Dynamics of Growth Zone Patterning in the Milkweed Bug *Oncopeltus fasciatus*

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Summary Statement: We provide a highly detailed description of the morphological and cellular processes involved in posterior segmentation in an insect with a basal phylogenetic position and conservative developmental mode.
Abstract

We describe the dynamic process of abdominal segment generation in the milkweed bug *Oncopeltus fasciatus*. We present detailed morphological measurements of the growing germband throughout segmentation. Our data are complemented by cell division profiles and expression patterns of key genes, including *invecd* and *even-skipped* as markers for different stages of segment formation. We describe morphological and mechanistic changes in the growth zone and in nascent segments during generation of individual segments and throughout segmentation, and examine the relative contribution of newly formed versus existing tissue to segment formation. While abdominal segment addition is primarily generated through cell rearrangement of a pool of undifferentiated cells, there is nonetheless proliferation in the posterior. By correlating proliferation with gene expression in the growth zone, we propose a model for the dynamics of the growth zone during segmentation in which the growth zone is functionally subdivided into two separate regions: a posterior region devoted to a slow rate of growth among undifferentiated cells and an anterior region in which segmental differentiation is initiated and proliferation inhibited.
Introduction

A segmented body plan is a fundamental feature of arthropods. Nevertheless, the mode of segment determination varies considerably among different taxa, even within insects (Lynch et al., 2012). The fruit fly *Drosophila melanogaster* has been used as the main model organism for understanding the mechanisms of segmentation. However, with all of its advantages, *Drosophila* exhibits a derived form of embryonic development, making it a poor representative of more common and widespread types of arthropod segmentation (Davis and Patel, 2002; Mito et al., 2010; Peel et al., 2005).

In *Drosophila*, all body segments are defined almost simultaneously, via a process starting in the early syncytial blastoderm stage of development, and mediated by a cascade of interacting transcription factors (Hartenstein and Chipman, 2015; Lawrence, 1992; Nüsslein-Volhard and Wieschaus, 1980). This mode of development is referred to as “long germ” development. In contrast to this, the development of many basally branching insects is characterized by a mode of segmentation known as “short germ” (Davis and Patel, 2002; Krause, 1939; Liu and Kaufman, 2005b; Sander, 1976) or sequential segmentation. In this mode of development, only some segments form in the blastoderm stage. These include the head segments and may include, depending on the species, some or all of the thoracic segments. The remaining segments (some or all of the thoracic segments as well as the abdominal segments) are defined sequentially, one segment after the other from anterior to posterior, from a cellularized region at the posterior of the embryo. This region is referred to as the “growth zone”, or as the “segment addition zone” (Janssen et al., 2010). Sequential segmentation in insects is believed to reflect the ancestral mode of segmentation in arthropods (Peel et al., 2005; Stahi and Chipman, 2016).

While the details of simultaneous segmentation have been studied extensively in *Drosophila*, much less is known about the mechanisms of sequential segmentation. Specifically, many questions remain regarding the cellular aspects of this process, which takes place in a very different tissue environment compared to *Drosophila* (Peel et al., 2005). The role of cell proliferation and migration in the formation of segments from the growth zone varies among species and modes of segmentation (Beermann et al., 2011; Chipman, 2008; Copf et al., 2004; McGregor et al., 2009; Nakamoto et al., 2015; Oberhofer et al., 2014; Peel et al., 2005; Ten Tusscher, 2013). While some require intensive cell proliferation (e.g. malacostracan crustaceans (Dohle and Scholtz, 1988;
Scholtz, 1992; Wolff and Scholtz, 2002)), others, such as the centipede Strigamia maritima, seem to rely mainly on a pre-established pool of cells (Brena and Akam, 2013; Chipman et al., 2004b). The relative contribution of these two sources of cells to newly formed segments among different arthropods is unknown.

The embryology of the milkweed bug Oncopeltus fasciatus, a short germ hemipteran, has been described using classical techniques (Butt, 1949) and it is now re-emerging as a model for basal insect development. Its ease of rearing, availability of molecular tools, and recently sequenced genome make Oncopeltus an appealing system. In the development of Oncopeltus, all head, gnathal and thoracic segments are specified during the blastoderm stage (Ben-David and Chipman, 2010; Birkan et al., 2011; Liu and Kaufman, 2005b; Stahi and Chipman, 2016), in a manner resembling segmentation in Drosophila. By contrast, formation of abdominal segments starts de novo from a pool of undifferentiated cells in the growth zone. The growth zone is formed by the migration and proliferation of cells in the posterior blastoderm (Liu and Kaufman, 2004). These cells first form a small invagination in the posterior pole, moving inwards as the formation of the primordial germband occurs. By the time the germband has formed, ingressed cells include those fated to become the head and the thoracic segments and an undetermined region of cells that will undergo elongation and sequential segmentation to give rise to the abdominal segments.

In this work, we describe the dynamic process of segment generation in detail, by combining a morphometric analysis of carefully timed specimens with spatial and temporal patterns of cell division and gene expression. We show that the growth zone is functionally subdivided into two separate regions: a posterior region of undifferentiated cells devoted to growth – both through contributions of pre-existing cells and through cell division – and a region of reduced cell division devoted to initiating the specification of segments. Each region correlates with specific expression patterns of segmentation genes. We find that during the addition of a single segment, the growth zone undergoes dynamic changes in shape. We also observe significant variability in the size of the growth zone between individual Oncopeltus embryos, suggesting that the mechanisms that regulate segment addition from the posterior are robust to variations in size.
Results

Dynamics of the growth zone and newly formed segments

We measured various morphological parameters in the growth zone and recently formed segments over time (Fig. 1A-B; Supplementary Files 1A-C). Throughout the germband stage, the growth zone extends posteriorly, while new segments emerge from its anterior end. We find that the size of the growth zone – defined as the area from the posterior of the embryo to the posteriormost stripe of the segment polarity gene *invected* (*inv*) – decreases gradually in all dimensions (Fig. 1C-E). To confirm that the decrease in size is not due to cell death, we carried out anti-caspase staining, and found no notable pattern of apoptosis in the growth zone during these stages (Supplementary File 2).

Conversely, once a segment has been formed (as defined by the expression of a new *inv* stripe), it continues to grow in size. Importantly, growth within a segment at this time occurs solely along the anterior-posterior axis (segment length; Fig. 1G), and not in width (Fig. 1F). By contrast, segment width, as measured along the *inv* stripe, varies little throughout the segmentation of the abdomen. With the exception of the small decrease in width of the 7\textsuperscript{th} abdominal stripe (establishing the 6\textsuperscript{th} abdominal segment), between stage A8 and A9 (Fig. 1F, p<0.01), segment width is the most consistent dimension we measured. Conversely, segment length increased significantly for every segment following its formation, and segment area increased significantly for all except the first and second segment (Fig. 1G-H). We note that although all the trends detailed above are clear, there is a fair amount of variability within each parameter.

Growth and segmentation

During development, the growth zone gradually decreases in size, due to the formation of new segments. However, while new segments are formed, the growth zone itself is in fact growing, but not at a rate that compensates for the loss of area due to segment formation, with the exception of the transition between stage A1 and A2 (Fig 1E). To assess the growth during a single stage, the average area of the growth zone at the current stage plus the area of the most recently formed segment was divided by the average area of the growth zone in the previous stage (Fig. 2A1-2). This value includes a simplification; it assumes that the thickness of the growth zone and the newly formed segment are equal, and that the thickness of both is roughly uniform over time. While
there may be a change in thickness in the transition from growth zone to segmental tissue, this would cause a slight underestimation or overestimation of the growth rate, but should not affect the pattern observed. In this calculation, a value of 1 indicates that the size of the new segment is equal to the area lost by the growth zone, and no additional growth has taken place. Conversely, a value >1 indicates that growth took place in the growth zone and/or latest segment during this stage.

Calculated this way, our data shows that per stage, the growth zone increases in size between 0.9% (± 3.7%, stage A3-4) and 9.8% (± 6.1%, stage A1-2) (Fig. 2B; note that error bars represent propagated standard error of a calculation and not the distribution of a direct measurement). While growth rates appear to differ from one stage to the next, these differences are not significant.

Nevertheless, this possibly discontinuous growth relative to segment number, in particular the change in growth rate from segment A1-A2 to A3-A4, raises the question how growth relates to segmentation rate. Specifically, we wanted to know if abdominal segmentation is a linear process, or whether the rate of segmentation changes between stages. Our ability to resolve this question is complicated by the combination of the reproductive biology of *Oncopeltus* and our method of egg collection. Because eggs are laid in large clutches, at a rate of approximately one egg every 1-2 minutes, a single clutch might constitute a large proportion of the eggs sampled in a time window. This would inadvertently bias the birthdate distribution within the sample time window, by essentially synchronizing a large part of the sample. Thus, we collected a second data set in which clutches were explicitly broken up and randomized over different time windows. The data show a linear rate of segmentation ($R^2=0.9151$), with a new abdominal segment forming every 1.5h (Fig. 2C). No evidence was found to indicate a deviation from the linear segmentation rate (Chi squared test p-value = 0.6738, see Methods).

**Gene expression in the growth zone**

We then asked how the morphology of the segmenting germband correlates with molecular processes. To address this, we followed the expression of four genes with documented roles in sequential segmentation in insects.
*inv* mRNA is expressed, as expected, in the posterior of each molecularly defined segment. *inv* is a paralog of *engrailed* and has very high sequence identity and in some species, identical expression, (Campbell and Caveney, 1989; Peel et al., 2006; Peterson et al., 1998). We, and others, use the *en/inv* expression pattern to define the border between the segmented germband and the unsegmented growth zone (Fig. 3).

Fig. 3A shows a sequence of *inv* stained embryos from the beginning of posterior segmentation (stage A1) up to the determination of the 9th abdominal segment (stage A9, at which point our analysis ended). Fig. 3B shows the dynamics of *inv* expression over the formation of a single segment (in this case, the first abdominal segment). For this analysis, a single clutch was collected within 30 minutes and individual embryos were sampled from this clutch every 30 minutes (note that there is some variability in the age of the embryos within a clutch, so the timing is not precise). The expression of an individual segmental *inv* stripe matures over the duration of an hour, appearing initially as a thin stripe that is discontinuous along the ventral midline, gradually thickening and maturing into a continuous stripe. While we did not carry out high temporal resolution analysis for the formation of other segments, the variation observed in the posterior-most stripe within our other samples indicates that this sequence of events is typical of other abdominal segments.

The time course in Fig. 3B also shows that the shape of the growth zone changes dramatically during the formation of a new segment. The growth zone starts out round and elongates to a teardrop shape as the *inv* stripe is consolidated. Although we have not followed the shape change during the addition of more posterior stripes, we suggest that this change in shape is indicative of a cyclic process of cells rearranging in the posterior to form the next segment, and that this process might be occurring during the formation of the other segments as well.

*caudal* (*cad*) mRNA is expressed in a stable and uniform manner in the posterior of the growth zone throughout development (Fig. 4A). Expression is strongest in the posterior of the growth zone, and diminishes in a gradient towards the anterior of the growth zone, where it is absent. There is almost no change in the extent or level of expression in the growth zone throughout the segmentation process. After all segments have formed, expression clears slightly from the very posterior of the growth zone, perhaps indicating that the process of segmentation is completed.
**even-skipped** (*eve*) mRNA expression (Fig. 4B) is characterized by two distinct areas of expression: in the posterior growth zone *eve* expression is uniform and stable throughout germband elongation and segmentation. In contrast, *eve* expression in the anterior growth zone displays a striped pattern. The number of stripes is variable and dynamic, with 3-4 stripes in the earlier stages of segmentation and only 2-3 stripes at later stages. The expression of *eve* in *Oncopeltus* has been previously described in detail by Liu and Kaufman (2005a).

**Delta** (*Dl*) mRNA has a more complex expression pattern (Fig. 4C) and is expressed in two distinct domains. The first is a speckled pattern marking pro-neural tissue in the head lobes, and continuing along the segmented germband in two medio-lateral rows of pro-neural cells, as previously described in other arthropods (Chipman and Stollewerk, 2006; Eriksson et al., 2013; Kainz et al., 2011; Stollewerk and Chipman, 2006). More relevant to segmentation, *Dl* is expressed in stripes in the anterior growth zone. 2-3 stripes are present in the anterior growth zone in early stages of segmentation and 1-2 stripes at late stages of the process. The stripes vary in strength of expression, and the position of the strongest stripe is variable between embryos collected within the same 2-hour time window. Closer examination reveals that there is sometimes an overlap between the segmental and pro-neural patterns, with stronger expression in lateral spots within the segmental stripes. Notably, there is almost no detectable *Dl* expression in the posterior growth zone during segmentation.

**Cell proliferation**

Labeling for phosphorylated histone 3 (PH3), to mark cells in mitosis, uncovers a simple yet striking pattern (Fig. 5A; Fig. 6A), in which cell proliferation is detected in the posterior part of the growth zone, followed by a gap or “window” of variable size and appearance in the anterior growth zone (indicated by an asterisk in Fig. 5), where there is clearly decreased cell proliferation. An increase in relative cell proliferation is detected anterior to this window in the segmented germband. No similar gap is observed in other embryonic regions.

In order to better describe the borders of this domain of decreased PH3 expression, and possibly its role in the segmentation process, the PH3 labeling was repeated in combination with in situ hybridization for the genes detailed above. The most notable link between PH3 staining and gene expression is the overlap between the
posterior proliferative zone and the expression of \textit{cad} and the posterior expression region of \textit{eve} (Fig. 5B,C). The domain of reduced proliferation correlates with the region of striped \textit{eve} expression and expression of \textit{inv} is always anterior to this domain. The borders of \textit{Dl} expression do not correlate consistently with the borders of this window (data not shown). These correlations are consistent throughout the entire process of abdominal segmentation.

To further quantify the distribution of PH3 positive cells with \textit{eve} mRNA expression, we analyzed 68 embryos ranging from 44-56hAEL labeled with both \textit{eve} and PH3. We chose the expression pattern of \textit{eve} for delimiting zones for detailed analysis of cell proliferation (Fig. 6B), since it provides a pattern defining three primary regions of interest: (i) posterior growth zone, defined by a solid staining in the most posterior part of the embryo, (ii) anterior growth zone (roughly equivalent to the pre-segmental region, as in Schröder et al. (2008)), defined by a striped expression pattern and (iii) the segmented germband, which extends anterior from the anteriormost \textit{eve} stripe.

Using a custom designed macro, we scored the total number of cells in each of those regions (counting nuclei labeled with DAPI), as well as the number of cells undergoing mitosis (counting cells positive for PH3). The ratio between these counts gives us the fraction of cells that are undergoing mitosis in the different regions (Fig. 6C). Indeed, as detected visually, the relative number of proliferating cells in the anterior growth zone is significantly lower (ANOVA, p<0.001) than the number in the posterior growth zone. The fraction of proliferating cells in the segmented germband is also significantly higher (ANOVA, p<0.001) than the anterior growth zone, as well as the posterior growth zone, though this difference is marginal (but significant: ANOVA, p<0.05). This pattern is consistent throughout abdominal segmentation (Supplementary File 3).

\textbf{Discussion}

The development of the abdominal segments in the hemipteran \textit{Oncopeltus fasciatus} occurs through the sequential addition of segments from a posterior growth zone. While there is a growing body of data on sequential segmentation in other arthropods (Brena and Akam, 2013; Chipman and Akam, 2008; Choe et al., 2006; Copf
et al., 2004; Damen et al., 2000; Janssen et al., 2010; Kainz et al., 2011; Lynch et al., 2012; McGregor et al., 2009; Nakamoto et al., 2015; Olesnicky et al., 2006; Sarrazin et al., 2012; Schoppmeier and Damen, 2005; Williams et al., 2012), we are the first to combine carefully timed morphometric measurements with gene expression patterns and measurements of cell division. This combined approach allows us a more precise understanding of the dynamic process of segment addition in *Oncopeltus*. In this work we have focused on posterior segmentation, aiming to give novel insights into the basic morphological dynamics during germband elongation and segmentation in the insect embryo, and the involvement of cell proliferation and key developmental genes to this process.

**Morphological changes in the growth zone**

The most obvious result from our morphological analysis of the growth zone is its steady decrease in size over the process of segment generation, as tissue leaves the growth zone to contribute to the nascent segments. This is hardly surprising, and although it has rarely been quantified, it is a well-known aspect of the process of segmentation. These results mirror the findings of Nakamoto et al. (2015) in the embryo of the red flour beetle *Tribolium castaneum*. The decrease in size in *Oncopeltus* is manifested mostly in the length of the growth zone, and to a lesser extent, in its width, leading to a constant decrease in area. Once new segments are defined molecularly, their width changes very little throughout the segmentation process. Meanwhile, their length and area increase slightly, indicating that there is additional growth after segment formation, as would be expected from the observed cell proliferation in the nascent segment.

**Variability**

One of the intriguing points arising from our measurements is the large variance in growth zone and segment size and shape. Since the mean value of segment width changes little, if at all, over development (Fig. 1F), its variability at different stages is a good indication of the overall variation that exists between individuals (Supplementary file 5). Variation in this one-dimensional parameter (i.e. width of the penultimate stripe) spanned an up to twofold difference between the smallest and largest embryo measured. Confounding factors in this observation could be
experimental, such as measurement or mounting errors. To account for measurement errors, all photos were taken at a standard magnification and all measurements were repeated three times and averaged. Mounting differences between slides were found to account for some of the variance in our measurements (roughly 21.5%, see Supplementary file 5), leaving a conservative 52% increase in this parameter between the smallest and largest animals. Some of this variability is no doubt related to the known variability in egg size in insects (Houchmandzadeh et al., 2002), however, we did not measure egg size prior to dissection of the germband. The variability suggests that the segmenting embryo is robust to large changes in the surface area of the growth zone.

**Short term changes in shape in the growth zone**

In Fig. 3B, we show dramatic changes in growth zone shape that occur during the formation of a single segment. These changes demonstrate that some variance is due to the sub-stages of segment formation, and show that the shape of the growth zone and of the nascent segments is dynamic during the addition of each segment. Although the cellular mechanisms responsible for this more rapid shape change were not a part of this study, we speculate that coordinated changes of cell shape and or cell rearrangements driven by actin-myosin contractions may play a role. Unraveling the complexities of the sub-phases of segment generation requires much higher temporal resolution than we have been able of achieve in the current study and must await future work.

**Rate of segment generation**

While being a reiterative process, abdominal segmentation may not occur at a steady rate (Fig. 2C). In *Oncopeltus*, we show that segmentation rate is linear (Fig. 2C). By contrast, a non-linear segmentation rate has been shown in *Tribolium* (Nakamoto et al., 2015). In *Tribolium*, sequential segment addition includes all the post-mandibular segments and the change of segmentation rate occurred during the transition from thoracic to abdominal segment addition. Because the change in segmentation rate correlated with a change in the behavior of marked blastoderm clones, Nakamoto et al. (2015) hypothesize that the two might be linked, and thus change in a coordinated
fashion during the transition from thoracic to abdominal segmentation. In *Oncopeltus*,
the constant segmentation rate is observed within the production of a single body tagma.

**The source of segmental tissue**

There is some debate over whether there is any growth (generation of new tissue) in the
growth zone, or whether all of the tissue that contributes to new segments is present from its origin (Chipman, 2008; Peel et al., 2005). Our data show that most of the tissue in newly formed segments in *Oncopeltus* is derived from existing growth zone tissue. However, there is a certain contribution of cell proliferation to this process, as the tissue recruited to the new segment (in most cases) is greater than the decrease in size of the growth zone and cells undergoing proliferation in the posterior of the growth zone are detected during all stages analyzed.

Most of the proliferation occurs at the posterior of the growth zone, at the point in the growth zone most distant from where the nascent segments form (Fig. 6A; Supplementary File 3). Conversely, tissue from the anterior part of the growth zone contributes to the formation of new segments. While the growth zone diminishes in size with the formation of each segment, it is replenished to a certain extent by cell proliferation in the posterior.

**A functional model for the arthropod growth zone**

Our data allows us to formulate a generalized model for *Oncopeltus* (Fig. 7), and use it as a basis of evolutionary comparison among arthropods. This model is consistent with partial data from several other arthropod species (Brena and Akam, 2012; Brena and Akam, 2013; Chipman and Akam, 2008; Nakamoto et al., 2015; Schröder et al., 2008; Williams et al., 2012).

We see the segmentation process taking place over three distinct embryonic domains. The posterior growth zone contains undifferentiated cells. This domain is characterized by the expression of *cad* and by stable expression of *eve*. At the cellular level, proliferative activity is detectable in this domain throughout segmentation in *Oncopeltus* with a similar result being reported for *Tribolium* (Sarrazin et al., 2012). Uniform expression of both *cad* and *eve* has been demonstrated in the posterior growth zone of many arthropods (Brown et al., 1997; Chipman and Akam, 2008; Copf et al.,
2003; Dearden and Akam, 2001; El-Sherif et al., 2014; Hughes and Kaufman, 2002; Mito et al., 2007; Patel et al., 1994; Shinmyo et al., 2005). We suggest that cad, and potentially eve, are responsible for maintaining the cells of the posterior growth zone in an undifferentiated state, and that this is a general feature of arthropod posterior growth zones. Wnt signaling is upstream of cad in several arthropods (Chesebro et al., 2013; McGregor et al., 2009; Shinmyo et al., 2005) and may be the initiator of growth zone function.

The anterior growth zone is where cells undergo a series of specification events leading up to their recruitment into nascent segments. This domain is characterized by the expression of genes in a dynamic pattern. In our results for Oncopeltus, and in the equivalent domain of other arthropods, these include orthologs of Drosophila pair-rule genes, and in some cases also Notch pathway genes (Brena and Akam, 2013; Chipman and Akam, 2008; Choe et al., 2006; Damen et al., 2000; El-Sherif et al., 2014; Eriksson et al., 2013; Mito et al., 2007; Mito et al., 2011; Patel et al., 1994; Pueyo et al., 2008; Stollewerk et al., 2003). In Tribolium, the dynamic expression pattern of the pair-rule genes has been shown to be a traveling wave of gene expression and in other species expression data is consistent with this idea (Chipman and Akam, 2008; El-Sherif et al., 2012; Pueyo et al., 2008; Schoppmeier and Damen, 2005). We suggest that this domain is equivalent to the “transition zone” of the centipede Strigamia maritima (Chipman et al., 2004a) and the pre-segmental region (PSR) of Tribolium castaneum as defined by Schröder et al. (2008). Using carefully timed embryos, in a combined analysis of gene expression with patterns of cell divisions, we found that the most conspicuous characteristic of this domain in Oncopeltus is the significantly decreased level of cell proliferation. We find evidence for a similar correlation of proliferative activity in the fairy shrimp, Thamnocephalus: cells of the anterior growth zone do not undergo DNA synthesis while those in the posterior growth zone do (as indicated by EdU incorporation, T.A. Williams, personal observation). We predict that a similar correlation between reduction in cell division and the onset of segmental specification will be found in other arthropods.

Cells of the anterior growth zone are sequestered into the third domain; the posterior segmented germband, where they start expressing segment polarity genes and differentiate into segmental tissue with distinct fates. Based on what has been shown in Tribolium, this sequestration most likely includes a significant contribution of cell
movement (Benton et al., 2013; Sarrazin et al., 2012), probably cell intercalation through convergent extension, but we have not attempted to follow such movements in the current analysis. In this domain, cell proliferation and growth continue. The boundary between the posterior segmented germband and the anterior growth zone is defined by the expression of \textit{inv/en}.

**Concluding remarks**

The term “growth zone” has fallen out of favor in recent years, since this term had traditionally been assumed to refer to a region of high proliferative activity used to generate a continual supply of cells for segment generation. More recent work has demonstrated the diversity of patterns of cellular activity within the growth zones of sequentially segmenting arthropods (Brena and Akam, 2013; Chipman, 2008; Chipman et al., 2004b; Copf et al., 2004; Dearden and Akam, 2001; Dohle and Scholtz, 1988; El-Sherif et al., 2012; Kainz et al., 2011; Nakamoto et al., 2015; Olesnicky et al., 2006; Pueyo et al., 2008; Scholtz, 1992; Williams et al., 2012), ranging from species that rely heavily on posterior proliferative activity, to those that rely more extensively on cellular rearrangements, to all manner of variation between these two extremes. We have shown that there is growth through cell proliferation, as well as contributions from pre-existing cells in the growth zone of the milkweed bug \textit{Oncopeltus fasciatus}. We argue for maintaining the term “growth zone” as a generally understood term for the area from which the germband grows – albeit using a diversity of cellular mechanisms.

Our analysis provides a highly detailed description of the processes involved in posterior segmentation by characterizing the cellular domain in which it arises, and linking cell division to expression of segmental regulators. Posterior segmentation is a defining feature of arthropods and clearly appeared early in their evolutionary history. A better comparative understanding of how known regulators operate within a diverse array of cellular contexts will contribute to our understanding of the evolution of the arthropod body plan.
Material and methods

Embryo preparation

Methods for the embryology of Oncopeltus (egg collection, fixation, dissection, in-situ hybridization, and imaging) are as previously described in Ben-David and Chipman (2010). Unless otherwise noted, embryos were collected in one-hour and two-hour windows, and placed in a 25°C incubator until fixation at the age of interest. In some cases, collections consisted of part of a clutch that was in the process of being laid.

Gene cloning and probe preparation

Two of the four probes used in this study were for genes cloned previously. The gene we refer to throughout this manuscript as *invected* (*inv*) was originally identified as *engrailed* and has appeared as such in previously published papers (Angelini and Kaufman, 2005; Erezyilmaz et al., 2009; Liu and Patel, 2010; Weisbrod et al., 2013). However, a reanalysis of this sequence following the sequencing of the full genome of *Oncopeltus* revealed that it is in fact the *engrailed* paralog *invected* (Peel et al., 2006). This sequence was extended using primers designed from genomic sequence to give a probe of 873 bp.

The sequence for *even-skipped* (*eve*) is based on Liu and Kaufman (2005a), and was extended using primers based on the full genomic sequence to give a probe of 770 bp.

Cloning of *caudal* (*cad*) was done with gene specific primers, designed according to an unpublished transcriptome, to give a probe of 513 bp, and later verified using genomic data. *Delta* (*Dl*) was originally cloned through degenerate PCR, then re-cloned using specific primers based on genomic sequence to give a probe of 712 bp.

Genbank accession numbers for the cloned sequences are: *Delta*: KU870474, *caudal*: KU870475

Probes were prepared with digoxigenin-labeled UTPs (Roche) using the DIG RNA Labeling Kit (Roche), with linearized T-easy plasmids (Promega) containing the target sequence as template (Ben-David and Chipman, 2010). The primers used for the probes are in Supplementary File 4.
**in situ hybridization and antibody staining**

In situ hybridization was carried out as described previously (Ben-David and Chipman, 2010), and developed using BM-Purple (Roche).

In double stainings with in situ hybridization, anti-phosphorylated histone H3 (PH3) antibody was added (1:500; Abcam ab14955), simultaneously to the alkaline phosphatase-anti DIG antibody (1:4000; Roche) in an overnight (4°C) incubation. Three to five washes were performed at room temperature the following day, and after a 30 min secondary block (10% normal horse serum (Vector labs), or normal goat serum (Thermo Scientific) the secondary antibody (Alexa 448, anti-mouse, 1:200; Invitrogen) was added for a two-hour incubation in dark conditions. Three to five washes were done, after which the in situ hybridization protocol was continued as usual.

**Growth zone measurements**

Growth zone measurements were done manually on captured images of embryos stained for the segment polarity gene *inv*, using a Fiji (Schindelin et al., 2012) macro designed to collect and organize the data. The dimensions that were measured included length, width, and area of the growth zone and of the two posterior-most segments (Fig. 1). The width of the growth zone was measured at its widest point. The segment widths were measured on the *inv* stripes. Each dimension was measured three times, and the average was used as a single data point. The areas of interest were measured in 235 embryos, covering all stages of abdominal segmentation (See Table 1, and Supplementary File 1). Each stage was represented by at least 16 embryos. For each embryo, temporal age was noted (in hours post egg laying), as well as its developmental stage defined as the number of abdominal segments expressing *inv*.

Measurements were processed and analyzed using custom Python (Van Rossum, 1995) and R (R. Development Core Team, 2008) scripts. Pairwise comparisons were performed using one-way ANOVA, and corrected for multiple testing (per dimension measured; e.g. growth zone width) with the Holm procedure (Holm, 1979).
**Segmentation rate**

Segmentation rate was assessed for a deviation from linear using a separately collected specifically designated sample of 123 embryos. Embryos from multiple clutches were collected over the space of one hour, then randomly assigned a time window (ranging from 44-55h after collection) and fixed at this time. The collection thus generated contains embryos whose ages are randomly spread within each 60-minute time window. From this data set, 9 embryos were sampled randomly in each time window, to generate a total sample of 108 embryos that can be assumed to be a uniformly distributed sample within the total age range of 44-56hAEL. If any stage of segmentation is shorter or longer than the others, then it should be over or underrepresented in this uniform sample (when excluding stages 1 and 9, as we cannot assume they start exactly at 44h and end exactly at 56h, respectively). This was tested using a Chi-squared goodness of fit test, using the R platform.

**Visualization of cell division**

Fluorescent images were acquired with an Olympus FV1200 spectral confocal system based on an IX-83 inverted microscope stand, using a 40X/NA=0.95 air objective. Confocal fluorescence images of DAPI and anti PH3-Alexa488, and non-confocal transmitted-light DIC images, were acquired. The DAPI channel used 405nm excitation and a 430nm low pass emission band, and the Alexa488 channel used 488nm excitation and a 505nm low pass emission band, acquired sequentially. Z-stacks were acquired with 1.5 µm spacing. Fields were tiled with no overlap in order to acquire large areas. Stacks were collapsed to a single image using a maximum intensity Z-projection.

Combined *in situ* and antibody labelling images were created by filtering out the BM-purple staining only and converting it to red pseudocolor using Adobe Photoshop, and combining that as a separate channel with the DAPI and PH3 channels.

For averaging cell division patterns, 35 images of different embryos from mixed stages (in the range of 46-54 hAEL; note that exact stage cannot be determined based on morphology alone) were aligned on the widest part of the growth zone. These images were subsequently averaged in FIJI and subjected to a Gaussian blur with sigma=1 µm to generate a heatmap of cell division in the growth zone.
Quantification of cell division

The total number of PH3-alexa-488 labeled cells, and the total number of DAPI-labeled cells, were calculated using the Fiji distribution of ImageJ (Schindelin et al., 2012; Schneider et al., 2012), and the 3D-Droplet Finder plugin (available at: http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:droplet_counter:start). Briefly, the raw images were leveled using a rolling-ball filter (radius=50), blurred (Gaussian-Blur, sigma=1.75 μm), and then run through the Droplet-Finder plugin set. A Python script was written to convert the output of the droplet finder plugin to a set of point regions of interest. The enabled us to mark the detected nuclei and visualize them with the 3D-viewer plugin. In a crowded 3D field, the cells could not be manually counted, so we cannot provide a quantitative comparison to “ground truth”. However, the segmentation was consistent with qualitative observation of the marked nuclei. For the more sparsely labeled PH3-alexa488 images, the automated detection was within 91.3(±15.2)% of ground-truth when compared to human observation.

We note that DAPI staining is not maintained throughout mitosis, and as such the total number of cells as counted by DAPI stained nuclei is underestimated by our count. Given that the proportion of cells in mitosis is already low (2-5%), the underestimation of the total number of cells falls within the range of error and was not further corrected.

The procedure was applied to three different areas in the growth zone, which were distinguished by the expression pattern of *eve* (Fig. 6B). This analysis was done on a sample of 45 embryos, stained for PH3 and *eve* RNA, separated into different 2-hour age groups, evenly spread out over our ages of interest from 44-56hAEL, as well as 23 additional embryos for which age was unknown (Supplementary File 3). One-way ANOVAs were done on the ratio of PH3/DAPI positive cells using R, comparing the proportion of cells in mitosis in the different areas of the growth zone, controlled for individual embryos, and corrected for multiple testing with the Holm procedure (Holm, 1979).
Acknowledgements
A pilot of the growth zone measurement was done by Sharon Lavon as part of her high school matriculation project. We thank Janice Hester for her valuable advice on our statistical analysis of segmentation rate. Three anonymous reviewers helped improve and clarify the manuscript.

Competing Interests
The authors declare no competing interests.

Author Contributions
TA carried out all experiments, collected, stained and imaged embryos, cloned the relevant genes and did most of the measurements. BMIV did most of the data analysis, wrote Python and R scripts and prepared most of the figures. TW, LMN and ADC designed the study. SDH contributed to study design and to data analysis. AW wrote the macro for quantification of cell division. TA, BMIV and ADC wrote the initial version of the paper, all authors contributed to multiple rounds of revision and data analysis. ADC supervised the work. All authors read the paper and approved the final version.

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Data Availability
Most custom FIJI macros and R and Python scripts used in this study are available at github.com/bvreede/growthzone. The macro used for the quantification of cell division is available from the authors.
References


Figures

**Figure 1: Measurements on growth zone and segments**

A) Cartoon of *O. fasciatus* embryo after segmentation is complete. Abdominal segments are color-coded, with the posterior *inv* stripe of each segment in a lighter shade. B) Cartoon of growth zone and newly formed segments, indicating the measurements done. C-E) Violin plots representing the distribution of measurements on the growth zone, by developmental stage (number of *inv* stripes). Pair-wise one-way ANOVAs were done to assess statistically significant changes in dimensions from one stage to the next. Asterisks indicate significant changes: * = p < 0.05; ** = p < 0.01; *** = p < 0.001. F-H) Violin plots representing the distribution of measurements on the two most recently formed segments, and the three most recently formed *inv* stripes, by developmental stage (number of *inv* stripes). The colors correspond to the segment colors in panel A. Pair-wise one-way ANOVAs were done to assess statistically significant changes in dimensions of the same segment from one stage to the next. Asterisks indicate significant changes: * = p < 0.05; ** = p < 0.01; *** = p < 0.001, and colors correspond to the segment in question. In panel F, asterisks above the plot concern the first pair of measurements of a segment (e.g. stage A1 vs A2 for segment 1), and below the plot are the final pair of measurements of a segment (e.g. stage A2 vs A3 for segment 1).
Figure 2: Growth and segmentation

A1) Cartoon showing the areas used for the calculation of growth. A2) growth at stage $n$ was measured by dividing the combined area of the growth zone and the most recently formed segment by the average area of the growth zone in stage $n-1$. A3) Due to the way growth is calculated (i.e. using the averages of all individuals in the same stage), the developmental time for which growth is calculated is offset with respect to the segmental stage. The schematic shows how stages used on the y-axis for panel C and the x-axis for panel B relate to each other. B) Growth as calculated shown in panel A, using measurement averages. Error bars indicate the (propagated) standard error of the mean. C) Staged embryos collected in 60-minute windows ($n=123$), from 44-45h to 55-56h AEL, as well as the linear model fitting this data (red line).
Figure 3: The changes in the growth zone throughout the segmentation process.
A) Embryos of increasing age (stages A3-A9) from left to right stained for the expression of inv. Note the gradual decrease in growth zone size as segmentation proceeds. B) Early embryos in stage A0-A1 (during the formation of the first abdominal segment). Embryos from a single clutch fixed in 30’ intervals demonstrate changes in
growth zone dimensions during the formation of a single segment. The growth zone begins round, gradually elongating, assuming a teardrop shape as the new segment slowly buds from the anterior growth zone. Abbreviations: hl: head lobe, md: mandibular segment, mx: maxillary segment, lb: labial segment, T3: third thoracic segment, A1: first abdominal segment.
Figure 4: Developmental gene expression patterns during germband segmentation.

Expression pattern of caudal (A), even-skipped (B) and Delta (C) mRNA at different developmental stages, from the earliest germband (~40 hours after laying) to the final stages of abdominal segmentation (~55 hours after laying). A) cad is expressed in a simple and stable manner in the posterior growth zone throughout germband segmentation. B) eve displays a more complex expression pattern, in which the entire posterior growth zone expresses eve, yet in the anterior growth zone eve is expressed in a dynamic striped pattern. C) Dl is expressed in the anterior growth zone in a varying number of stripes, with no expression in the posterior growth zone. More anteriorly, Dl is expressed in the nervous system seen as two mediolateral lines of punctate expression, extending posterior from the head lobes to (but not including) the growth zone. GZ: growth zone, hl: head lobe, md: mandibular segment, mx: maxillary segment, lb: labial segment.
Figure 5: Cell division and gene expression in the growth zone.
Double stainings of anti-PH3 (green) as an indicator of cell division with in situ hybridization for segmentation genes (red pseudocolor, detected using brightfield). DAPI is used as nuclear counterstain in all stainings (blue). Brackets labeled ‘GZ’ mark growth zone area; brackets labeled with an asterisk mark a gap in cell proliferation in the anterior growth zone. The precise age of the embryos is not known, but they are all towards the end of posterior segmentation (~50 hours after laying). A) Embryo stained with anti-PH3 and DAPI without in situ staining. The gap in cell proliferation is noticeable in the anterior GZ (asterisk). B) cad staining correlates with area with increased PH3 marked cells. Note that the anterior red staining is an artifact of the image merge process and is not seen in single stained embryos (compare Fig. 4A). C) eve in the posterior overlaps with cad expression. The striped expression pattern of eve
corresponds to an area with decreased PH3 staining. D) *inv* marks the anterior border of the growth zone and the boundary between high PH3 staining (anteriorly) and low PH3 staining (posteriorly).
Figure 6: Cell proliferation in different areas of the germband

A) Heat map of cell proliferation in the germband. Merged image of 35 PH3-stained germbands in various stages, aged from 46-54hAEL, aligned at the widest part of the growth zone, translated to a look-up table (legend below the panel). The dotted lines indicate where the zone of low proliferation starts and ends. B) Cartoon of even-skipped expression, indicating the different areas for which cell proliferation was calculated in panel C. C) Proportion of cells in mitosis in each area (see panel B), calculated using the number of cells positive for PH3 divided by the number of nuclei labeled by DAPI staining. Asterisks indicate statistically significant differences between areas: * = p < 0.05; ** = p < 0.01; *** = p < 0.001.
Figure 7: A model of the *Oncopeltus* growth zone

The segmentation process takes place over three distinct domains. The posterior growth zone is characterized by the expression of *caudal* and the stable expression of *eve* and probably other pair-rule gene orthologs (jointly indicated in yellow). It is also characterized by a relatively high level of cell division (indicated schematically by densely packed large dots). The anterior growth zone is characterized by the dynamic expression of pair-rule gene orthologs, in some other arthropods Notch pathway ligands have a similar expression domain (jointly indicated by light blue stripes). Cell division levels are significantly lower than the domains anterior and posterior to it (indicated schematically by sparsely distributed small dots). Cell movements in this domain lead to the constriction of the growth zone and to the extension of the posterior growth zone posteriorly. The dynamic cyclical expression of genes in this domain leads to the sequential sequestering of the anteriormost tissue into the segmented germband. The posterior of the germband is defined by the expression of *inv* (indicated by a red stripe). Cell division levels in this area are higher than those in the anterior growth zone (indicated schematically by densely packed large dots).
Supplementary file I

Dataset of abdominal segmentation in *Oncopeltus fasciatus*

Three datasets are enclosed to this paper:

- Supplementary file 1A: Raw data after measurements on imaged *O. fasciatus* embryos. Each image was measured three times, consecutively. The location of the measurements (a1-a3; w1-w4; l5-l8) are indicated in the image below. All units are in µm.

- Supplementary file 1B: Processed data: entries are averaged from three measurements done in the raw data. The measurements are again indicated in the image below.

- Supplementary file 1C: Additional data on segment number and age, used for the calculation of segmentation rate.

The R² of all items in the dataset was calculated and plotted on a heatmap, below:
Supplementary File 2: Apoptosis in the growth zone

In order to confirm whether apoptosis has a role in the changing size of the growth zone, we have stained against the apoptotic marker caspase (Using the same protocol as for anti-PH3 staining, but with the following reagents: Primary antibody: abcam13847, dilution 1:2000. Secondary antibody: anti rabbit HRP, DAB substrate). Examining the embryo while still in the yolk, reveals an abundance of apoptotic cells surrounding the embryo, presumably in extra-embryonic tissues. This can be seen in the un-dissected embryo from different angles (A-A’’) (examples pointed out by arrows). However, when embryos are dissected out of the yolk, the embryonic tissue is seen to be almost completely free of apoptotic cells: (B) early germband, approximately 48 hAEL (C) late germband, approximately 64 hAEL. Very few apoptotic cells are observed in the gnathal thoracic, or abdominal segments. A somewhat greater amount of apoptosis is detected in the head lobe, and may be associated with the formation of the nervous system. In later embryos, we notice apoptosis concentrated in the tip of the developing limbs (panel C, marked by arrows). Very few apoptotic cells are scattered in the mesoderm cells of the growth zone. The number of apoptotic cells in the growth zone is more than an order of magnitude lower than the number of dividing cells (i.e. 43.03 +/- 16.50, n=68). We thus consider apoptosis to be a negligible factor in growth zone dynamics. hl – head lobes, md, mandibular segment, mx – maxillary segment, lb – labial segment.
Supplementary file III

Mitosis in the growthzone of *Oncopeltus fasciatus*

Supplementary file 3A contains the data of all measurements on mitosis in the *O. fasciatus* growth zone and germband, collected as described in the methods section of the accompanying manuscript. Briefly, data was collected over three sections of the germ band (also see Fig. 6B):

- posterior growth zone, characterized by solid *even-skipped* (*eve*) expression;
- anterior growth zone, characterized by striped *eve* expression;
- germband anterior to the growth zone, characterized by the absence of *eve* expression.

In each of those sections, the number of cells stained by DAPI and by anti-PH3 (a marker of cell division), were counted using a custom FIJI macro (available from the authors). The ratios were calculated and explicitly presented in the dataset as well as plotted in Fig. 6 and below.

In addition to the collective data, as presented in Fig. 6C, for 45 embryos (out of 68 in the total dataset) ages were included in the analysis. (Note that staging was not possible because this requires *engrailed* staining, which would have conflicted with *eve* necessary for the division into relevant sections.) This allowed us to assess whether the pattern we observed (i.e.: the proportion of cells in mitosis is low in the anterior growth zone compared to the posterior growth zone and the germband) holds true throughout abdominal segmentation.

Paired one-way ANOVAs were performed in R to assess statistical significance of the difference between mitosis ratios in tissues (see Methods in the manuscript; the linear model controlled for individual embryos, and p-values were corrected for multiple testing using the Holm procedure). Significant p-values (<0.1) are reported below the plot.

![Mitosis in the germ band](image)

<table>
<thead>
<tr>
<th></th>
<th>posterior GZ vs. anterior GZ</th>
<th>anterior GZ vs. germband</th>
<th>posterior GZ vs. germband</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (hAEL)</td>
<td>44  46  48  50  52  54  56</td>
<td>44  46  48  50  52  54  56</td>
<td>44  46  48  50  52  54  56</td>
</tr>
<tr>
<td>proportion of cells in mitosis</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.001</td>
<td>n/s</td>
</tr>
<tr>
<td>n embryos</td>
<td>9   7   6   7   6   10</td>
<td>9   7   6   7   6   10</td>
<td>9   7   6   7   6   10</td>
</tr>
</tbody>
</table>
Similarly, embryos ranging from 46-54hAEL were split up in 2h time windows to generate heatmaps (as in Fig. 6A).
**Supplementary File 4: Primers used.**

**eve:**
F: AGGGTGCTGGAGCGGAGGGG  
R: GCCGCAGGACAAACTTGGATT

**Delta:**
F: AGTGCCCTCCATCCGCTGT  
R: GCTGTTGACGGCTCCTTG

**cad:**
F: TCACACCAGACTCCAGGAAA  
R: AAACAGTGCTGAAAAGATAC

**inv:**
F: TCAATCGGATGTAGTAGGA  
R: TCGGCAAAGGTCTTGGCCAT
Supplementary file 5

Variability in the data

In the dataset, we see a large amount of variation in the sizes of embryos at each stage. This is an intriguing observation, as the process of development needs to be robust to these size variations, if they are real. To determine whether this is the case, we need to assess the amount of experimental noise, and its influence on the variance in our measurements. This means we need to assess (1) the amount of variability between embryos, and (2) to what extent this can be attributed to experimental parameters, such as (a) measurement error or (b) mounting error.

Measurement errors (a) were accounted for by performing three separate measurements on each image. Each datapoint in the dataset used is the average of these measurements.

To account for mounting error (b), we need to assess what part of the variance between measurements can be attributed to different slides that may have mounting differences between them. To start, we need to find a parameter that is not dynamic over the development of the embryo. A good candidate is segment width, which does not change significantly in the transition from the second to third stripe in any stage (Fig 1F), making this measurement our best proxy for variability.

Assessing segment width as a proxy for embryonic size

Without live imaging we cannot fully exclude a cyclical dynamic, whereby the width of the segment decreases and increases again (or increases and decreases again) within a single stage. However, if this were the case, we would expect a strong correlation between segment length (which increases within the stage) and segment width, in a second order polynomial regression.

#between stripe2 and seg2

```r
M1 <- lm(stripe.2.width~X2nd.segment.length+(X2nd.segment.length)^2+X2nd.segment.length:segments+segments,data=sup1b)
summary(M1)
```

```r
## Call:
## lm(formula = stripe.2.width ~ X2nd.segment.length + (X2nd.segment.length)^2 +
## X2nd.segment.length:segments + segments, data = sup1b)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -34.849  -10.119   0.878   8.605  37.045
##
## Coefficients:
##             Estimate Std. Error t value Pr(>|t|)
## (Intercept)  87.723     25.031   3.505 0.000579 ***
## X2nd.segment.length  5.070      2.912   1.741 0.083405 .
## segments4       40.501     47.976   0.844 0.399705
## segments5      115.175     36.309   3.172 0.001783 **
## segments6       60.936     34.061   1.789 0.075313 .
## segments7      115.175     36.309   3.172 0.001783 **
## segments8       60.936     34.061   1.789 0.075313 .
## segments9      115.175     36.309   3.172 0.001783 **
## X2nd.segment.length:segments4  -5.847      5.714  -1.023 0.307545
## X2nd.segment.length:segments5   -12.968     4.134  -3.137 0.001997 **
```
In fact, the R² of these regressions is 0.04 between the length of the penultimate segment and the stripe preceding it (p<0.1), and 0.13 (p<0.001) between the length of the penultimate segment and the stripe that follows. Thus, it is unlikely that a cyclical pattern exists and is responsible for intra-stage variability.

```r
M2 <- lm(stripe.3.width ~ X2nd.segment.length + (X2nd.segment.length)^2 + X2nd.segment.length:segments + segments, data = sup1b)
summary(M2)
```
Quantifying variation

Using variation in the penultimate stripe as a proxy for overall variability, we see that, indeed, this variation is extensive. The total mean and variance of this parameter is:

```r
# mean stripe 2 width:
mean(sup1b$stripe.2.width,na.rm=TRUE)
## [1] 127.2376

# total variance in stripe 2 width
var(sup1b$stripe.2.width,na.rm=TRUE)
## [1] 225.2739
```

Separated by stage, the metric looks like this:

![Penultimate engrailed stripe](image)

The effect of mounting on variance in segment width

Fitting the parameter in a linear mixed model, with segments and slide as random effects, can tell us what part of the variance can be attributed to differential mounting (i.e. slides).

```r
library(lme4)

## Loading required package: Matrix
M <- lmer(stripe.2.width ~ 1+(1|segments)+(1|slide), data=sup1b)
summary(M)

## Linear mixed model fit by REML ['lmerMod']
## Formula: stripe.2.width ~ 1 + (1 | segments) + (1 | slide)
## Data: sup1b
```
## REML criterion at convergence: 1776.9

## Scaled residuals:
```
Min 1Q Median 3Q Max
-2.56231 -0.65085 0.02652 0.59947 2.64669
```

## Random effects:
```
<table>
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<th>Name</th>
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<th>Std.Dev.</th>
</tr>
</thead>
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<td>48.54</td>
<td>6.967</td>
</tr>
<tr>
<td>segments</td>
<td>(Intercept)</td>
<td>20.97</td>
<td>4.579</td>
</tr>
<tr>
<td>Residual</td>
<td></td>
<td>173.32</td>
<td>13.165</td>
</tr>
</tbody>
</table>
```

Number of obs: 219, groups: slide, 17; segments, 8

## Fixed effects:
```
<table>
<thead>
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<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125.082</td>
<td>2.608</td>
<td>47.96</td>
</tr>
</tbody>
</table>
```

The model indicates that the variance attributable to slide ID is 21.55% (48.54/225.27; 225.27 is the total variance for stripe width, see ‘Quantifying variation’). Segments account for a further 9.31% (20.97/225.27) of variance.

**Conclusion**

Using the residual standard deviation of this model, the distribution of stripe width is 127.24 ± 13.17 μm (127.24 is the mean stripe width, see ‘Quantifying variation’); this means that 95% of the measurements are between 100.9μm and 153.58μm (2 standard deviations from the mean), giving a 53% increase in size between the smallest and largest measurement of 95% of embryos, when accounting for measurement error and mounting errors.
Supplementary file 1A

Click here to Download Dataset file 1A

Supplementary file 1B

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Supplementary file 1C

Click here to Download Dataset file 1C

Supplementary file 3A

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