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Segmentation gene expression patterns in *Bactrocera dorsalis* and related insects: regulation and shape of blastoderm and larval cuticle

WORRAMIN SUKSUWAN[#], XIAOLI CAI, LERTLUK NGERNSIRI[#] and STEFAN BAUMGARTNER*

Dept. of Experimental Medical Sciences, Lund University, Lund, Sweden

ABSTRACT The oriental fruit fly, *Bactrocera dorsalis*, is regarded as a severe pest of fruit production in Asia. Despite its economic importance, only limited information regarding the molecular and developmental biology of this insect is known to date. We provide a detailed analysis of *B. dorsalis* embryology, as well as the expression patterns of a number of segmentation genes known to act during patterning of *Drosophila* and compare these to the patterns of other insect families. An anterior shift of the expression of gap genes was detected when compared to *Drosophila*. This shift was largely restored during the step where the gap genes control expression of the pair-rule genes. We analyzed and compared the shapes of the embryos of insects of different families, *B. dorsalis* and the blow fly *Lucilia sericata* with that of the well-characterized *Drosophila melanogaster*. We found distinct shapes as well as differences in the ratios of the length of the anterior-posterior axis and the dorsal-ventral axis. These features were integrated into a profile of how the expression patterns of the gap gene *Krüppel* and the pair-rule gene *even-skipped* were observed along the A-P axis in three insects families. Since significant differences were observed, we discuss how *Krüppel* controls the *even-skipped* stripes. Furthermore, we discuss how the position and angles of the segmentation gene stripes differed from other insects. Finally, we analyzed the outcome of the expression patterns of the late acting segment polarity genes in relation to the anlagen of the naked-cuticle and denticle belt area of the *B. dorsalis* larva.

KEY WORDS: *Bactrocera dorsalis, segmentation, Kruppel, even-skipped*

The oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: *Tephritidae*) is a major cause of damage of fruit production in tropical and sub-tropical regions, often leading to a total failure of crop production (Vargas *et al*., 2015). It has in fact been classified as belonging to the most destructive category of insects (category A pests) (Vargas *et al*., 2015). *B. dorsalis* is widely prevalent in many countries in Asia, but also in the U.S. state of Hawaii. Since accidental introduction in the 1940's, *B. dorsalis* has established itself as a common pest. The insects have also occasionally been detected in the U. S. mainland, e. g. in California and Florida. Attempts have been made to eradicate the pest during four major infestations between 1960 and 1997. In Asia, eradication was not possible for monetary reasons initially. Consequently, damage on crop production was so severe that eradication programs were ultimately implemented to combat this insect.

Phylogenetically, *Tephritidae* is a family of fruit flies, located immediately adjacent of that of *Drosophilidae* (Yong *et al*., 2016). Both families belong to the sub-section *Acalyptratae*, and are referred to as "fruit flies", while the *Drosophilidae* are often referred to as "the common fruit fly". To make *Tephritidae* more distinct from *Drosophilidae*, they are often called "peacock flies". Both families maintain a similar body shape, however, *Tephritidae* are usually larger than *Drosophilidae* and are often more colorful with pictured wings. The third family that will be described below is *Calliphoridae*, a family that is phylogenetically even closer to *Tephritidae* than to *Drosophilidae* (Andere *et al*., 2016). Within the *Calliphoridae*, we will focus on the blow fly *Lucilia sericata*.

Abbreviations used in this paper: En, engrailed; Eve, even-skipped; Hb, hunchback; Kr, Krüppel; otd, orthodenticle.

***Address correspondence to:** Stefan Baumgartner. Dept. of Experimental Medical Sciences, Lund University, BMC D10, S-22184 Lund, Sweden. e-mail: Stefan.Baumgartner@med.lu.se

[#]Present address: Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, Thailand.

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Within the family of *Tephritidae*, several other insect members have been described to some detail such as *Ceratitis capitata* (the Mediterranean fruit fly) (Scolari *et al*., 2014) and *B. oleae* (the olive fly; Mavragani-Tsipidou, 2002). Common to this family is that all three fly species represent the most destructive of fruit pests. *Ceratitis capitata*, in particular, because of its wide distribution over the world, its ability to tolerate cooler climates better than most other species of tropical fruit flies and its wide range of hosts is ranked first among economically important fruit fly species.

The body of the adult *B. dorsalis* is about 8 mm in length, with a wingspan of about 7 mm. Its body color is quite variable: the thorax is mainly dark brown with yellow stripes, while the abdomen is light brown with two black stripes perpendicular to the anterior-posterior (A-P) axis, and a black stripe along the midline. More than 150 kinds of fruits and vegetables are known to be attacked by *B. dorsalis*. Mango, papaya and avocado are the preferred food sources (Leblanc *et al*., 2012).

Embryogenesis and the formation of segments in insects follows two modes: in short-germband development, segments are added sequentially by adding them to the posterior of a growing embryo (reviewed by Davis and Patel, 2002). The blastoderm embryo occupies only a small fraction of the egg (the remainder consists of yolk and extra-embryonic tissue). Anterior segments are already determined during the blastoderm stage, while posterior segments are added only after gastrulation. In contrast, in insects using long-germband development (where *B. dorsalis* belongs to), the body axis is established already during oogenesis and segments are formed through subdividing the embryo into equally-sized subdomains (Davis and Patel, 2002). Most long-germband embryos take up a large proportion of the egg, and segments are already determined before gastrulation begins. No tissue growth is involved in this process. The morphological formation of segments occurs much later in development; segmental boundaries are visible only at the extended germband stage.

In *Drosophila*, the process of segmentation was described in detail and revealed that at the molecular level, a hierarchy of segmentation genes was crucial for setting up the anterior-posterior axis (Peel *et al*., 2005). Since segmentation is the common denominator of all insects, attempts have been made to integrate segmentation gene expression patterns of different insects into gene network models to detect common modes of regulation and to explain differences that were observed. The best defined gene regulation model is the gap-gene network model analyzed in dipteran insects (Crombach *et al*., 2016, Jaeger, 2011) which states that early activation and placement of gap gene expression domains show significant quantitative differences, yet the final patterning output of the system, i. e. the expression of pair-rule genes, is essentially identical in the species investigated, referred to as " system drift" (Crombach *et al*., 2016). However, information is still limited in regard to comparative gene expression sets in insect families which would allow to test this network model.

An important question is how do the gap genes regulate the pair-rules genes and is this regulation conserved between insect families? For the former question, some progress was made, mainly in *Drosophila*, due to the availability of mutants and the possibility of manipulating this insect. Major focus was on the gap genes *Krüppel*, *hunchback*, *giant* and *knirps*, and how these genes regulated the different stripes of the pair-rule gene *even-skipped* (Goto *et al*., 1989; Harding *et al*., 1989; Stanojevic *et al*., 1991; Small *et al*., 1996). These reports extended our understanding how the interplay of gap genes control the different *even-skipped* stripes, and defined an initial set of transcriptional activators and repressors for each of the *even-skipped* stripe enhancers. For example, *even-skipped* stripe 2 appeared because it was activated by the *bicoid* and *hunchback* gradients, but repressed by *giant* anteriorly to its margin and by *Krüppel* posteriorly to its margin, giving rise to a 4-cell-wide expression of *even-skipped* stripe 2 (Small *et al*., 1996). To further understanding as to whether or not *even-skipped* enhancers were conserved between insects families, *even–skipped* enhancers were identified in scavenger flies, *Sepsidae* (Hare *et al*., 2008), but the sequence identity of the enhancers to those of *Drosophila* was unexpectedly low. This report demonstrated that the identification of regulatory sequence by simple sequence comparison in other insects would not be successful, unless yet-unestablished functional studies would be performed as well. To circumvent this issue, *in silico* approaches were developed to model the transcription of *Drosophila even-skipped* stripe 2 (Ilsley *et al*., 2013). To date, the only method to reliably detect similarities or differences of how gap genes activate the pair-rule genes in different insect families is to compare the relative positions of gap genes and pair-rule gene stripes along the AP-axis and to determine whether or not a particular gap gene can exert control on a particular pair-rule gene stripe.

As far as the analyses of expression patterns of segmentation genes in *Tephritidae* is concerned, only limited information is available. One report identified plasticity in expression with *orthodenticle* (*otd*), an early segmentation gene in the medfly, *Ceratitis capitata* and the caribfly, *Anastrepha suspensa* (Schetelig *et al*., 2008), suggesting that changes in *otd* expression can occur even in closely related taxa. Another report showed that the maternal gene *nanos* of the medfly was expressed in a similar pattern as *Drosophila* forming a posterior mRNA gradient, but not identifying plasticity between the two families (Ogaugwu and Wimmer, 2013).

Despite its significant impact on the world-wide fruit market, little is known about the molecular and developmental biology of *B. dorsalis*. Recently, some progress was made in developing genomic resources for this species after the complete sequencing of the genome and some detailed analysis (Calla and Geib, 2015; Geib *et al*., 2014; Sim *et al*., 2015). In addition, RNA sequencing of the transcriptome of genes involved in sexual maturation and mating led to the identification of important genes involved in the sexual development of the *B. dorsalis* female, which consequently may help to develop sterility programs (Zheng *et al*., 2016). Concurrently, a draft sequence of a relative of *B. dorsalis*, *B. tryoni*, was made available in 2014 (Gilchrist *et al*., 2014). However, only limited information on the embryology and developmental biology of *Bactrocera* is available, with the exception of a superficial description of the biology of *B. tau* (Singh, 2010) and that of another *Tephritid*, *B. tryoni* (Anderson, 1964).

To supplement the information currently available in describing *B. dorsalis* embryology, we have characterized its embryonic development in detail. We describe the embryonic development of this species, present a panel of expression patterns of early segmentation genes and compare these to the know expression patterns of other established model insects. To address the question whether or not the control of the pair-rule genes by the gap genes is conserved between three insect families, we superimposed the expression patterns of the gap gene *Krüppel* to those of the pair-

rule gene *even-skipped* and draw conclusions how *Krüppel* can control the *even-skipped* stripes in the different insect families. We also noted that the angle and behavior of the *even-skipped* stripes are distinct from those of previously characterized insects with respect to the A-P axis. Furthermore, we show that the shape of the *B. dorsalis* egg and the behavior of the nuclei during the blastoderm stage is considerably different from that of previously characterized insects. Lastly, we analyzed the outcome of the expression patterns of the late acting segment polarity genes in relation to the anlagen of the naked-cuticle and denticle belt area of the *B. dorsalis* larva.

Results

Embryogenesis of B. dorsalis

To date, only sparse information of *B. dorsalis* embryogenesis and postembryonic development exists. Some information exist for two closely-related species, *B. tau* (Singh, 2010) and *B. tryoni* (Anderson, 1964), respectively. For this reason, we have analyzed and summarized relevant stages of the *B. dorsalis* life cycle in Fig. 1. We refer to the nomenclature of the stages of *Drosophila* embryogenesis as previously described (Campos-Ortega and Hartenstein, 1985) and also to those of the blow fly *Lucilia sericata* (Mellenthin *et al.*, 2006; Blechert *et al.*, 2011). Embryonic development of *B. dorsalis* proceeds as a long germband insect (Davis and Patel, 2002). As in most insects, the egg is surrounded by a vitelline membrane and a chorion, however, in contrast to *Drosophila*, no dorsal appendages are observed. Once the chorion is removed, an egg is revealed which is about 0.8 mm long and 0.3 mm in diameter (Fig. 1). The ratio of the length to width, however, is quite different from that of *Drosophila* rendering the egg prone to pressure and physical manipulations. The ventral side as well as the dorsal side are usually curved, a feature not found in *Drosophila*. The posterior tip is often pointed (Fig. 1D) giving the egg a distinct form, compared to those of *Drosophila* or *Lucilia* (Fig. 7B).

After fertilization, the first nuclear cleavages are very similar to those of *Drosophila* where the nuclei divide in the interior of the yolk (Fig. 1 A,B) until they migrate to the periphery to form a syncytial blastoderm (Fig. 1B). In contrast to *Drosophila* where the pole cells (the future germ cells) are formed at nuclear cycle (nc) 10 at the posterior pole, the pole cells are only formed at nc 12 in *B. dorsalis* (Fig. 1C). As in *Drosophila*, after nc 10, four more nuclear cycles follow until the stage of cellular blastoderm is reached (Fig. 1 C,D). Interestingly, during nc 14, no elongation of nuclei occurs as is observed in *Drosophila*, hence nuclei remain rather round during this nc (Fig. 1D). At about 6 hours after egg deposition, the first signs of gastrulation become obvious (Fig. 1E). However, the shape of the cells still does not change and they remain round. Anteriorly, a prominent circumferential furrow is observed which corresponds to the cephalic furrow in *Drosophila*. Ventrally, the mesoderm starts to invaginate (Fig. 1E). On the lateral side, up to 4 folds are observed. This is due to the fact that the radius of the

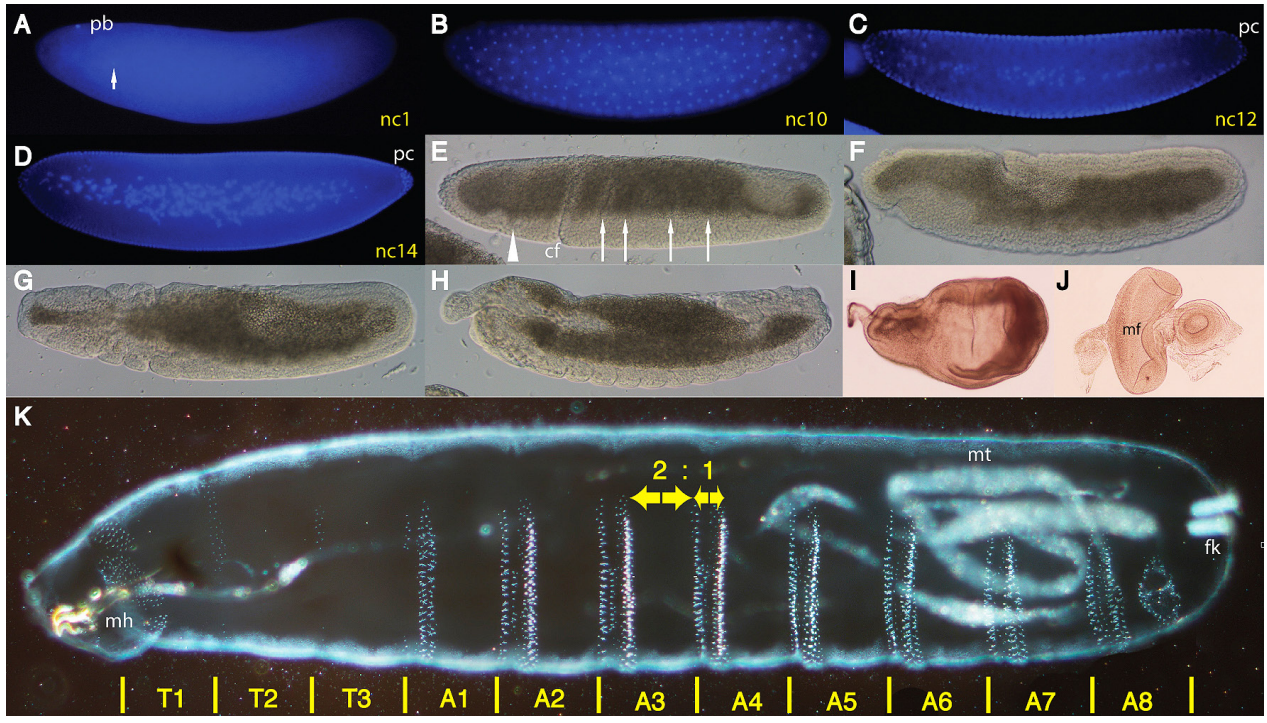

Fig. 1. Embryogenesis and imaginal discs of *B. dorsalis*. (A-K) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted.* **(A-D)** *DAPI staining, nuclear cycles (nc) are indicated in yellow.* **(A)** *Nuclear cycle (nc) 1 embryo staining showing the polar body (pb) and the zygotic nucleus (arrow).* **(B)** *Nc 10 embryo. Note absence of pole cells.* **(C)** *Nc 12 embryo. Note the presence of the pole cells (pc) as a small number of cell at the posterior tip.* **(D)** *Nc 14 showing the pole cells (pc).* **(E-J)** *Bright field microscopy.* **(E)** *Gastrulating embryo showing the cephalic furrow (cf) and up to 4 posterior transverse fold (arrows). Anteriorly, an anterior fold is observed (arrowhead).* **(F)** *Extended germband embryo.* **(G)** *Embryo during germband retraction.* **(H)** *Germband retracted embryo.* **(I)** *3rd instar wing disc.* **(J)** *3rd instar eye-antennal disc showing the morphogenetic furrow (mf).* **(K)** *Dark field picture of a 1st instar larva with annotation of the segments and segmental borders (in yellow). The ratio of the distance of naked cuticle versus denticle belts is indicated above A3. Abbreviations: mh, mouth hook; mt, malphigian tubules; fk, filzkörper.*

curve leading to germband extension is smaller in *Bactrocera* than in *Drosophila* with respect to the dimension of the body axes. 10 hours into development, the germband is fully extended (Fig. 1F), and its shape appears quite similar to that of *Drosophila* or *Lucilia*. At approximately 14 hours of development, the germband starts to retract (Fig. 1G) taking about 10 hours to complete retraction (Fig. 1H). The following morphogenetic movements such as head involution or dorsal closure proceed as they do in *Drosophila*, however, the speed by which they progress is considerably slower, as noted by (Vargas, 2000; Anderson, 1964). Only after approximately 48 hours at 25°C, the larva hatches (Fig. 1K) showing an identical number of segments compared to *Drosophila* or *Lucilia*, a head, 3 thoracic segments, 8 abdominal segments and a tail (Jurgens, 1987; Martinez Arias, 1993; Mellenthin *et al.*, 2006). The patterns of the denticle bands are distinct from that of *Drosophila* (Moline *et al.*, 1999) or that of *Lucilia* (Mellenthin *et al.*, 2006), showing a gap between weaker anterior rows and stronger posterior rows (Fig. 1K), the latter caused by thicker individual denticle hairs. The ratio of the distance of naked cuticle to that of the denticle bands was about 2:1, a feature distinct from other insects (Fig. 1K; Mellenthin *et al.*, 2006).

B. dorsalis undergoes 3 larval stages, as does *Drosophila* and *Lucilia*. The larval stages last about 8 days at 24°C (Vargas, 2000). These values are also larger than those of *Drosophila* or *Lucilia*. During the end of the 3rd instar larval stage, *B. dorsalis* larvae show a peculiar behavior inherent to most *Tephritidae*: the larvae bend their body by arresting their muscles and releasing the tension which enables them to jump as far as 50 centimeters and as high as 30 centimeters. This behavior is interpreted as an attempt to leave the habitat and to find a secluded place for pupation. The pupal stage takes about 12 days at 24°C (Vargas, 2000). Hence, the complete life cycle of *B. dorsalis* can take as long as 19 days at 24° (Vargas, 2000), 40-50% longer than in *Drosophila*, mostly due to the prolonged larval and pupal stage.

To determine if *B. dorsalis* utilizes imaginal discs to form the precursors for the adult structures, 3rd instar larvae were dissected to look for conspicuous discs such as wing and an eye-antennal discs to investigate if they resemble those of *Drosophila* (Fig. 1I, J). A wing disc is similar in shape to that of *Drosophila* (Fig. 1I) and so is an eye-antennal disc (Fig. 1J). Photoreceptor cells follow a similar developmental fate as in *Drosophila* or *Lucilia*, as revealed by the existence of a morphogenetic furrow (Fig. 1J). Altogether, this data demonstrates that imaginal disc development of *B. dorsalis* and probably that of most *Tephritidae* resembles strongly that of *Drosophilidae* or *Calliphoridae*.

B. dorsalis segmentation genes

In order to analyze *Bactrocera* segmentation gene expression, we first screened a database (Geib *et al.*, 2014) for the presence of orthologous segmentation genes of the prime model system *Drosophila melanogaster*. In *Drosophila*, the process of segmentation was described in detail showing that a hierarchy of segmentation genes is crucial for setting up the anterior-posterior axis (Peel *et al.*, 2005). In *Bactrocera*, not all genes of this hierarchy were found (Geib *et al.*, 2014). For example, the maternal gene *bicoid* (*bcd*), shown to be a feature of higher Diptera only, is not present in the *Bactrocera* genome. In *Drosophila*, *bicoid* is expressed in a protein gradient along the A-P axis (Driever and Nüsslein-Volhard, 1988), preceded by the formation of a mRNA gradient (Frigerio *et al.*, 1986; Spirov *et al.*, 2009; Fahmy *et al.*, 2014). The protein gradient serves as morphogen gradient to pattern the anterior-posterior axis. In insects where *bicoid* is lacking, it was proposed that two genes described in *Drosophila* as gap genes, the *hunchback* (*hb*) and the *orthodenticle* (*otd*) act cooperatively to pattern the anterior-posterior axis, instead (Peel *et al.*, 2005; Schetelig *et al.*, 2008).

With this in mind, we have analyzed the expression patterns of important *Bactrocera* members of the segmentation gene hierarchy (Nüsslein-Volhard and Wieschaus, 1980). Of the class of genes at the top of the hierarchy, we analyzed the *hb* and *otd* gene, followed by a member of the gap gene class, the *Krüppel* (*Kr*) gene. The pair-rule gene class is represented by the *even-skipped* (*eve*) gene, while the segment polarity gene class by the *engrailed* (*en*) gene.

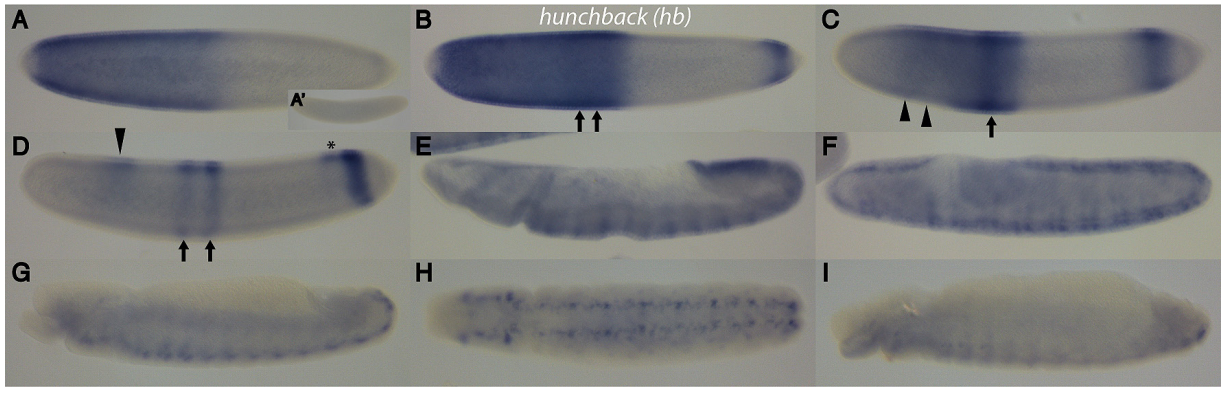

Fig. 2. Embryonic expression of *B. dorsalis hunchback* (*hb*). (A-I) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted.* **(A)** *Nc 13 embryo,* hb *expression is in a broad anterior domain.* **(A')** *Early cleavage stage embryo, no maternal* hb *expression is detected.* **(B)** *Early nc 14 embryo, an additional posterior band emerges. In the anterior broad band, the posterior end increases transcription (arrows).* **(C)** *Late nc 14 embryo, the anterior end of the broad domain looses transcripts (arrowheads). In the posterior part, a distinct band becomes visible (arrow).* **(D)** *Early gastrulation embryo, the broad anterior band has resolved into a dorsal band (arrowhead) and two adjacent bands (arrows), while the posterior band shows a dorsally-expressed spot (asterisk) and the residual strong posterior band.* **(E)** *Germband extension, a repetitive pattern of ectodermal stripes emerges.* **(F)** *Extended germband, transcripts are in a segment-specific pattern in specific neuroblasts.* **(G,H)** *Germband retracted embryo, lateral* **(G)** *and ventral* **(H)** *view, respectively, a repetitive segmental pattern in neuroblasts is observed.* **(I)** *Late embryogenesis, transcripts persist mainly in the ventral chord.*

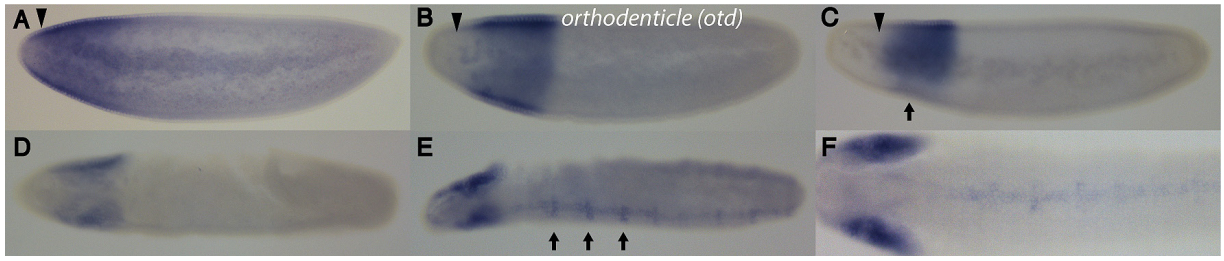

Fig. 3. Embryonic expression of *B. dorsalis orthodenticle (otd)*. (A-F) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted.* **(A)** *Early nc 14 embryo,* otd *is expressed in a broad domain, leaving the anterior-most tip free (arrowhead).* **(B)** *Late nc 14 embryo, a large area of the tip does no longer show* hb *transcripts (arrowhead).* **(C)** *Early gastrula embryo, anteriorly, transcripts disappear (arrowhead), as well as in the ventral-most region (arrow).* **(D)** *Embryo during germband extension,* hb *transcripts are detected in two large lateral patches.* **(E)** *Extended germband embryo, the anterior patches remain, while midline cells show transcripts with a segmental pattern (arrows).* **(F)** *Extended germband, ventral view, the lateral patches remain as well as expression in midline cells.*

Expression of hunchback (hb)

Expression of *Bactrocera hb* commenced at nuclear cycle (nc) 13 where a broad domain extending was detected from about 5% to 55% egg length (EL, where the anterior tip is defined as 0% and the posterior tip as 100%; Fig. 2A). In *Drosophila*, this expression is similar, although the anterior domain included the whole anterior tip (Bender *et al*., 1988; Tautz and Pfeifle, 1989), FlyBase (Attrill *et al*., 2016). In contrast to *Drosophila*, however, no ubiquitous maternal *hb* contribution in *Bactrocera* was detected during the early nuclear stages (Fig. 2A'). At early nc 14, the posterior end of the broad *hb* domain showed increased levels of transcription (Fig. 2B, arrow), while at the posterior end a posterior stripe appeared. During later stages of nc 14, the anterior end of the broad domain showed decreased levels of *hb* (Fig. 2C, arrowheads) and the posterior band increased in intensity. At early gastrula (Fig. 2D), expression of the anterior domain almost completely ceased, with the exception of two distinct stripes in the middle (Fig. 2D, arrows) and an anterior dorsal patch (Fig. 2D, arrowhead). The posterior band remained strong with the anterior end showing expression only dorsally (Fig. 2D, asterisk). In *Drosophila*, a similar pattern was observed (Attrill *et al*., 2016), however, only one stripe was found in the middle and the stripe was shown shifted to the anterior, in comparison to *Bactrocera*. During germband extension (Fig. 2E), a repetitive pattern of *hb* stripes evolved, with the posterior-most expression showing strongest expression. At extended germband (Fig. 2F), strong expression was observed in neuroblasts in a segmented pattern. During germband retraction (Fig. 2 G,H), *hb* expression was observed in neuroblasts. During late embryogenesis (Fig. 2I), many neurons in the ventral nerve chord showed strong *hb* expression.

Expression of orthodenticle (otd)

Expression of *otd* initiated as a broad circumferential anterior domain at early nc 14, ranging from 2% to 34% EL (Fig. 3A) and leaving the tip free (Fig, 3A, arrowhead), thus clearly later than *B. dorsalis hb* (Fig. 2A) and making *otd* a possible target of regulation by *hb*. During nc 14 (Fig. 3B), the anterior edge shifted to about 11% EL (Fig. 3B, arrowhead), while the posterior edge remained

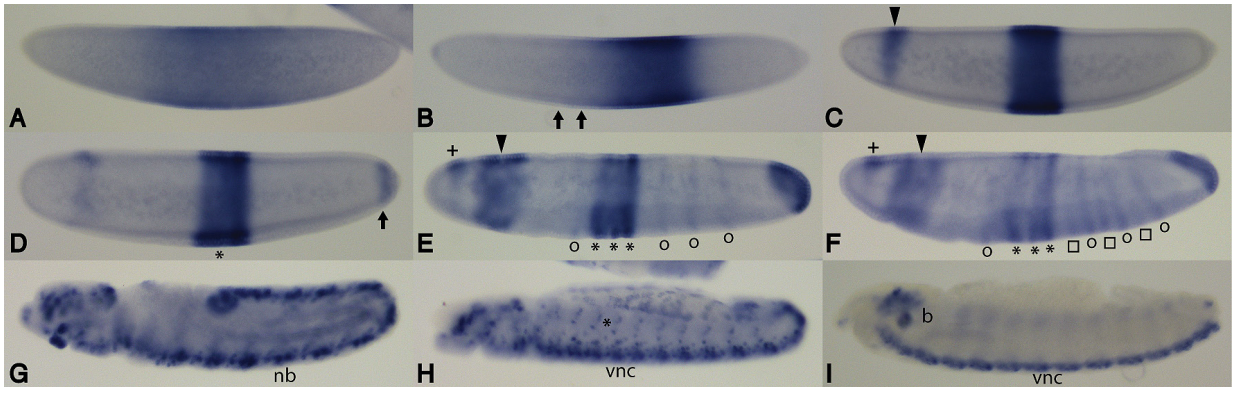

Fig. 4. Embryonic expression of *B. dorsalis Krüppel (Kr)*. (A-I) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted.* **(A)** *Nc 13 embryo, a broad* Kr *band is observed.* **(B)** *Early nc 14 embryo,* Kr *transcripts disappear at the anterior end of the broad band (arrows).* **(C)** *Late nc 14 embryo, a central domain has emerged and anteriorly, a dorsal "cap" appears (arrowhead).* **(D)** *Early gastrula embryo, a posterior band appears (arrow). The middle band begins to split up (asterisk).* **(E)** *Embryo at beginning of germband extension, the band in the middle shows clear splitting into 3 bands (asterisk) and 3 new weak bands appear posteriorly and one anteriorly to the central band (open circles). Anteriorly, the dorsal cap becomes stronger (arrowhead). Even more anteriorly, a new cap band appears (+).* **(F)** *Embryo during germband extension, slightly older than that in* **(E)**, *posterior to the middle band which remains split as 3 separate bands, a series of new bands (squares) appear posteriorly which alternate with those marked by open circles making a total of 10 consecutive bands with alternate intensities.* **(G)** *Extended germband embryo, strong transcription is observed in neuroblasts (nb).* **(H)** *Retracted germband embryo,* Kr *transcripts are seen in the ventral nerve cord (vnc) and in certain muscle precursor cells (asterisk).* **(I)** *Embryo shortly before hatching, transcripts are detected in the ventral nerve chord (vnc) and in the brain (b).*

constant. During early gastrula (Fig. 3C), transcripts disappeared further from the anterior edge (Fig. 3C, arrowhead) and comprised a band from 17-34% EL. At the ventral side, the presumptive mesodermal anlagen was devoid of any transcripts (Fig. 3C, arrow), similar to that in *Drosophila* (Finkelstein *et al.*, 1990; Attrill *et al.*, 2016). During germband extension (Fig. 3D), the anterior domain remained. At extended germband (Fig. 3 E,F), a two-cell wide stripe with lateral extensions emerged (Fig. 3E, arrows). These cells corresponds to the midline cells that marked the outermost cells of the invaginating mesoderm. A similar expression in midline cells was also observed in *Drosophila* (Finkelstein and Perrimon, 1990; Attrill *et al.*, 2016). However, while transcription of *otd* continues in *Drosophila*, *otd* transcripts in *B. dorsalis* ceased after the extended germband stage. Contrary to our expectation, we noted that in two other *Tephritidae*, the medfly *Ceratitis capitata* and the caribfly *Anastrepha suspensa*, maternal *otd* expression was observed (Schetelig *et al.*, 2008). Moreover, medfly *otd* showed a wide band of expression at the early blastoderm expression, and only at cellular blastoderm, all three *Tephritidae otd* patterns converged to an identical broad anterior band of about 17-34% EL. Therefore, there is considerable plasticity of *otd* gene regulation between close relatives within the same family, also noted by (Schetelig *et al.*, 2008).

Expression of Krüppel (Kr)

Kr expression in *B. dorsalis* started with a broad band in the middle of the embryo from about 30% EL to 75% EL with tapered expression on either side at nc 13 (Fig. 4A). Its occurrence paralleled that of *hb* (Fig. 2A). At early nc 14, the anterior-most part of the band diminished (Fig. 4B, arrows) and transcripts were observed in a band from 45% to 70% with a sharp posterior boundary. At late nc 14 (Fig. 4C), a band from 45% to 60% with sharp anterior and posterior boundaries emerged, while anteriorly, a new band appeared whose width tapered off on the ventral side (Fig. 4C, arrowhead). At early gastrula stage (Fig. 4D), a posterior band appeared and the broad middle band began to split (Fig. 4D, asterisk). Slightly later, at the anterior end, another band appeared (Fig. 4E, marked with +), while the initial anterior bands broadened and segregated.

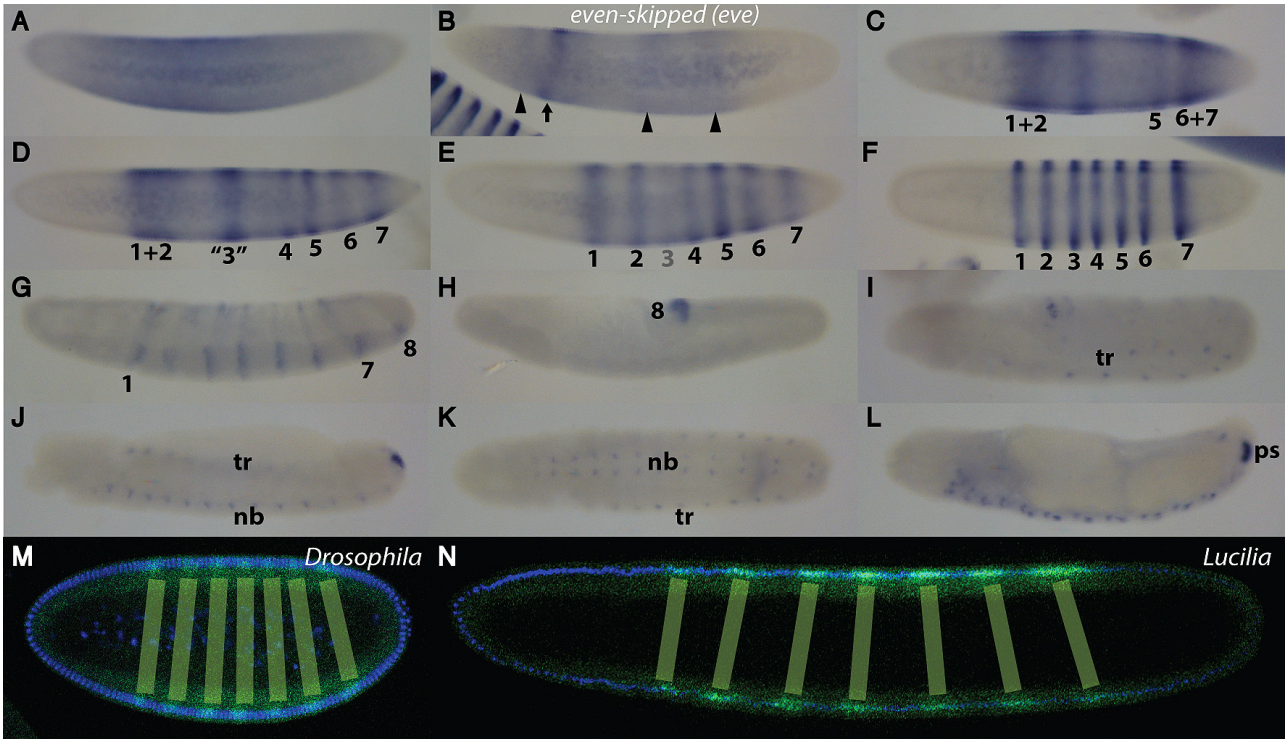

Fig. 5. Embryonic expression of *B. dorsalis even-skipped (eve)*. (A-N) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted.* **(A)** *Early nc 14 embryo,* eve *is expressed in a broad central domain with diffuse ends.* **(B)** *Early nc 14 embryo slightly later than in* **(A)**, *an anterior band (arrow) appears, along with further weak bands (arrowheads).* **(C)** *Mid nc 14 embryo, the weak bands become stronger and anteriorly, another broad band appears, forming bands 1, 2, 5, 6 and 7.* **(D)** *Mid nc 14, slightly older than* **(C)**, *6 bands with different widths and intensities evolve. Bands 4-7 have final width and positions, bands 1-3 are still not determined. Numbering of stripes according to the final 7 stripe-pattern in* **(F)**. **(E)** *Nc 14 embryo close to cellularization, all bands are established, band 3 is considerably weaker.* **(F)** *Early gastrula embryo, 7 bands with equal intensity have evolved.* **(G)** *Embryo during germband extension, posterior to band 7, band 8 appears. Bands 1-7 become weaker.* **(H)** *Extended germband embryo, only the posterior-most band 8 remains.* **(I)** *Embryo at beginning of germband retraction, the posterior band is still visible. Single cells, presumably in tracheal precursors (tr), start to express* eve. **(J)** *Germband retracted embryo, a repetitive pattern in certain neuroblasts (nb) and tracheal cells (tr) emerges.* **(K)** *Ventral of a similar staged embryo as in* **(J)**, *expression is conspicuous in neuroblasts and tracheal cells.* **(L)** *Late embryogenesis,* eve *expression is in the ventral nerve chord in neuroblasts and the posterior spiracle (ps).* **(M)** *Confocal picture of a late nc 14 of a* D. melanogaster *embryo, Eve protein expression is in green, along with DAPI (blue) to reveal the nuclei. The angle of the 7 stripes with respect to the A-P axis is indicated in shaded green.* **(N)** *Confocal picture of a late nc 14* L. sericata *embryo, Eve protein expression is in green, along with DAPI (blue) to reveal the nuclei. The angle of the 7 stripes with respect to the A-P axis is indicated in shaded green.*

The band in the middle divided into 3 bands (Fig. 4E, marked by asterisk). Posterior to the middle bands, 3 weak new stripes appeared and anteriorly one additional band was detected (Fig. 4E, marked by "o"). During germband extension (Fig. 4F), a faint set of stripes (Fig. 4F, marked by squares) appeared between the 3 posterior bands marked with "o". Hence, at this stage, a row of 10 distinct bands with different intensities emerged, subdividing the region at 40% to 80% EL of the embryo into a striped pattern, reminiscent of segment polarity gene expression (Fig. 6). During extended germband (Fig. 4G), all segments exhibited strong *Kr* expression, mainly in specific neuroblasts (nb). During germband retraction (Fig. 4H), strong *Kr* expression was in neuroblasts of the ventral nerve cord (vnc) and in certain muscle precursor cells (asterisk). During late embryogenesis (Fig. 4I), *Kr* was strongly expressed in the brain (b) and the ventral nerve chord (vnc).

Kr expression has been analyzed in a variety of insects: *Drosophila melanogaster* (Jaeckle *et al.*, 1986; Gaul *et al.*, 1987), *Musca domestica* (Sommer and Tautz, 1991), *Clogmia albipunctata* (Rohr *et al.*, 1999; Garcia-Solache *et al.*, 2010), *Oncopeltus fasciatus* (Liu and Kaufman, 2004), *Tribolium castaneum* (Bucher and Klingler, 2004), *Nasonia vtiripennis* (Olesnicky *et al.*, 2006), *Episyrphus balteatus* (Lemke *et al.*, 2010), *Apis mellifera* (Wilson *et al.*, 2010), *Lucilia sericata* (Blechert *et al.*, 2011) and *Bombyx mori* (Nakao, 2015). In comparison to the aforementioned insects, the evolution of the complex *B. dorsalis Kr* expression pattern at blastoderm and early gastrulation resembled that of *L. sericata Kr* (Blechert *et al.*, 2011), as similar banding patterns were seen in the anterior, middle (Fig. 4D) and biphasic onset of the posterior weak stripes (Fig. 4E, F).

Expression of even-skipped (eve)

Expression of *eve* began at early nc 14 (Fig. 5A) revealing a broad band with diffuse ends. At early nc 14 (Fig. 5B), an anterior strong band was visible (Fig. 5B, arrow), along with some minor bands (Fig. 5B, arrowheads). At mid nc 14 (Fig. 5C), a more defined banding pattern appeared with broad anterior and posterior bands, respectively. In the middle, a strong but transitional band appeared. Thereafter, but still during mid nc 14 (Fig. 5D), the posterior broad band split up, and a new band, band 4, appeared. Anteriorly, the broad band (band 1+2) remained together, while the identity of the middle band (referred to as "3") still remained obscured. Close to cellularization (Fig. 5E), all bands resolved, with the exception of band 3 which was still weak. At early gastrulation (Fig. 5F), 7 bands with a width of 3 cells evolved without regular spacing. Notably, band 7 was more posteriorly-located. During germband extension (Fig. 5G), a new band 8 appeared which was the only one to remain at extended germband (Fig. 5H). During germband retraction (Fig. 5I), a segmental pattern at certain tracheal cells emerged while the posterior band remained defined. At retracted germband (Fig. 5 J,K), the pattern remained unchanged in the tracheal cells, while neuroblasts showed *eve* transcripts in a repetitive pattern. During late embryogenesis (Fig. 5L), *eve* transcription remained high in neuroblasts and the posterior spiracles.

To compare the relative position along the A-P axis and the spatial geometry of the *eve* stripes in some related phyla, *D. melanogaster* and *L. sericata* embryos were stained with monoclonal antibody 2B8, known to detect the Eve protein in distant insect families. While 2B8 did not detect Eve in *B. dorsalis* (data not shown), it revealed 7 stripes in *D. melanogaster* (Fig. 5M) and *L. sericata* (Fig. 5N). Interestingly, in comparison to *B. dorsalis* (Fig. 5F) where the stripes were perpendicular to the A-P axis, the angles of the anterior-most and posterior-most stripes were tilted towards the dorsal side, respectively (Fig. 5 M,N). While this could easily be explained in the case of *D. melanogaster* by the fact that the dorsal side was rather straight and the ventral side rounded (Fig.

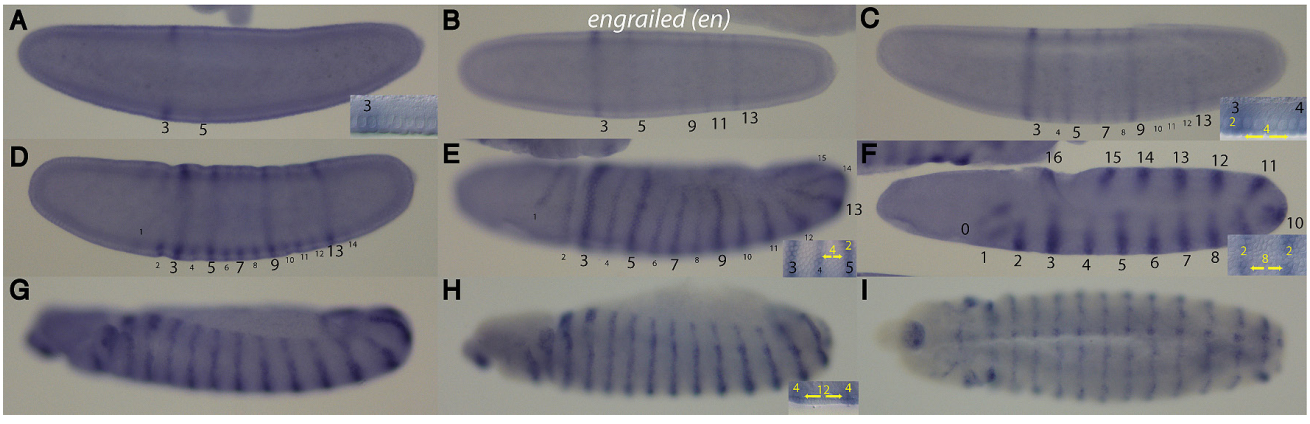

Fig. 6. Embryonic expression of *B. dorsalis engrailed (en)*. (A-I) *All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted. Numbering of stripes is according to the final 17 stripe-pattern in* **(F)**. **(A)** *Late nc 14 embryo,* en *transcripts are detected in a 2-cell wide stripe 3 and posteriorly a weaker stripe 5. Insert shows width of 2 cells in stripe 3.* **(B)** *Nc14, cellular blastoderm embryo, posterior to stripe 5, stripes 9, 11 and 13 appear. Stripe 7 is delayed.* **(C)** *Early gastrula embryo, odd-numbered stripes 3-13 become established. In between, even-numbered stripes 4-12 appear with the exception of stripe 10 which is delayed. Insert shows the width of stripe 3 of 2 cells, as well as the interstripe section of 4 cells (indicated by arrows) and stripe 4.* **(D)** *Early gastrula embryo, slightly later than in* **(C)**, *stripes 1, 2 and 14 appear. The stripe intensity is not equal, the odd-numbered ones are stronger than the even-numbered ones.* **(E)** *Germband extension embryo, posterior to stripe 14, stripe 15 appears. The intensity of the stripes is still not equal. Insert shows the area of stripe 3 to 5 with width of 2 cells for the stripes and 4 cells for the interstripe region.* **(F)** *Extended germband embryo, 16 stripes with equal intensity have evolved. Insert shows the area of stripe 4 to 5 with width of 2 cells for the stripes and 8 cells for the interstripe region.* **(G)** *Embryo during germband retraction, the number of stripes remains identical.* **(H,I)** *Germband retracted embryos, lateral and ventral view, respectively. Insert in* **(H)** *shows the area of stripe 8 to 9 with a width of 4 cells for the stripes and 12 cells for the interstripe region. All stripes are close to the posterior border of each segment.*

5M), difficulties arose to explain the observation in the case of *L. sericata* (Fig. 5N) which has a similar body shape and A-P/D-V axis ratio as *B. dorsalis* (Fig. 5F).

Expression of engrailed (en)

B. dorsalis en expression was first detected at late nc 14 in a two-cell wide stripe and a posterior weaker stripe (Fig. 6A). The stripes correspond to stripes 3 and 5 and were within the position of the first appearance of the striped expression of *eve* (insert of Fig. 6A). Shortly thereafter, at cellularization (Fig. 6B), stripes 9, 11 and 13 emerged, comprising the first wave of *en* expression in odd-numbered bands. During early gastrula (Fig. 6C), the odd-numbered stripes were complemented by weaker even-numbered stripes in between, a pattern which progressed through germband extension (Fig. 6 D,E) with 2 cells expressing *en* followed by a gap of 4 cells, making a total of 6 cells/segment (insert in Fig. 6E). At extended germband, even- and odd-numbered stripes reached identical intensities (Fig. 6F). Anteriorly, stripes 0 and posteriorly stripes 15 and 16 became visible, making a total of 17 stripes. The width of the *en* bands was 2 cells and the interstripe region 8 cells (insert in Fig. 6F). Hence, one segment comprised of about 10 cells at this stage. During germband retraction (Fig. 6G), a striped pattern in the ectoderm in the posterior of each segment was visible, which persisted during the remaining stages of embryogenesis (Fig. 6 H,I). The number of *en*-expressing cells increased to 4 cells and the interstripe region to 12 cells, making a total of 16 cells/segment (insert in Fig. 6H).

Evo-Devo: comparative expression analysis reveals distinct interpretation of the gap signal at the pair-rule level

A survey in the literature revealed that the expression of *Kr* was investigated in many insects, a comprehensive list is presented above. Hence, it serves as a prime marker to understand how gene expression correlates with the geometry and layout of the insect embryo. Of these aforementioned insects, only a handful were long germband insects: *Drosophila melanogaster* (Gaul *et al.*, 1987), *Musca domestica* (Sommer and Tautz, 1991), *Episyrphus balteatus* (Lemke *et al.*, 2010) and *Lucilia sericata* (Blechert *et al.*, 2011). This collection of data nevertheless permitted the establishment of a map of their expression domains with respect to the A-P axis (Fig. 7A). This comparative map revealed that the majority of long germband insects showed *Kr* expressed in a band between 40-53/57% EL, with *L. sericata* showing a distinct anterior shift (30-45% EL, (Blechert *et al.*, 2011) and *B. dorsalis* a marginal posterior shift (45-60% EL).

Surprisingly, when *eve* banding patterns were compared (Fig. 7B), these considerable differences at the gap-gene level were almost completely restored: The anterior *L. sericata eve* bands were still more anterior compared to the "reference" bands of *D. melanogaster* and *B. dorsalis*, however, the percentage of the difference compared to *Kr* diminished. Interestingly, the posterior-most bands were at similar levels, despite the difference of 12-15% at the posterior end of the *Kr* bands. We conclude that a compensation of the relative position of the segmental anlagen must have occurred during the stage from the gap gene to the pair-rule gene level which is more pronounced at the posterior part of the embryo.

To address the question if gap genes showed a conserved behavior of controlling the pair-rule genes between the three insect families, we superimposed the *Kr* expression domains to the *eve* stripes (Fig. 7B). We noted significant differences: in *Drosophila*, the anterior margin of the *Kr* band is congruent to the posterior margin of *eve* stripe 2, consistent with the notion that *Kr* demarcates the posterior end of *eve* stripe 2 (Small *et al.*, 1996). In *Lucilia*, we noted the dramatic anterior shift of the *Kr* domain (Blechert *et al.*, 2011), but when this domain was superimposed to that of *eve*, the anterior margin of the *Kr* band was congruent to the posterior margin of *eve* stripe 1, and not to that of stripe 2. In *Bactrocera*, however, the anterior margin of the *Kr* band was congruent to the posterior margin of *eve* stripe 2, as in *Drosophila*, suggesting a similar control. What was common to all three insect families was the fact that the *Kr* band straddled two adjacent *eve* stripes. Data

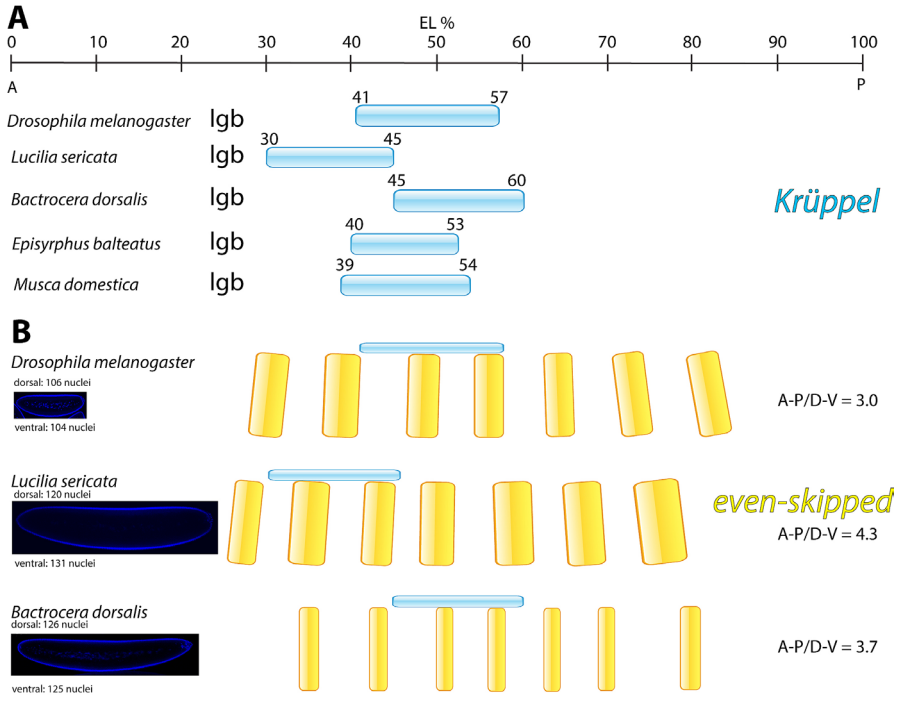

Fig. 7. Schematic representation of relative expression domains of *Krüppel* and *even-skipped* in long germband insects. (A) *Expression domains in regard to the A-P axis in percentage of egg length (% EL) of* Kr *(blue) in insect families, as reported (Gaul* et al.*, 1987; Rohr* et al.*, 1999, Sommer and Tautz, 1991; Lemke* et al.*, 2010; Blechert* et al.*, 2011) along with those of* B. dorsalis. *Note the distinct anterior shift in* L. sericata *(Blechert* et al.*, 2011). lgb indicates "long germband insect".* **(B)** *Expression domains of* even-skipped *(yellow) and superimposed* Krüppel *(blue) in regard to the A-P axis in % EL in* D. melanogaster *(Fig. 5M),* L. sericata *(Fig. 5N) and* B. dorsalis *(Fig. 5F). Representative nuclear DAPI stainings of embryos are indicated and are shown to scale. The number of nuclei on the dorsal as well as ventral side is indicated. Angles of the bands regarding the A-P axis are indicated in yellow as they appear in the respective insect. The ratio of the A-P/D-V axis is indicated on the right side. Note the prefect perpendicularity of the bands in* B. dorsalis *with respect to the A-P axis, compared to the other insects.*

from *Episyrphus balteatus* indicated that the *eve* stripes were at similar positions as those of *Drosophila* (Lemke and Schmidt-Ott, 2009), but since the *Kr* band was narrower in comparison to that of *Drosophila* (Fig. 7A), it did not encompass *eve* stripe 4 as in *Drosophila* and consequently, only a single stripe, *eve* stripe 3 was contained within the *Kr* domain.

Evo-Devo: comparative analysis of the shape of the embryos and consequences for the segmentation gene expression at blastoderm stage

Insects embryos have distinct overall shapes where the primary determinant of the shape is the ratio of the A-P axis versus that of the dorsal-ventral (D-V) axis. To investigate the cause for this, the number of cells for the A-P axis on the dorsal and ventral side were counted in 3 insect systems, *D. melanogaster*, *L. sericata* and *B. dorsalis*. It should be noted that not only the cell number, but also the size and shape of the individual cells contribute to the overall shape of the insect eggs (Blechert *et al*., 2011, Mellenthin *et al*., 2006). For *Drosophila*, on average 106 ± 3 nuclei on the dorsal side versus 104 ± 3 nuclei on the ventral side were counted, for *Lucilia* 120 ± 4 nuclei on the dorsal side versus 131 ± 5 nuclei on the ventral side, and for *Bactrocera* 126 ± 4 on the dorsal side and 125 ± 3 nuclei on the ventral side (Fig. 7B). The ventral side of the *Drosophila* egg was more rounded, despite the fact that both sides have similar numbers of cells. At close examination, this difference is due to the fact that dorsal cells are highly columnar and the amount of lateral cytoplasm is small, enabling a straight dorsal surface (Fig. 7B). Conversely, ventral cells were slightly constricted at the basal side which enabled a rounded surface. The form of the insect embryo is already determined during the late stages of oogenesis, an observation extended to all long-germband insects. Furthermore, the migration of the nuclei to the periphery during nc 9-10 is a microtubule-driven process not involving changes to the shape of the embryo. Hence, the nuclei of the *Drosophila* embryo adapted to their environment once they have reached their final position at the cortex. The situation in the *Lucilia* egg, however, which also shows a rounded ventral surface, is not as pronounced as in *Drosophila*. Due to its elongated shape, it appears to follow another strategy. The number of nuclei on the ventral side is larger than that of the dorsal side, consequently, leading to almost even spacing on both sides. *Bactrocera* embryos have similar numbers of nuclei on either side and a slightly rounded ventral side which appears to be controlled in a similar way as in *Drosophila*.

To address the question whether the different number of cells on the dorsal and ventral side have implications for the expression patterns, a comparison of the angles of the *eve* stripes with respect to the A-P axis in all 3 insects was conducted. It was noted that the *eve* stripes in *Drosophila* and *Lucilia* were not always perpendicular to the A-P axis, particularly the first and the last stripes. While this behavior can be explained by the curved nature of the ventral side in *Drosophila* (Fig. 5M), a reasonable explanation could not be given for the elongated *Lucilia* egg (Fig. 5N). In contrast, a similarly shaped egg such as the *Bactrocera* egg revealed almost perfectly arranged perpendicular stripes (Fig. 5 D-F). Notably, all *B. dorsalis* segmentation genes analyzed exhibited this behavior at blastoderm stage (Fig. 2-6).

As far as the overall shape of the embryos, there were distinct A-P/D-V ratios between the insects. *Drosophila*, a relatively compact insect egg showed a ratio of 3.0, while *Bactrocera* showed a ratio of 3.8 and *Lucilia* as the largest of the 3 eggs had a ratio of 4.3 (Fig. 7). It is important to note that the ratio has an impact on the mechanical stability of the egg: rounder eggs are more stable than elongated eggs. This fact becomes an issue when the egg is manipulated, e.g. by pricking it with a needle during genetic transformation where *Lucilia* and *Bactrocera* are particularly vulnerable, compared to *Drosophila* (unpublished observations).

Discussion

We have analyzed the expression of some of the important early A-P axis patterning genes in *B. dorsalis* and found some similarities to known expression patterns in *Drosophila*, but also some distinct features associated with this oriental fruit fly. Furthermore, we described, in detail, its embryogenesis and imaginal disc development.

We noted some similarities of the appearance of the *B. dorsalis* egg to that of *L. sericata* or *D. melanogaster*. All three insects showed an identical number of segments, including a head, 3 thoracic segments, 8 abdominal segments, and a tail (Fig. 1K). The *engrailed* gene was an excellent marker for segment number as well as identity, hence, comparison of the *en* stripe numbers and position might give some clues as to the subdivision of the insect. As evident from Fig. 6, *B. dorsalis* revealed more *en* stripes than *L. sericata* (Mellenthin *et al*., 2006) or *D. melanogaster* (Attrill *et al*., 2016). This is due to a stripe in the head, designated "0" (Fig. 6F) and stripe 16 at the posterior end, while the number and position of the stripes in the trunk seemed constant. These different numbers of stripes may be attributed to different functions of tissues. For example, *B. dorsalis* females harbor an ovipositor while a true equivalent of this organ is not found in *L. sericata* or *D. melanogaster*.

It is important to note that in *Drosophila*, not all cells of the cellular blastoderm contribute to the later larval body structure, referred to as the "fate map of the blastoderm" (Technau and Campos-Ortega, 1985) The precursor cells of T1 (Fig. 1K) were located at about 35% EL, and A8 at ~ 80% EL, while regions more anterior were precursors for head structures and elements of the internal digestive system such as anterior midgut or esophagus. At the molecular level, parasegments were defined as the metameric units to subdivide the blastoderm embryo, whereby the *eve* stripes define all odd-numbered parasegments (Martinez-Arias and Lawrence, 1985). In this respect, it is noteworthy that in *Drosophila*, parasegment 3 which is defined by *eve* stripe 2 or *en* stripe 3 corresponds to the anterior half of T1. Hence, neither *eve* stripe 1 nor *en* stripes 1 & 2 contribute to the cuticle pattern, but rather to head structures. Likewise, at the posterior end, *eve* stripe 7 and *en* stripe 15 constitute A8. When looking at the relative position of the *eve* stripes among the 3 different insects, it became evident that the anlagen of the larval cuticle cells were located at distinct regions of the blastoderm (Fig. 7B). In this respect, if the *eve* stripes serve as a landmark to what extent the blastoderm cells will become progenitors for the exoskeleton of the larva, only about 45% of the surface of the *Bactrocera* blastoderm cells (Fig. 6F) will contribute to the exoskeleton which is lower than in *Drosophila* (56%) or *Lucilia* (53%).

As far as the major toolkit of segmentation genes was concerned, *Bactrocera* lacked maternal *bcd* which is a major player on the top of the hierarchy of segmentation genes in many insects. It is a

generally accepted view that in higher Dipterans, *bcd* arose through a duplication of an ancestral *Hox3*/*zerknüllt* gene. In more basal Diptera where *bcd* is lacking, it was suggested that the function of *bcd* is exerted by a maternally - as well as zygotically-expressed *Hox3*/*zerknüllt* gene (Stauber *et al.*, 2002). In higher Dipterans, these functions are now separated into two functions exerted by maternally-expressed *bcd* and a zygotically-expressed *zerknüllt* gene (Stauber *et al.*, 2002). In short germband insects such as *Tribolium* where *bcd* is lacking as well, it was proposed that *hb* and *otd* together exert the function of *bcd* (Schroder, 2003).

Very recently, a report in the midge *Chironomus* showed that *panish*, a protein containing a cysteine-clamp DNA-binding motif, can exert similar functions as does *bcd* in *Drosophila* (Klomp *et al.*, 2015). Like *bcd*, *panish* was strongly expressed at the anterior tip of the fertilized embryo as a result of maternal deposition, and like *bcd*, formed a mRNA gradient at blastoderm stage. RNAi-mediated knock-down of *panish* revealed a bicaudal phenotype similar to strong *bcd* mutations. These results showed that evolution possibly established further systems that enabled patterning the anterior end and that *bcd* and *panish* were limited to specific families of flies. Of note, *panish* was not found in the *Bactrocera* genome nor was it detectable in two closely-related chironomid species which suggested that *panish* arose only very recently (Klomp *et al.*, 2015).

The next class of genes in the hierarchy of the segmentation, the gap gene class showed both *hb* and *Kr* genes conserved. Compared to *Drosophila* where the first expression was observed during nc 12 (Knipple *et al.*, 1985; Bender *et al.*, 1988), *Bactrocera hb* and *Kr* showed expression from nc 13 on, suggesting that blastoderm identity followed a distinct regulation. During later embryogenesis, however, identical organs were labeled suggesting that the later functions of both genes were retained. The third gap gene, *otd*, also showed its expression delayed by one nc, compared to *Drosophila* (Finkelstein and Perrimon, 1990).

Down the hierarchy follows the pair rule gene class (Nüsslein-Volhard and Wieschaus, 1980) where the *eve* gene was analyzed. Compared to *Drosophila*, the number of *eve* stripes remained conserved, however, it was the evolution of the stripes that was clearly different in the two insects (MacDonald *et al.*, 1986; Fig. 5). Again, transcription of *eve* in *Bactrocera* was delayed by one nc, compared to *Drosophila* (Macdonald *et al.*, 1986). In addition, eye-catching was the fact how these 7 stripes were aligned along the A-P axis between the two insects at cellular blastoderm (Fig. 5F vs. Fig. 5M). These appeared oblique at either end in *Drosophila* or *Lucilia* (Fig. 5 M,N), while in *Bactrocera*, all segmentation genes were expressed perpendicular to the A-P axis (Fig. 2-6). We should also bear in mind that the number of cells on the dorsal versus the ventral side was only marginally different in all 3 insects, hence the outcome in the perpendicularity of the stripes must have a different origin.

Our comparative analysis in Fig. 7. demonstrates that there are marked differences across insect families of how the gap genes exert their control on pair-rule genes, evidenced by the position of the *Kr* band along the AP axis in comparison to the *eve* stripes. *eve* stripe 2 is a paradigm of how the maternal input, together with the gap genes, control the pair-rule genes (Small *et al.*, 1996). While *Bactrocera* and *Drosophila* showed similar overlap of the *Kr* bands with reference to the *eve* stripes where the anterior border of the *Kr* band was precisely adjacent to the posterior border of *eve* stripe 2 (Fig. 7B), the position of the *Lucilia Kr* band was distinct and was moved exactly one *eve* stripe unit to the anterior. Moreover, *eve* stripe 4 which in *Drosophila* and *Bactrocera* is probably activated by *Kr* (Fig. 7B) does not overlap any longer with the *Kr* band in *Lucilia*. Likewise, in *Episyrphus balteatus*, *eve* stripe 4 does not seem to involve *Kr* regulation either, as the posterior part of the *Kr* band does no longer overlap with *eve* stripe 4 (Lemke *et al.*, 2010, Lemke and Schmidt-Ott, 2009). From this comparative analysis, we can conclude that regulation of *eve* stripes 2-4 by *Kr* is not conserved among insect families.

During blastoderm stage at nc 14, we noted a particular behavior for *B. dorsalis* nuclei, as they did not undergo an elongation step as in *L. sericata* or *D. melanogaster*. In *Drosophila*, elongation of the nuclei is initiated by the formation of an inverted bask of microtubules that originate from the centrosomes that are located in the periplasm next to the nuclei (Foe *et al.*, 2000, Foe *et al.*, 1993). These microtubules guide the invagination of a furrow which migrates from the apical to the basal side of the nuclei, until they wrap the nuclei into a cell. The leading edge of the furrow is driven by an actin-myosin interaction that migrates, from the apical to the basal side, along the microtubules, until the cellular membrane encapsules the nucleus fully, thus forming a cell. The elongated form of the cells at cellular blastoderm allow cellular constrictions which are one of the driving forces for gastrulation. In contrast, the *B. dorsalis* cells hardly elongated during nc 14 and instead stayed largely round (Fig. 1D). This was true even for gastrulation which creates difficulties for cells to change the cellular morphology and to assign constrictions that are important for gastrulation. We presume that in *Bactrocera*, the mechanisms leading to the driving forces for ventral furrow formation or germband extension must be different ones, compared to *D. melanogaster* of *L. sericata*.

During the analysis of segmentation genes in *Lucilia* (Mellenthin *et al.*, 2006), we noticed that the ratio of the area of naked cuticle to that of the denticle bands varied dramatically when comparing the large *Lucilia* fly to that of the small *Drosophila*. In *Lucilia*, we measured a ratio of 3:1, while the one in *Drosophila* was 1.5:1 (Mellenthin *et al.*, 2006). We also noted that the naked cuticle area was particularly dependent on the patterning activity of the segmentation gene *wingless* which in *Lucilia* showed signaling activity over 3 times the distance compared to *Drosophila* (Mellenthin *et al.*, 2006). Further, using mathematical calculations, it was shown that a 20-fold increase of the Wingless producing cells still was not enough to compensate for the larger distance between the sender and receiver cell, unless a facilitated movement of the Wingless protein was allowed (Mellenthin *et al.*, 2006). In the case of the *Bactrocera* cuticle, we noted that the ratio was 2:1 (Fig. 1K) which was closer to that of the small *Drosophila*, and not in the range of the larger *Lucilia*. Hence, the ratio of the naked cuticle/denticle belts does not increase linearly with the size of the insect. Rather, these ratios likely represent adaptations of the systems to their habitat or their ability to exert movements. We therefore reasoned that these differences in these ratios should immediately be reflected in the expression patterns of the naked cuticle-patterning genes of these biological systems. In this case, the *en* gene is good marker gene, as it allows to define several issues important for insect segmentation, this for the following reasons: (i) its anterior expression border defines the parasegmental border, a feature which is conserved across most insect phyla (Mellenthin *et al.*, 2006), (ii) the number of *en*-expressing cells is a good estimate how large the naked cuticle will be (Mellenthin *et al.*, 2006), (iii)

the number of *en* bands is a good marker for estimation of how many segments a fly has (Baumgartner *et al*., 1987; Mellenthin *et al*., 2006). When compared to *Lucilia* where 6 cells were reported to show *en* expression at extended germband (Mellenthin *et al*., 2006), *Bactrocera en* was expressed in a much less wider band, comprising only 2 cells (Fig. 6 F). Hence, the capacity to pattern the cuticle is limited, taken into account the size of the insect. In fact, the much smaller *Drosophila* embryo also revealed 2 cells expressing *en* which corroborated the notion that size did not matter.

Materials and Methods

Maintenance of B. dorsalis

B. dorsalis flies were maintained in 25 x50 cm round Plexiglas cages and fed with a constant source providing 3 parts of sugar, 1 part of yeast hydrolysate and water. For larval stages, a mixture 1000 g, sugar, 100 g yeast hydrolysate, 50 g yeast extract and 50 g peptone, supplemented by banana or apple pieces was used. To prevent escape of larvae during the "jumping phase" of larval stages, containers were always covered with a cover during this time.

Embryo collection

Bactrocera flies were exposed to standard food for 10 days. For embryo collection, a smaller plastic beaker was prepared with many small holes (diameter > 1 mm) which was positioned over a freshly-cut apple piece. To enable a precise embryo collection, a pre-collection phase of 2 hours was employed where eggs that were deposited were discarded. In some cases, an apple, cut into a halves, was used for embryo collection, where the embryos were collected by removal of the shell and collecting the embryos from the inner surface of the shell.

Identification of orthologous genes in B. dorsalis

The *Bactrocera* orthologues of segmentation genes were identified through standard BLAST searches of a database where access was provided by Scott Geib. This database is accessible via the NCBI *Bactrocera dorsalis* Annotation Release 100. Note: the maternal *bicoid* gene is not present in the *Bactrocera* genome. *B. dorsalis hunchback* has accession number XM_011208844, *orthodenticle* XM_011202351.1, *Krüppel* XM_011207908.1, *even-skipped* XM_011210455.1 and *engrailed* XM_011216118.1. *hunchback* was amplified by PCR primers GCGAAATCACTACAAGATCAG (forw) and TAAATACTTAGGAACGTAACC (rev), *orthodenticle* with CTCGACAGAAGCCTTAATGGC (forw) and TTATTCGCATTGCCTCCAGCG (rev), *Krüppel* with ACCCCCATAACCGTGCCGATG (forw) and CTACTCCATTAGGGTGGTTTG (rev), *even-skipped* with GCGATCAATTGACACGTCTGG (forw) and AAGACTCGGTTTTGTAGGGCT (rev), engrailed with CTCCGCGTTTACTACAACGCC (forw) and GGGACGATCGCTGTAGCGCGT (rev). Each PCR fragment was sequenced to verify its origin.

In situ hybridization

Templates for Riboprobes were generated using a 0-4 h *B. dorsalis* cDNA library (L. Ngernsiri, unpublished) as templates and T7 RNA polymerase-binding sites on the reverse primer from the above identified *B. dorsalis* segmentation genes. These DNA templates were purified, sequenced and used as templates using a DIG-labeling kit (Roche) as described (Fahmy *et al*., 2014). A non-related sense probe was used as a negative control. *In situ* hybridization followed a protocol according to (Fahmy *et al*., 2014).

Immunohistochemistry

D. melanogaster and *L. sericata* embryos were heat-fixed and stained with a monoclonal antibody against the Even-skipped protein, (mab 2B8, DSHB) at a concentration of 1:250, counterstained with DAPI and monitored on a Zeiss LSM 710 confocal microscope.

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