In *Nasonia*, this applies to the mRNA of the of the type I BMP receptor *thickveins* (Özüak et al. 2014a), and in *Apis* to the mRNA of the ligand *dpp* (Wilson et al. 2011). The proteins produced by these mRNAs might remain dorsally anchored, resulting in polarized BMP signaling in the early embryo. Four BMP signaling components are zygotically expressed in the dorsal half of the *Nasonia* blastoderm embryo, including the BMP type II receptor *punt 1*, the membrane anchored BMP modulator *cv2a*, and the secreted BMP modulators *tsg2* and *ltl* (Pers et al. 2016). This strongly suggests that BMP signaling forms a self-regulatory network sensitive to polarizing maternal inputs in *Nasonia*.

Taken together, current evidence suggests that in contrast to all other insects studied so far, *Nasonia* possesses a bipolar DV patterning system, which depends on mRNAs localized to the dorsal side of the oocyte, which act either directly on BMP at the dorsal side or indirectly (via EGF signaling to the follicular epithelium) on Toll at the ventral side of the embryo. Thus, the

amount of spatial information for DV axis formation provided during oogenesis in *Nasonia* is even higher than in *Drosophila* since both the embryonic dorsal and ventral midline are specified by maternal inputs and this occurs via a highly precise mechanism of mRNA localization.

A global transcriptome analysis has identified more than 100 genes differentially expressed along the DV axis in *Nasonia* with only a small number being shared between fly and wasp despite the striking similarity of the blastoderm fate map (Pers et al. 2016). Many of the divergent components are cytoskeletal and adhesion molecules likely to be related to the divergent cell and tissue behavior during gastrulation. However, TFs and signaling components were also identified that are only DV regulated in *Nasonia*, indicating major differences between the GRNs of both insects. Three differentially expressed genes showed unexpected responses upon BMP and Toll KD, indicating that they are targets of both signaling pathways. This regulatory behavior might be a remnant from an earlier GRN in which Toll provided polarity along the entire DV axis.

The BMP and Toll target genes also include a set of 15 novel genes that are characterized by the presence of ankyrin domains and lack orthologs outside of the wasp superfamily Chalcidoidea. These CLANK genes (Chalcidoidea Lineage specific ANKyrin domain-encoding genes) presumably originated by horizontal gene transfer from a prokaryote in a common ancestor of chalcid wasps (Pers & Lynch 2018). Interestingly, they have been functionally integrated into the DV GRN as their loss impairs gene expression along the DV axis. Thus, the CLANKs provide a stunning example of how the DV GRN can evolve robustness by integrating new elements.

ONCOPELTUS: TOLL AS A POLARITY CUE FOR SELF-ORGANIZING BMP SIGNALING

The analysis of DV GRNs in the three holometabolous insects studied so far has shown that in species more distantly related to *Drosophila*, the function of Toll signaling becomes less complex and more spatially restricted while the role of BMP expands along the DV axis. While in all three holometabolous insects Toll remains strictly required for mesoderm specification, in hemimetabolous insects this restriction no longer applies.

The first hemimetabolous insects subject to functional studies of DV patterning were the milkweed bug, *Oncopeltus fasciatus*, and the kissing bug, *Rhodnius prolixus* (Berni et al. 2014, Sachs et al. 2015). These species belong to the Hemiptera, a taxon within the Acercaria (or Paraneoptera), which represents the sister group to the Holometabola (Misof et al. 2014) (**Figure 2*b***). The Acercaria share a number of derived reproductive features with the Holometabola, like meroistic ovaries and the formation of large germ rudiments (Büning 1994, Roth 2004). However, all species exhibit short germ segmentation and an ancestral (katatreptic) mode of extraembryonic membrane formation (Panfilio 2008).

In contrast to *Nasonia*, Toll signaling is required in *Oncopeltus* to establish polarity along the entire DV axis as loss of Toll leads to completely apolar embryos that lack all ventral structures and show uniform BMP signaling around the DV circumference (**Figure 2*f***) (Sachs et al. 2015). This might indicate that Toll's role is more relevant for DV patterning in *Oncopeltus* than in *Nasonia*. However, the analysis of BMP signaling components reveals a different picture. Like in *Drosophila* and *Tribolium*, the ventrally expressed BMP inhibitor Sog is an important functional part of the *Oncopeltus* DV patterning system (**Figure 2*e***). Indeed, the loss of *sog* in *Oncopeltus* leads to completely dorsalized embryos, which also lack the mesoderm and are indistinguishable from embryos lacking Toll signaling (Sachs et al. 2015). Conversely, loss of BMP signaling leads to completely ventralized embryos in which mesodermal genes are expressed evenly around the DV circumference (**Figure 2*g***). Uniform expression of mesodermal genes is established instantaneously and is not the result of the progressive expansion of a ventrally restricted domain like in *Nasonia*. Thus, *Oncopeltus* represents the first case of an insect in which both loss of Toll and loss of BMP

signaling lead to completely apolar embryos with opposite, uniformly dorsalized and ventralized phenotypes, respectively. *Oncopeltus* embryos lacking both Toll and BMP signaling also show an expansion of the mesoderm, revealing that even the most ventral cell fates of the DV axis can be established in absence of Toll signaling (Sachs et al. 2015). A similar phenotype has recently been observed for *Rhodnius prolixus* (Berni et al. 2023), a hemipteran species whose lineage split from that of *Oncopeltus* approximately 250 million years ago (Johnson et al. 2018), suggesting that the ability of mesoderm formation in the absence of Toll signaling is broadly conserved within the Heteroptera, one of the major branches of the Hemiptera. Considering that all cell fates along the DV axis can form in absence of Toll signaling, what, then, is the role of Toll in *Oncopeltus*?

In *Oncopeltus*, BMP signaling is a potent negative regulator of *sog* transcription (**Figure 2*e***). This interaction establishes a double negative feedback loop: BMP signaling represses the transcription of its own inhibitor, Sog, and thus is self-amplifying. In early blastoderm stages, *dpp* and *sog* are activated uniformly in a Toll-independent manner. Weakly graded *cact* transcription along the DV axis indicates that Toll signaling forms a shallow gradient spanning the entire embryonic circumference (Sachs et al. 2015) (**Figure 2*d***). Toll enhances *sog* expression at the ventral side. We assume that this leads both to ventral repression of BMP signaling and to a flux of BMP-Sog complexes to the dorsal side, where Sog cleavage (by Tolloid) releases BMP and increases BMP signaling. Since BMP signaling represses *sog* expression, the asymmetry initiated by Toll is dynamically enhanced, leading to a stable patterning output (**Figure 2*d*,*e***). According to this scenario, Toll signaling provides only polarity information for a self-organizing BMP signaling network, which is responsible for patterning the embryonic DV axis.

Theoretical modeling confirms that this network has self-regulatory properties and is largely independent from the shape of Toll signaling inputs, which may display only minute DV asymmetries (Sachs et al. 2015). Moreover, the patterning output shows almost perfect scaling as the sizes of the *sog* and BMP signaling domains are adjusted to the dimensions of the egg. Given the scaling behavior, the model can account for one of the famous experiments from classical insect embryology: embryonic twinning after egg fragmentation in the leaf hopper *Euscelis incisus* (formerly *plebejus*) (Cicadomorpha, Hemiptera) (Sander 1971).

Direct or indirect repressive inputs of BMP signaling on *sog* represent a highly conserved regulatory motif. The *sog* ortholog *chordin* is negatively controlled by BMP signaling during DV axis formation in many animal phyla, including sea anemones (Cnidaria), spiders, hemichordates, and vertebrates (Akiyama-Oda & Oda 2006, Genikhovich et al. 2015, Inomata et al. 2013, Lowe et al. 2006). This negative feedback is crucial for size regulation and scaling but frequently requires additional components (Inomata et al. 2013). The *Oncopeltus* DV system appears to represent a minimal network able to produce scaled pattern outputs.

The pattern of BMP signaling established in blastoderm *Oncopeltus* embryos appears to be very simple, composed of a plateau of high signaling at the dorsal side and a broad region lacking detectable BMP signaling at the ventral side (**Figure 2*d***). Thus, there is little fine-grained spatial information at the blastoderm stage compared with the holometabolous insects studied so far. We have to assume that all further subdivision along the DV axis occurs at later stages. Accordingly, so far, no genes have been identified that show narrow expression domains at the dorsal side of blastoderm embryos. Laterally expressed genes like *vnd*, *ind*, and *msh* show stripe-like expression only after gastrulation in extending germband embryos (Chen 2015, Sachs et al. 2015).

What are the ovarian polarity cues that provide asymmetry to Toll signaling in *Oncopeltus*? Given that *pipe* plays no role in the ovary of *Tribolium* and *Nasonia*, it comes as a surprise that *pipe* is expressed in the follicular epithelium and is required for DV patterning in *Oncopeltus* (Chen 2015, Pechmann et al. 2021). This suggests that the Pipe-dependent way *Drosophila* polarizes Toll signaling represents an ancient mechanism predating the arrival of holometabolous

development. Interestingly, *pipe* seems to be uniformly expressed in the follicular epithelium of *Oncopeltus* (**Figure 2*c***). However, a weak asymmetry correlated with the asymmetrically localized oocyte nucleus cannot be excluded. The observation of broad *pipe* expression is in line with evidence for shallow Toll signaling gradients in the early *Oncopeltus* embryo.

In summary, *Oncopeltus* DV patterning employs Toll neither as a signaling gradient with direct, concentration-dependent patterning functions (*Drosophila* and *Tribolium*) nor as a local inducer of ventral cell fates (*Nasonia*), but only as a polarity cue for a BMP signaling network with regulatory properties known from DV patterning in other animals. Thus, Toll might have been integrated into a largely autonomous BMP-based patterning system in order to orient the future embryonic axis with regard to the egg axis. As broadly active Toll signaling would suffice to provide polarity information, this situation might have evolved from a state in which uniform Toll signaling was associated with protecting insect eggs against pathogens (see below).

CONVERGENT FUNCTIONS OF TOLL IN THE CRICKET *GRYLLUS BIMACULATUS*

The mainly BMP-based system of *Oncopeltus* resembles DV patterning in other animal lineages and thus might be close to the ancestral state for DV patterning in insects. To explore this possibility, other insect lineages have to be studied. The Acercaria, to which *Oncopeltus* belongs, together with the Holometabola, form one branch (Eumetabola) of the Neoptera. The cricket *Gryllus bimaculatus* (Orthoptera) is a representative of the other large neopteran branch, the highly divers Polyneoptera (Wipfler et al. 2019) (**Figure 2*b***). The Polyneoptera have retained many ancestral morphological and developmental traits, like the panoistic ovary and small germ rudiments, which form by extensive nuclear or cell migration at the surface of large, yolk-rich eggs (Büning 1994, Donoughe et al. 2022, Nakamura et al. 2010, Roth 2004).

In *Gryllus*, DV polarity depends on Toll signaling like in *Oncopeltus*, *Tribolium*, and *Drosophila* (Pechmann et al. 2021). Loss of Toll signaling leads to apolar, dorsalized embryos (**Figure 2*f***), and germ rudiment condensation occurs symmetrically at the posterior pole, resulting in rotationally symmetric germ rudiments, which lack all ventral cell fates and show uniform high levels of BMP signaling. In contrast, embryos lacking BMP signaling retain considerable polarity (**Figure 2*g***). Indeed, ventral and ventrolateral cell fates are not affected. Mesoderm and mesectoderm specification appear to be normal (Pechmann et al. 2021). Only the dorsal half of the embryo has lost polarity due to a loss of dorsal and an even expansion of lateral cell fates. This phenotype is similar to that observed in *Tribolium* and *Drosophila* upon loss of BMP signaling (**Figure 2*g***) and indicates that in *Gryllus*, Toll specifies and positions ventral cell fates without contribution of BMP signaling. Indeed, the BMP activity gradient is restricted to the dorsal half of uniform blastoderm embryos and has a fairly steep profile compared with *Oncopeltus*, again more similar to *Drosophila* (Pechmann et al. 2021).

Another surprise highlighting similarities to *Drosophila* relates to oogenesis. Although *Gryllus* possesses the ancestral panoistic ovary type, *pipe* is expressed in the follicular epithelium like in *Oncopeltus*. However, different from *Oncopeltus*, *pipe* expression is clearly polarized, being repressed in follicle cells closer to the oocyte nucleus (Pechmann et al. 2021) (**Figure 2*c***). Like in *Drosophila*, *pipe* repression depends on EGF signaling. The clear asymmetry of *pipe* expression underscores the assumption that the DV system of *Gryllus* provides a higher degree of maternal patterning information to the embryo compared with *Oncopeltus*. Overall, the *Gryllus* DV system exhibits striking similarities to *Drosophila*. Toll signaling in *Gryllus* may well act as a morphogen, which specifies ventral and ventrolateral cell fates in a concentration-dependent manner (**Figure 2*d*,*e***). The similarity to *Drosophila* can be explained by two evolutionary scenarios: Either Toll's patterning function in *Gryllus* and *Drosophila* is the result of convergent evolution or a *Drosophila*-like system

arose early in insect evolution and was extensively altered in independent lineages including shifts from Toll-based to BMP-based patterning (Pechmann et al. 2021).

A number of molecular and developmental aspects strongly favor the scenario of convergent evolution. First, the establishment of polar BMP signaling is clearly dependent on Toll in *Gryllus*, implying that Toll directly or indirectly controls components of the BMP network. However, the mechanism is largely obscure since *Gryllus* has lost the BMP inhibitor Sog and apparently uses a derived lineage-restricted mechanism of BMP polarization (Pechmann et al. 2021). Second, comparative embryology has uncovered features of early polyneopteran development, which represent derived states (apomorphies) found neither in basally branching wingless (Archaeognatha and Zygentoma) and palaeopteran (Odonata and Ephemeroptera) insects (**Figures 2*b***, **4**) nor in many Acercaria (Wipfler et al. 2019).

After considering this evidence, we currently assume that the increased role of Toll in *Gryllus* is the result of independent evolution. To gain more support for this assumption, a broader species sampling is required. Recently, the mayfly (Ephemeroptera) *Cloeon dipterum* was established as a lab model, which now allows the study of a representative of the Palaeoptera, the basal-most branch of the winged insects (Almudi et al. 2019) (**Figure 2*b***). As an outgroup to the winged insects, the zygentoman firebrat, *Thermobia domestica* offers a promising experimental system (Hughes et al. 2004, Ohde et al. 2018) (**Figure 4**).

INNATE IMMUNITY AND THE EVOLUTION OF DV PATTERNING IN INSECTS

With the exception of *Gryllus*, the analysis of DV axis formation in insects suggests that in species more distantly related to *Drosophila*, Toll's direct patterning role decreases while BMP signaling becomes more relevant. Outside the insects, so far no DV patterning role of Toll has been reported. Thus, it is conceivable that Toll signaling was integrated into an ancestral BMP-based patterning system during early insect or hexapod evolution. What was Toll's function prior to its recruitment for DV signaling? In *Drosophila*, nine Toll receptors are involved in a variety of developmental processes, including signaling during cell competition and cell adhesion during germband elongation and compartment boundary maintenance (Anthoney et al. 2018, Umetsu 2022). Thus, Toll's role in DV patterning might be derived from a prior developmental function in the ovary or early embryo. Of all nine Toll receptors, it is, however, Toll-1 that governs most of the immune responses as well as DV patterning (Ferrandon et al. 2007). This association appears to hold also for the other insects we have studied (Benton et al. 2016). Thus, the link between immunity and patterning has been stable throughout considerable parts of insect evolution, although Toll receptor diversity would allow for subfunctionalization. I therefore suggest that the recruitment of Toll for DV patterning represents a case of neofunctionalization where the developmental function was co-opted from a prior role in immunity. Extraembryonic development in insects provides some hints of how this might have occurred.

The segregation between the serosa and germ rudiment is the first cell type distinction during embryogenesis in most insects, with the presumptive serosa covering typically a large part of the egg surface (Roth 2004) (**Figure 4**). After gastrulation, the serosa covers the entire yolk and embryo (**Figure 4**) and fulfills a number of protective functions for the egg. With few exceptions, the serosa produces a cuticle, which supplements the eggshell in providing mechanical stability and regulating fluid and gas exchange with the environment (Jacobs et al. 2013, Rezende et al. 2016).

In addition, the serosa has been shown to protect the egg against pathogens following an early observation in *Tribolium* (Chen et al. 2000). Infection of control and serosa-less eggs shows that the serosa up-regulates a wide range of immune genes and provides resistance to pathogens (Jacobs et al. 2014, Jacobs & van der Zee 2013). Similar experiments in *Drosophila*, which lacks a serosa,

reveal that *Drosophila* eggs are unable to mount an immune response. The spectrum of genes induced in the serosa upon infection in *Tribolium* is similar to that of larval and adult immune responses and includes components of Toll- and IMD signaling, the melanization cascade, production of reactive oxygen species, and antimicrobial peptides (AMPs). The analysis also suggests that a number of immune genes like Dl/NF-κB are constitutively active in the serosa.

These studies were extended to *Oncopeltus* and gave similar results, including the up-regulation of Toll signaling components and AMPs in the serosa (Jacobs et al. 2022). Finally, the grasshopper *Locusta migratoria* also constitutively expresses Dl/NF-κB in the serosa and induces AMP expression in the serosa upon immune challenge (Jacobs et al. 2022). In summary, these studies together with findings in the lepidopteran *Manduca sexta* (Gorman et al. 2004, Shan et al. 2022) suggest that serosal immune competence is an ancestral feature of insects.

The discovery of serosal immunity brings the immune and patterning functions of Toll in close temporal proximity. For example, in *Tribolium*, Dl/NF-κB is up-regulated in the presumptive serosa cells while its embryonic function is just being turned down (Chen et al. 2000). The temporal closeness by itself poses an interesting regulatory problem. How can Toll target genes with Dl/NF-κB binding sites distinguish inputs derived from patterning or immune signals, which are active at adjacent or overlapping developmental stages? The temporal closeness of Toll's patterning and immune function might also cause evolutionary trade-offs: An expanded role in patterning may favor a reduced immune function, with *Drosophila* representing an extreme example of a highly complex patterning and a totally absent immune function of Toll in the early embryo. In contrast, an expanded and earlier immune function might be encountered in embryos in which Toll's patterning role is strongly reduced or absent (*a*) because of secondary loss or (*b*) because they represent ancestral lineages prior to the recruitment of Toll for DV patterning.

The second alternative is of particular significance for understanding the origin of Toll's patterning role. It would be interesting to identify examples of basal insects or non-insect hexapods, which ancestrally lack Toll's DV function. How do they polarize their DV axis, and how is the embryonic axis related to the egg axis? Are there cases in which the egg lacks bilateral symmetry typical for pterygote insects? Finally, when does the embryo show immune competence? The question of timing is crucial to explain the functional change from immunity to patterning. In insects studied so far, serosa formation and the activation of immune genes do not precede, but rather follow, the processes of DV axis formation for which Toll is required. How, then, could Toll's patterning function be derived from an immune function? Three possibilities can be considered. (*a*) A heterochronic shift could lead to an earlier expression of Toll signaling components. A transition from serosal to blastoderm expression would provide the opportunity for Toll signaling to have an impact on target genes involved in DV patterning like *twist* and *sog*. Heterochrony has been widely used to explain how changes in the timing of developmental events may result in new phenotypes and new links in GRNs (Dobreva et al. 2022). In our case, an existing BMP-based DV patterning system would gain more robustness by being anchored to the eggshell through a signaling process (Toll) that senses egg polarity and thus allows an early establishment of bilateral symmetry. (*b*) Maternal provisioning might supply the egg with components for pathogen defense prior to the initiation of embryonic development and serosa formation. In some extant insects, immune components or signals that activate immune genes in the early embryo are transferred maternally to the egg. This happens during transgenerational immune priming (Vilcinskas 2021). For example, in the beetle *Tenebrio molitor*, mothers provision the egg with immune-related proteins like AMPs, resulting in increased immune protection of the egg (Tetreau et al. 2020). Likewise, ancestral hexapods might have supplied their eggs maternally with Toll signaling components. Constitutive maternal Toll signaling might have resulted in AMP secretion into the perivitelline cleft, providing protection against pathogens at the earliest stages of embryonic

development. Maternal Toll signaling could then be co-opted to control DV patterning genes. A weak polarization of signals emerging from the eggshell would yield the selective advantage of a more stable alignment of egg and embryonic axes. (*c*) The evolutionary history of the serosa and its physiological roles suggests yet another scenario, explained in more detail below.

During hexapod evolution, the ability to form a continuous serosal epithelium enveloping the entire egg emerged at the base of the winged insects (Pterygota). At this point in evolution, insect embryos gained the capacity to secrete a continuous serosal cuticle, which could mechanically strengthen and physiologically modulate the maternally produced eggshell (Panfilio 2008, Rezende et al. 2016) (**Figure 4**). The sister groups of the winged insects (Zygentoma and Archaeognatha) show variability in ventral closure of the serosa (Masumoto & Machida 2006). The formation of the serosal cuticle in these species is complemented by a cuticular plug closing the ventral gap and highlighting the importance of cuticular continuity (**Figure 4**). Outside the insects, the non-insect hexapods (Diplura, Collembola, and Protura) lack the abilities of serosal folding over the embryo and cuticle secretion by serosal cells (Rezende et al. 2016). However, they also possess an embryo-derived cuticle underneath the eggshell, which provides desiccation resistance (Tomizuka & Machida 2015, Vargas et al. 2021). Interestingly, this cuticle is produced by cells of the uniform blastoderm and hence is called the blastodermal cuticle (**Figure 4**). Cuticle production by blastodermal cells is a counterintuitive phenomenon as the hallmark of the blastoderm is its undifferentiated state. In order to secrete a cuticle, blastodermal cells have transiently to acquire properties of terminally differentiated cells like those of the serosa or the epidermis. However, blastodermal cuticle production is not unique to non-insect hexapods, but it has been described for many arthropod groups and was likely a crucial factor for the transition from aquatic to terrestrial lifestyles (Rezende et al. 2016).

If blastoderm cells are able to undergo a reversible transition to cuticle formation, it is conceivable that they transiently acquire the other characteristic of serosal or body wall cells: immune competence. I suggest that blastoderm cells of non-insect hexapods secreted not only a cuticle but also immune related components like AMPs. For this purpose, they utilized immune signaling pathways including Toll signaling. With the evolutionary transition to insects proper (Ectognatha = Archaeognatha, Zygentoma, and Pterygota), the serosa emerged as an epithelium, which took over cuticle formation and protection against microbes. Hence, blastoderm cells were released from the transient burden of activating GRNs of late differentiated states. At the same time, components of immune signaling that had been active in the blastoderm could be integrated into early developmental GRNs, providing the opportunity for an inroad of Toll signaling into DV axis formation. This third scenario for neofunctionalization of Toll is particularly appealing because it combines features of scenarios one and two. Blastodermal cuticle secretion linked to the capacity of pathogen defense is the result of a heterochronic shift and, like maternal provisioning, has the potential to supply Toll signaling components prior to embryonic patterning. An analysis of the immune competence and early DV gene expression in embryos from non-insect hexapods will constitute a first step for analyzing the proposed scenario.

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LITERATURE CITED

Akiyama-Oda Y, Oda H. 2006. Axis specification in the spider embryo: *dpp* is required for radial-to-axial symmetry transformation and *sog* for ventral patterning. *Development* 133:2347–57

Almudi I, Martin-Blanco CA, Garcia-Fernandez IM, Lopez-Catalina A, Davie K, Aerts S, Casares F. 2019. Establishment of the mayfly *Cloeon dipterum* as a new model system to investigate insect evolution. *EvoDevo* 10:6

Anderson KV, Bokla L, Nüsslein-Volhard C. 1985a. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: the induction of polarity by the Toll gene product. *Cell* 42:791–98

Anderson KV, Jurgens G, Nüsslein-Volhard C. 1985b. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the Toll gene product. *Cell* 42:779–89

Anderson KV, Nüsslein-Volhard C. 1984. Information for the dorsal-ventral pattern of the *Drosophila* embryo is stored as maternal mRNA. *Nature* 311:223–27

Anthoney N, Foldi I, Hidalgo A. 2018. Toll and Toll-like receptor signalling in development. *Development* 145(9):dev156018

Araujo H, Bier E. 2000. *sog* and *dpp* exert opposing maternal functions to modify Toll signaling and pattern the dorsoventral axis of the *Drosophila* embryo. *Development* 127:3631–44

Benton MA. 2018. A revised understanding of *Tribolium* morphogenesis further reconciles short and long germ development. *PLOS Biol.* 16:e2005093

Benton MA, Frey N, Nunes da Fonseca R, von Levetzow C, Stappert D, et al. 2019. Fog signaling has diverse roles in epithelial morphogenesis in insects. *eLife* 8:e47346

Benton MA, Pechmann M, Frey N, Stappert D, Conrads KH, et al. 2016. Toll genes have an ancestral role in axis elongation. *Curr. Biol.* 26:1609–15

Berni M, Fontenele MR, Tobias-Santos V, Caceres-Rodrigues A, Mury FB, et al. 2014. Toll signals regulate dorsal-ventral patterning and anterior-posterior placement of the embryo in the hemipteran *Rhodnius prolixus*. *EvoDevo* 5:38

Berni M, Mota J, Bressan D, Ribeiro L, Martins G, et al. 2023. A pro-BMP function exerted by *Rhodnius prolixus* Short gastrulation reveals great diversity in the role of BMP modulators during embryonic development. *Open Biol.* 13:230023

Bier E, De Robertis EM. 2015. BMP gradients: a paradigm for morphogen-mediated developmental patterning. *Science* 348:aaa5838

Brennan JJ, Gilmore TD. 2018. Evolutionary origins of Toll-like receptor signaling. *Mol. Biol. Evol.* 35:1576–87

Buchta T, Özüak O, Stappert D, Roth S, Lynch JA. 2013. Patterning the dorsal–ventral axis of the wasp *Nasonia vitripennis*. *Dev. Biol.* 381:189–202

Büning J. 1994. *The Insect Ovary*. New York: Springer

Cheatle Jarvela AM, Trelstad CS, Pick L. 2023. Anterior-posterior patterning of segments in *Anopheles stephensi* offers insights into the transition from sequential to simultaneous segmentation in holometabolous insects. *J. Exp. Zool. B Mol. Dev. Evol.* 340:116–30

Chen G, Handel K, Roth S. 2000. The maternal NF-κB/dorsal gradient of *Tribolium castaneum*: dynamics of early dorsoventral patterning in a short-germ beetle. *Development* 127:5145–56

Chen Y-T. 2015. *The role of Toll signaling in dorsoventral axis formation in the milkweed bug Oncopeltus fasciatus*. PhD Thesis, Univ. Cologne, Cologne, Ger.

Church SH, Donoughe S, de Medeiros BAS, Extavour CG. 2019. Insect egg size and shape evolve with ecology but not developmental rate. *Nature* 571:58–62

Clark E, Peel AD, Akam M. 2019. Arthropod segmentation. *Development* 146(18):dev170480

da Fonseca RN, van der Zee M, Roth S. 2010. Evolution of extracellular Dpp modulators in insects: the roles of *tolloid* and *twisted-gastrulation* in dorsoventral patterning of the *Tribolium* embryo. *Dev. Biol.* 345:80–93

da Fonseca RN, von Levetzow C, Kalscheuer P, Basal A, van der Zee M, Roth S. 2008. Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle *Tribolium castaneum*. *Dev. Cell* 14:605–15

Daigneault J, Klemetsaune L, Wasserman SA. 2013. The IRAK homolog Pelle is the functional counterpart of IκB kinase in the *Drosophila* Toll pathway. *PLOS ONE* 8:e75150

Deignan L, Pinheiro MT, Sutcliffe C, Saunders A, Wilcockson SG, et al. 2016. Regulation of the BMP signaling-responsive transcriptional network in the *Drosophila* embryo. *PLOS Genet.* 12:e1006164

Dobreva MP, Camacho J, Abzhanov A. 2022. Time to synchronize our clocks: Connecting developmental mechanisms and evolutionary consequences of heterochrony. *J. Exp. Zool. B Mol. Dev. Evol.* 338:87–106
Donoughe S, Hoffmann J, Nakamura T, Rycroft CH, Extavour CG. 2022. Nuclear speed and cycle length co-vary with local density during syncytial blastoderm formation in a cricket. *Nat. Commun.* 13:3889
El-Sherif E, Lynch JA, Brown SJ. 2012. Comparisons of the embryonic development of *Drosophila*, *Nasonia*, and *Tribolium*. *Wiley Interdiscip. Rev. Dev. Biol.* 1:16–39
Ferrandon D, Imler JL, Hetru C, Hoffmann JA. 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat. Rev. Immunol.* 7:862–74
Fitzgerald KA, Kagan JC. 2020. Toll-like receptors and the control of immunity. *Cell* 180:1044–66
Gavin-Smyth J, Wang YC, Butler I, Ferguson EL. 2013. A genetic network conferring canalization to a bistable patterning system in *Drosophila*. *Curr. Biol.* 23:2296–302
Genikhovich G, Fried P, Prunster MM, Schinko JB, Gilles AF, et al. 2015. Axis patterning by BMPs: cnidarian network reveals evolutionary constraints. *Cell Rep.* 10(10):1646–54
Gorman MJ, Kankanala P, Kanost MR. 2004. Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. *Insect Mol. Biol.* 13:19–24
Handel K, Grünfelder CG, Roth S, Sander K. 2000. *Tribolium* embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. *Dev. Genes Evol.* 210:167–79
Hartenstein V. 1993. *Atlas of Drosophila Development*. Cold Spring Harbor, NY: Cold Spring Harb. Lab. Press
Haskel-Ittah M, Ben-Zvi D, Branski-Arieli M, Schejter ED, Shilo BZ, Barkai N. 2012. Self-organized shuttling: generating sharp dorsoventral polarity in the early *Drosophila* embryo. *Cell* 150:1016–28
Hilker M, Meiners T, eds. 2002. *Chemoecology of Insect Eggs and Egg Deposition*. Oxford, UK: Blackwell
Hinton HE. 1981. *The Biology of Insect Eggs*. Oxford, UK: Pergamon
Hong JW, Hendrix DA, Papatsenko D, Levine MS. 2008. How the Dorsal gradient works: insights from postgenome technologies. *PNAS* 105:20072–76
Hughes CL, Liu PZ, Kaufman TC. 2004. Expression patterns of the rogue Hox genes *Hox3/zen* and *fushi tarazu* in the apterygote insect *Thermobia domestica*. *Evol. Dev.* 6:393–401
Inomata H, Shibata T, Haraguchi T, Sasai Y. 2013. Scaling of dorsal-ventral patterning by embryo size-dependent degradation of Spemann's organizer signals. *Cell* 153:1296–311
Irizarry J, McGehee J, Kim G, Stein D, Stathopoulos A. 2020. Twist-dependent ratchet functioning downstream from Dorsal revealed using a light-inducible degron. *Genes Dev.* 34:965–72
Jacobs CG, Rezende GL, Lamers GE, van der Zee M. 2013. The extraembryonic serosa protects the insect egg against desiccation. *Proc. Biol. Sci.* 280:20131082
Jacobs CG, Spaink HP, van der Zee M. 2014. The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. *eLife* 3:e04111
Jacobs CGC, van der Hulst R, Chen YT, Williamson RP, Roth S, van der Zee M. 2022. Immune function of the serosa in hemimetabolous insect eggs. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 377:20210266
Jacobs CGC, van der Zee M. 2013. Immune competence in insect eggs depends on the extraembryonic serosa. *Dev. Comp. Immunol.* 41:263–69
Jazwinska A, Rushlow C, Roth S. 1999. The role of *brinker* in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* 126:3323–34
Johnson KP, Dietrich CH, Friedrich F, Beutel RG, Wipfler B, et al. 2018. Phylogenomics and the evolution of hemipteroid insects. *PNAS* 115:12775–80
Klingler M, Bucher G. 2022. The red flour beetle *T. castaneum*: elaborate genetic toolkit and unbiased large scale RNAi screening to study insect biology and evolution. *EvoDevo* 13:14
Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* 86:973–83
Lowe CJ, Terasaki M, Wu M, Freeman RM Jr., Runft L, et al. 2006. Dorsoventral patterning in hemichordates: insights into early chordate evolution. *PLOS Biol.* 4:e291
Lynch JA, Peel AD, Drechsler A, Averof M, Roth S. 2010. EGF signaling and the origin of axial polarity among the insects. *Curr. Biol.* 20:1042–47
Lynch JA, Roth S. 2011. The evolution of dorsal-ventral patterning mechanisms in insects. *Genes Dev.* 25:107–18

Manning AJ, Rogers SL. 2014. The Fog signaling pathway: insights into signaling in morphogenesis. *Dev. Biol.* 394:6–14

Masumoto M, Machida R. 2006. Development of embryonic membranes in the silverfish *Lepisma saccharina* linnaeus (insecta: Zygentoma, Lepismatidae). *Tissue Cell* 38:159–69

Maxton-Küchenmeister J, Handel K, Schmidt-Ott U, Roth S, Jäckle H. 1999. Toll homolog expression in the beetle *Tribolium* suggests a different mode of dorsoventral patterning than in *Drosophila* embryos. *Mech. Dev.* 83:107–14

Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394–97

Misof B, Liu S, Meusemann K, Peters RS, Donath A, et al. 2014. Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346:763–67

Morisato D. 2001. Spätzle regulates the shape of the Dorsal gradient in the *Drosophila* embryo. *Development* 128:2309–19

Muhammad MSD. 2018. *Large scale RNAi screen in Tribolium reveals novel genes involved in dorsoventral pattern formation*. PhD Thesis, Univ. Cologne, Cologne, Ger.

Nakamura T, Yoshizaki M, Ogawa S, Okamoto H, Shinmyo Y, et al. 2010. Imaging of transgenic cricket embryos reveals cell movements consistent with a syncytial patterning mechanism. *Curr. Biol.* 20:1641–47

Nie L, Cai SY, Shao JZ, Chen J. 2018. Toll-like receptors, associated biological roles, and signaling networks in non-mammals. *Front. Immunol.* 9:1523

Nüsslein-Volhard C. 2022. The Toll gene in *Drosophila* pattern formation. *Trends Genet.* 38:231–45

O'Connor MB, Umulis D, Othmer HG, Blair SS. 2006. Shaping BMP morphogen gradients in the *Drosophila* embryo and pupal wing. *Development* 133:183–93

Ohde T, Takehana Y, Shiotsuki T, Niimi T. 2018. CRISPR/Cas9-based heritable targeted mutagenesis in *Thermobia domestica*: a genetic tool in an apterygote development model of wing evolution. *Arthropod Struct. Dev.* 47:362–69

O'Neill LA, Golenbock D, Bowie AG. 2013. The history of Toll-like receptors—redefining innate immunity. *Nat. Rev. Immunol.* 13:453–60

Özüak O, Buchta T, Roth S, Lynch JA. 2014a. Ancient and diverged TGF-β signaling components in *Nasonia vitripennis*. *Dev. Genes Evol.* 224:223–33

Özüak O, Buchta T, Roth S, Lynch JA. 2014b. Dorsoventral polarity of the *Nasonia* embryo primarily relies on a BMP gradient formed without input from Toll. *Curr. Biol.* 24:2393–98

Panfilio KA. 2008. Extraembryonic development in insects and the acrobatics of blastokinesis. *Dev. Biol.* 313:471–91

Pechmann M, Kenny NJ, Pott L, Heger P, Chen YT, et al. 2021. Striking parallels between dorsoventral patterning in *Drosophila* and *Gryllus* reveal a complex evolutionary history behind a model gene regulatory network. *eLife* 10:e68287

Pers D, Buchta T, Özüak O, Roth S, Lynch JA. 2022. Expression and function of Toll pathway components in the early development of the wasp *Nasonia vitripennis*. *J. Dev. Biol.* 10(1):7

Pers D, Buchta T, Özüak O, Wolff S, Pietsch JM, et al. 2016. Global analysis of dorsoventral patterning in the wasp *Nasonia* reveals extensive incorporation of novelty in a regulatory network. *BMC Biol.* 14:63

Pers D, Lynch JA. 2018. Ankyrin domain encoding genes from an ancient horizontal transfer are functionally integrated into *Nasonia* developmental gene regulatory networks. *Genome Biol.* 19:148

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 282:2085–88

Reeves GT, Stathopoulos A. 2009. Graded dorsal and differential gene regulation in the *Drosophila* embryo. *Cold Spring Harb. Perspect. Biol.* 1:a000836

Rezende GL, Vargas HCM, Moussian B, Cohen E. 2016. Composite eggshell matrices: chorionic layers and sub-chorionic cuticular envelopes. In *Extracellular Composite Matrices in Arthropods*, ed. E Cohen, B Moussian, pp. 325–66. New York: Springer

Roth S. 2004. Gastrulation in other insects. In *Gastrulation: From Cells to Embryos*, ed. C Stern, pp. 105–21. Cold Spring, NY: Cold Spring Harb. Lab. Press

Roth S, Lynch JA. 2009. Symmetry breaking during *Drosophila* oogenesis. *Cold Spring Harb. Perspect. Biol.* 1:a001891

Roth S, Schupbach T. 1994. The relationship between ovarian and embryonic dorsoventral patterning in *Drosophila*. *Development* 120:2245–57
Roth S, Stein D, Nüsslein-Volhard C. 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* 59:1189–202
Rousso T, Lynch J, Yogev S, Roth S, Schejter ED, Shilo B-Z. 2010. Generation of distinct signaling modes via diversification of the Egfr ligand-processing cassette. *Development* 137:3427–37
Rushlow CA, Han K, Manley JL, Levine M. 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell* 59:1165–77
Sachs L, Chen Y-T, Drechsler A, Lynch JA, Panfilio KA, et al. 2015. Dynamic BMP signaling polarized by Toll patterns the dorsoventral axis in a hemimetabolous insect. *eLife* 4:e05502
Sander K. 1971. Pattern formation in longitudinal halves of leaf hopper eggs (Homoptera) and some remarks on the definition of "embryonic regulation." *Wilhelm Roux Arch. Entwickl. Mech.* 167:336–52
Sandmann T, Girardot C, Brehme M, Tongprasit W, Stolc V, Furlong EE. 2007. A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev.* 21:436–49
Schloop AE, Bandodkar PU, Reeves GT. 2020a. Formation, interpretation, and regulation of the *Drosophila* Dorsal/NF-κB gradient. *Curr. Top. Dev. Biol.* 137:143–91
Schloop AE, Carrell-Noel S, Friedman J, Thomas A, Reeves GT. 2020b. Mechanism and implications of morphogen shuttling: lessons learned from Dorsal and Cactus in *Drosophila*. *Dev. Biol.* 461:13–18
Schmidt-Ott U, Kwan CW. 2022. How two extraembryonic epithelia became one: serosa and amnion features and functions of *Drosophila*'s amnioserosa. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 377:20210265
Schmidt-Ott U, Lynch JA. 2016. Emerging developmental genetic model systems in holometabolous insects. *Curr. Opin. Genet. Dev.* 39:116–28
Schmitt-Engel C, Schultheis D, Schwirz J, Strohlein N, Troelenberg N, et al. 2015. The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. *Nat. Commun.* 6:7822
Shan T, Wang Y, Dittmer NT, Kanost MR, Jiang H. 2022. Serine protease networks mediate immune responses in extra-embryonic tissues of eggs in the tobacco hornworm, *Manduca sexta*. *J. Innate Immun.* 15:365–79
Stappert D, Frey N, von Levetzow C, Roth S. 2016. Genome-wide identification of *Tribolium* dorsoventral patterning genes. *Development* 143:2443–54
Stathopoulos A, Levine M. 2002. Linear signaling in the Toll-Dorsal pathway of *Drosophila*: activated Pelle kinase specifies all threshold outputs of gene expression while the bHLH protein Twist specifies a subset. *Development* 129:3411–19
Stein DS, Stevens LM. 2014. Maternal control of the *Drosophila* dorsal-ventral body axis. *Wiley Interdiscip. Rev. Dev. Biol.* 3:301–30
Steward R. 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* 59:1179–88
Tetreau G, Dhinaut J, Galinier R, Audant-Lacour P, Voisin SN, et al. 2020. Deciphering the molecular mechanisms of mother-to-egg immune protection in the mealworm beetle *Tenebrio molitor*. *PLOS Pathog.* 16:e1008935
Tomizuka S, Machida R. 2015. Embryonic development of a collembolan, *Tomocerus cuspidatus* Börner 1909: with special reference to the development and developmental potential of serosa (Hexapoda: Collembola, Tomoceridae). *Arthropod Struct. Dev.* 44:157–72
True JR, Haag ES. 2001. Developmental system drift and flexibility in evolutionary trajectories. *Evol. Dev.* 3:109–19
Umetsu D. 2022. Cell mechanics and cell-cell recognition controls by Toll-like receptors in tissue morphogenesis and homeostasis. *Fly* 16:233–47
van der Zee M, Stockhammer O, von Levetzow C, da Fonseca RN, Roth S. 2006. Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. *PNAS* 103:16307–12
Vargas HCM, Panfilio KA, Roelofs D, Rezende GL. 2021. Increase in egg resistance to desiccation in springtails correlates with blastodermal cuticle formation: eco-evolutionary implications for insect terrestrialization. *J. Exp. Zool. B. Mol. Dev. Evol.* 336:606–19
Vilcinskas A. 2021. Mechanisms of transgenerational immune priming in insects. *Dev. Comp. Immunol.* 124:104205

Wang YC, Ferguson EL. 2005. Spatial bistability of Dpp-receptor interactions during *Drosophila* dorsal-ventral patterning. *Nature* 434:229–34

Wheeler SR, Carrico ML, Wilson BA, Skeath JB. 2005. The *Tribolium* columnar genes reveal conservation and plasticity in neural precursor patterning along the embryonic dorsal-ventral axis. *Dev. Biol.* 279:491–500

Wilson MJ, Abbott H, Dearden PK. 2011. The evolution of oocyte patterning in insects: multiple cell-signaling pathways are active during honeybee oogenesis and are likely to play a role in axis patterning. *Evol. Dev.* 13:127–37

Wilson MJ, Kenny NJ, Dearden PK. 2014. Components of the dorsal-ventral pathway also contribute to anterior-posterior patterning in honeybee embryos (*Apis mellifera*). *EvoDevo* 5:11

Wipfler B, Letsch H, Frandsen PB, Kapli P, Mayer C, et al. 2019. Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. *PNAS* 116:3024–29