

# 1 **Arthropod segmentation**

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## 9 **Summary**

10 There is now compelling evidence that many arthropods pattern their segments using a clock-and-  
11 wavefront mechanism, analogous to that operating during vertebrate somitogenesis. In this review,  
12 we discuss how the arthropod segmentation clock generates a repeating sequence of pair-rule gene  
13 expression, and how this is converted into a segment-polarity pattern by “timing factor” wavefronts  
14 associated with axial extension. We argue that the gene regulatory network that patterns segments  
15 may be relatively conserved, although the timing of segmentation varies widely, and double-  
16 segment periodicity appears to have evolved at least twice. Finally, we describe how the repeated  
17 evolution of a simultaneous (*Drosophila*-like) mode of segmentation within holometabolan insects  
18 can be explained by heterochronic shifts in timing factor expression plus extensive pre-patterning of  
19 the pair-rule genes.

20

## 21 **Introduction**

22 Arthropods are an ecdysozoan phylum defined by their segmented bodies and jointed limbs. True  
23 arthropods (euarthropods) comprise three living clades: Chelicerata (spiders, scorpions and mites),  
24 Myriapoda (centipedes and millipedes), and Pancrustacea (crustaceans and insects). The closest  
25 relatives of arthropods are onychophorans (velvet worms) and tardigrades (water bears); together  
26 these phyla form the segmented superphylum Panarthropoda (**Fig. 1A**).

27 The great diversity of arthropod species is testament to the evolutionary potential of a segmented  
28 body plan: a modular organisation of fundamentally similar units arrayed serially along the  
29 anteroposterior (AP) axis (Hannibal and Patel, 2013). Arthropod segments, and their associated  
30 appendages, have diversified remarkably through adaptation to specific functions, such as feeding,  
31 locomotion, or reproduction. In addition, segment number can vary enormously, from fewer than  
32 twenty in insects and malacostracan crustaceans, to over a hundred in certain centipedes and

33 millipedes, resulting in a wide spectrum of organismal forms (Brusca et al., 2016). With over a million  
34 named species, arthropods have colonised and exploited almost every environment on Earth, thanks  
35 in no small part to the evolution of segmentation.

36 Our understanding of how segments are patterned in arthropod embryos has traditionally been  
37 heavily influenced by study of the fruit fly *Drosophila melanogaster*. Over the past two decades,  
38 research into sequentially-segmenting species has complemented the well-established *Drosophila*  
39 model, resulting in the discovery of an arthropod “segmentation clock”, and an outline of conserved  
40 and divergent aspects of arthropod segment patterning networks. In the light of these findings,  
41 recent studies have re-examined segmentation in *Drosophila*, uncovering new subtleties and  
42 interpreting their evolutionary significance.

43 In the sections that follow, we provide a general overview of arthropod segmentation and review  
44 our current understanding of three key issues: (1) the nature of the arthropod segmentation clock;  
45 (2) how the “pair-rule” genes pattern segments; and (3) the evolution of *Drosophila*-style  
46 simultaneous segmentation from a sequentially-segmenting ancestral state. We also reflect on the  
47 origins of arthropod segmentation (**Box 1**) and the control of segment number (**Box 2**). As we have  
48 chosen to focus on the time window when segments are actively being patterned, we do not discuss  
49 earlier AP patterning processes, such as axis specification, or later ones, such as segment  
50 morphogenesis.

51

## 52 **Overview of arthropod segmentation**

### 53 *Segments and parasegments*

54 In arthropods, morphological segmentation is built upon a more fundamental developmental unit,  
55 the “parasegment” (Martinez-Arias and Lawrence, 1985). Parasegment boundaries are established  
56 during embryogenesis by “segment-polarity” genes such as *engrailed* and *wingless*, which are  
57 expressed in a series of persistent stripes along the AP axis. Interestingly, parasegments are offset  
58 slightly from morphological segments: parasegment boundaries fall at the anterior edge of each  
59 *engrailed* domain and line up with the middle of each appendage, while segment boundaries fall at  
60 the posterior edge of each *engrailed* domain and lie in between the appendages (**Fig. 1B**). Analogous  
61 to vertebrate “resegmentation” (each vertebra being formed from portions of two different somite  
62 pairs), this developmental phase shift makes sense if the role of the parasegments is chiefly to  
63 organise the nervous system and associated appendicular structures, while the role of morphological

64 segmentation is to protect these centres and form exoskeletal articulations between them (Deutsch,  
65 2004).

66 Each segment-polarity gene is expressed at a particular position within a segmental unit, and the  
67 overall arrangement is remarkably conserved across Panarthropoda (Damen, 2002; Janssen and  
68 Budd, 2013). A central goal of segmentation research is to understand how upstream regulatory  
69 processes establish this important pattern within the embryo.

70

### 71 *Sequential segmentation and the segment addition zone*

72 Most arthropods pattern their segments sequentially, from head to tail, coupling the segmentation  
73 process to progressive axial extension (Sander, 1976). They usually specify some number of anterior  
74 segments in the blastoderm, but the majority of the segments emerge rhythmically from a posterior  
75 “segment addition zone” (SAZ) after the blastoderm to germband transition. The SAZ retracts  
76 posteriorly as new segments are added to the trunk, generally shrinking in size, until the embryo  
77 reaches full germband extension (**Fig. 1C**).

78 “SAZ” is now preferred over the traditional term “growth zone”, because it makes no assumption of  
79 localised and continuous cell proliferation in the posterior of the embryo (Janssen et al., 2010). The  
80 material for new segments is generally provided by a combination of cell division and convergent  
81 extension, but – as in vertebrates – the relative contributions of these cell behaviours to axial  
82 elongation vary widely across species (Auman et al., 2017; Benton, 2018; Benton et al., 2016; Mito et  
83 al., 2011; Nakamoto et al., 2015; Steventon et al., 2016). Accordingly, while cell division may in some  
84 species be coordinated with segment addition, segment patterning processes do not appear to be  
85 mechanistically dependent on the cell cycle (Cepeda et al., 2017), aside from in special cases such as  
86 malacostracan crustaceans. This group exhibits a highly derived mode of segmentation in which  
87 patterning occurs through regimented asymmetrical divisions of rows of posterior cells (Scholtz,  
88 1992).

89 While the shape, size, and proportions of the SAZ vary considerably across species, certain features  
90 are conserved. Segment-polarity stripes emerge at the anterior of the SAZ, and Wnt is expressed at  
91 its posterior (Williams and Nagy, 2017). Between these limits, we define the “anterior SAZ” as the  
92 portion of the SAZ that contains segments in the process of being patterned, and the “posterior SAZ”  
93 as the portion that contains cells not yet assigned to any particular prospective segment. These  
94 functionally-defined regions correlate with the differential expression of key developmental  
95 transcription factors; for example, Caudal (the arthropod homolog of the vertebrate Cdx proteins)

96 appears to be specifically associated with the posterior SAZ (Auman et al., 2017; Clark and Peel,  
97 2018).

98 Importantly, SAZ identity is transient and dynamic for any given cell. With the generation of each  
99 new segment, newly-patterned tissue “leaves” the anterior SAZ, which is simultaneously  
100 “replenished” by cells from the posterior SAZ. (Whether cells flow anteriorly out of the SAZ or the  
101 SAZ retracts posteriorly along the embryo depends on one’s choice of reference frame.) Thus, a cell  
102 which starts out within the posterior SAZ, expressing one set of genes, will at some point end up  
103 within the anterior SAZ, expressing a different set of genes, and finally within the segmented  
104 germband, expressing yet another (**Fig. 1C**). This provides a mechanistic explanation for the tight  
105 coupling between axial elongation and the segmentation process, because the changing expression  
106 levels of SAZ-associated factors such as Caudal are likely to trigger coordinated expression changes  
107 in segment patterning genes as the SAZ retracts (Clark and Peel, 2018; El-Sherif et al., 2014).

108

#### 109 *Segment patterning by a clock-and-wavefront mechanism*

110 Arthropod segmentation is frequently compared to vertebrate somitogenesis (reviewed in Hubaud  
111 and Pourquié, 2014; Oates et al., 2012). While segments and somites are not homologous  
112 morphological structures, it is now becoming clear that both arthropods and vertebrates have  
113 converged on a “clock-and-wavefront” strategy (Cooke and Zeeman, 1976) to pattern their AP axis.  
114 Temporal periodicity is generated by an oscillator (the “clock”), and progressively translated into  
115 spatial periodicity by a second signal (the “wavefront”), which travels along an axis and freezes (or  
116 reads out) the phase of the clock.

117 In vertebrates, the clock consists of cycles of gene expression in the presomitic mesoderm (PSM),  
118 while in arthropods it consists of cycles of gene expression in the posterior ectoderm. In both the  
119 vertebrate anterior PSM and the arthropod anterior SAZ, the oscillations are slowed by the  
120 retraction of posterior signals associated with axial extension, converting them into a series of  
121 stripes. These stripes then pattern other genes, which determine the AP polarity of somites (in  
122 vertebrates) or segments (in arthropods).

123 Curiously, the periodicity of the segmentation clock is not fixed across arthropods. Most groups  
124 pattern a single new segment for each cycle of the clock (as do vertebrates), but some species  
125 pattern two segments in each cycle, meaning that their clock has a double-segment (or “pair-rule”)  
126 periodicity (Chipman et al., 2004; Sarrazin et al., 2012) .

127

128 *Other modes of segmentation*

129 The sequential mode of segmentation is widespread and almost certainly ancestral within  
130 arthropods. However, across species, the timing of segmentation can vary dramatically relative to  
131 other developmental events.

132 For example, arthropod embryos differ widely in the number of segments they pattern at the  
133 blastoderm stage, versus afterwards during germband extension. In insects, this variation is roughly  
134 correlated with a spectrum of “germ types” defined in the pre-molecular era (Davis and Patel, 2002;  
135 Krause, 1939), but for simplicity and generality, we have chosen to eschew such terminology in this  
136 review. Instead, we will refer to sequential segmentation (usually occurring in a germband, under  
137 the control of a segmentation clock) versus simultaneous segmentation (usually occurring in a  
138 blastoderm, downstream of non-periodic spatial cues). The mechanisms underlying simultaneous  
139 segmentation are discussed in more detail below.

140 Outside of the insects, many arthropod groups undergo post-embryonic segmentation, i.e. delay the  
141 development of a portion of the AP axis until after hatching. In crustaceans with naupliar larvae, for  
142 example, only the head segments are patterned in the embryo, and trunk segments develop  
143 sequentially from a SAZ-like region after the larva has begun feeding (Anderson, 1973). Other, less  
144 extreme, examples are found within myriapods: these pattern the head and the first trunk segments  
145 in the embryo, but may add one or more trunk segments after each moult (Blower, 1985).

146 Our focus here is on the segmentation of the trunk (i.e. the axial patterning of the gnathal, thoracic,  
147 and abdominal segments), but note that there are other parts of the arthropod body that are  
148 segmented by different mechanisms, such as the anterior head (Posnien et al., 2010) or the jointed  
149 appendages (Angelini and Kaufman, 2005a). Within the trunk itself, the mechanisms we describe  
150 specifically control ectodermal segmentation; mesodermal segmentation occurs later, apparently  
151 directed by inductive signals from the segmented ectoderm (Azpiazu et al., 1996; Green and Akam,  
152 2013; Hannibal et al., 2012). Finally, there is evidence that dorsal segmentation in millipedes is  
153 decoupled from ventral segmentation, which later leads to segment fusions (Janssen, 2011; Janssen  
154 et al., 2004).

155

156 *Segment patterning genes*

157 Most of the arthropod segmentation genes we know about were originally identified from a genetic  
158 screen in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). *Drosophila* represents an extreme  
159 example of simultaneous segmentation, patterning all but its most terminal segments in the

160 blastoderm. It has taught us a lot about how segmentation genes regulate one another's expression  
161 (Akam, 1987; Nasiadka et al., 2002), but studies in other arthropods were (and are) necessary to  
162 reveal how these networks relate to more ancestral modes of segmentation (Peel et al., 2005).

163 In *Drosophila*, as in other arthropods, the segment-polarity genes are patterned by the "pair-rule"  
164 genes, which code for various transcription factors. In *Drosophila*, the pair-rule genes are expressed  
165 in stripes in the blastoderm, but in sequentially-segmenting species they are also expressed in the  
166 SAZ (Patel et al., 1994). In general, the pair-rule genes that turn on earliest in *Drosophila* ("primary"  
167 pair-rule genes) are expressed in the posterior SAZ in sequentially-segmenting species, and may  
168 oscillate, while those that turn on later ("secondary" pair-rule genes) are expressed in the anterior  
169 SAZ. The periodicity of pair-rule gene expression can be segmental or double-segmental depending  
170 on the species (in *Drosophila* it is double-segmental, hence the term "pair-rule"), but the genes are  
171 always referred to as the "pair-rule genes" regardless. There has been some confusion over the  
172 years as to which *Drosophila* pair-rule genes should be classed as primary and which as secondary or  
173 even tertiary. However, the most recent analysis (Schroeder et al., 2011), which classifies only *paired*  
174 (*prd*) and *sloppy-paired* (*slp*) as secondary, and all of *hairy*, *even-skipped* (*eve*), *runt*, *odd-skipped*  
175 (*odd*) and *fushi tarazu* (*ftz*) as primary, meshes well with the comparative evidence.

176 In *Drosophila*, the primary pair-rule genes are patterned by the "gap" genes, which code for another  
177 set of transcription factors. In *Drosophila*, these genes are expressed in broad, partially-overlapping  
178 domains along the length of the blastoderm, but in sequentially-segmenting species some portion of  
179 this pattern is generated over time, in the SAZ (**Box 2**). Gap genes in sequentially-segmenting species  
180 do not seem to be important for directing pair-rule gene expression. They do, however, appear to  
181 play a relatively conserved role in patterning the Hox genes, which regulate segment identity  
182 (Hughes and Kaufman, 2002a; Marques-Souza et al., 2008; Martin et al., 2016)

183

#### 184 **BOX 1: The evolutionary origins of arthropod segmentation**

185 The major segmented phyla – arthropods, annelids, and chordates – are evolutionarily distant and  
186 separated by many unsegmented groups. While losses of segmentation are possible in evolution  
187 (e.g. from spoon worms and peanut worms within annelids), we are sceptical about the existence of  
188 a segmented urbilaterian ancestor that could have given rise to all three phyla (Couso, 2009).  
189 Instead, segmentation appears to have evolved repeatedly during animal evolution, involving  
190 various developmental mechanisms (Graham et al 2014).

191 Some of the developmental commonalities between different segmented phyla may reflect  
192 bilaterian homologies that predate segmentation itself, such as elongation of the body from a  
193 posterior zone (Jacobs et al 2005, Martin and Kimelman 2009). Other similarities may reflect the  
194 convergent adoption of generic patterning strategies, such as molecular oscillators (Richmond and  
195 Oates 2012). Finally, certain similarities may reflect the parallel redeployment of ancient molecular  
196 mechanisms (Chipman, 2010), and therefore require both homology and convergence to fully  
197 explain. For example, segment boundary formation in some, but not all, annelids shows striking  
198 similarities to parasegment boundary formation in arthropods (Dray et al., 2010; Prud'homme et al.,  
199 2003; Seaver et al., 2001; Seaver and Kaneshige, 2006). Probably, this boundary specification  
200 mechanism evolved before trunk segmentation, possibly in the context of patterning the head and  
201 anterior nervous system (Vellutini and Hejzol, 2016).

202 The evolutionary success of segmented phyla emphasizes the adaptive value of diversified  
203 metameric structures, but it does not explain why segmentation evolved in the first place. One long-  
204 standing hypothesis stresses the advantages of a segmented body for generating coordinated waves  
205 of muscular activity to drive locomotion (Clark, 1964). Given that most of the earliest arising  
206 segmented lineages have many similar segments, this seems a likely explanation for the initial  
207 origins of serial repetition along the body axis, which was likely the forerunner for metameric  
208 segmentation. Under this scenario, repetition would be expected first in the nervous system and  
209 body wall musculature. Interestingly, onychophorans have distinct mesodermal somites, and show  
210 clear parasegmental boundaries in the limbs and nervous system (Eriksson et al., 2009), but show no  
211 obvious segmentation of the body wall ectoderm.

212

## 213 **BOX 2: Regulation of segment number**

214 In arthropods, segment number is determined by the total number of pair-rule stripes (and the  
215 periodicity with which they regulate segment-polarity genes). In simultaneously-segmenting insects  
216 such as *Drosophila*, individual pair-rule stripes are positioned by gap factors at specific locations  
217 along the AP axis, hardcoding segment number. In sequentially-segmenting species, segment  
218 number instead depends on the temporal duration of segmentation, divided by the period of the  
219 segmentation clock.

220 Gap genes appear to play some role in controlling the duration of segment addition (Cerny et al.,  
221 2005; Nakao, 2016). Over time, gap genes are expressed sequentially within the SAZ, their turnover  
222 driven by cross-regulatory interactions (Boos et al., 2018; Verd et al., 2018). This process, effectively

223 a developmental “timer”, shows intriguing similarities to the “neuroblast clock” (Isshiki et al., 2001;  
224 Peel et al., 2005). It evidently exerts some control over the body plan, since perturbing *hunchback*  
225 expression can both decrease (Liu and Kaufman, 2004a; Marques-Souza et al., 2008; Mito et al.,  
226 2005) and increase (Boos et al., 2018; Nakao, 2016) segment number in sequentially-segmenting  
227 insects. These phenotypes are not well understood, but might result from gap genes directly or  
228 indirectly regulating cell behaviour within the SAZ. Such effects are unlikely to be mediated via the  
229 Hox genes, since significant perturbations of Hox gene expression in insects and crustaceans have  
230 not been found to affect segment number (Angelini et al., 2005; Martin et al., 2016; Stuart et al.,  
231 1991).

232 Despite varying widely among arthropods, segment number is usually fixed within a species.  
233 However, there are certain groups, such as geophilomorph centipedes, where naturally occurring  
234 variation might provide clues as to how this number evolves (Kettle and Arthur, 2000; Vedel et al.,  
235 2008; Vedel et al., 2010). Another interesting question is how species which undergo post-  
236 embryonic segmentation coordinate segment patterning with the moult cycle. Ecdysone-related  
237 genes play segmentation roles in some embryos (Erezyilmaz et al., 2009; Heffer et al., 2013),  
238 suggesting these two processes might be deeply related.

239

## 240 **Nature of the arthropod segmentation clock**

### 241 *Oscillating gene expression in the SAZ*

242 Some segmentation genes exhibit extremely variable expression patterns in the posterior SAZs of  
243 fixed embryos, suggesting that they continually turn on and off over time. In the beetle *Tribolium*,  
244 split-embryo experiments have confirmed that this variability results from a temporally dynamic  
245 “segmentation clock” within individuals rather than spatially variable expression between individuals  
246 (Sarrazin et al., 2012). Expression dynamicity has also been demonstrated in *Tribolium* by comparing  
247 the average patterns of finely-staged cohorts of embryos, visualising discrepancies between the  
248 transcript and protein domains of a given gene, and gaining an understanding of cell dynamics within  
249 the SAZ via live imaging (Benton, 2018; El-Sherif et al., 2012; Sarrazin et al., 2012). In other species,  
250 gene expression dynamics within the SAZ have rarely been studied in detail. However, convincing  
251 “pseudo time-series” assembled from carefully-staged *Strigamia* (centipede) and *Parasteatoda*  
252 (spider) embryos imply that oscillatory dynamics are widespread (Brena and Akam, 2013; Schönauer  
253 et al., 2016).



254 Candidate gene approaches in species including *Tribolium*, *Strigamia*, the millipede *Glomeris*, and a  
255 second spider, *Cupiennius*, indicate that oscillating SAZ genes include the primary pair-rule genes  
256 *hairy*, *eve*, *runt* and *odd* (Choe et al., 2006; Damen et al., 2005; Green and Akam, 2013; Janssen et  
257 al., 2011). (The segmentation role of *ftz* is less widely conserved (Pick, 2016).) In addition, Notch  
258 signalling components appear to oscillate in many clades (see below), as do *prd* and *hedgehog* in  
259 spiders (Davis et al., 2005; Schoppmeier and Damen, 2005a; Schwager, 2008). However, since there  
260 has not yet been an exhaustive screen for cyclic expression, we don't know how many other genes  
261 may have been missed.

262 Measurements from *Tribolium* (El-Sherif et al., 2012; Nakamoto et al., 2015; Sarrazin et al., 2012)  
263 and *Strigamia* (Brena and Akam, 2012) suggest an oscillation period in these species of ~3 hours at  
264 18-20°C (or equivalently ~6 hrs at 13 °C or ~1.5 hours at 30°C, as segmentation speed scales with  
265 developmental rate). Adjusted for temperature, these numbers are comparable to the fastest  
266 segmenting vertebrates, such as zebrafish or snakes (Gomez et al., 2008). Interestingly, the rate of  
267 segment addition is not constant throughout development (Brena and Akam, 2013; Nakamoto et al.,  
268 2015). This implies that there is stage-specific variation in the oscillation period, the axial elongation  
269 rate, and/or the dynamics of tissue maturation in the SAZ (Schröter et al., 2012; Soroldoni et al.,  
270 2014).

271 At present, the mechanistic basis for the oscillations is not well understood. Nonetheless, it is useful  
272 to think about contributing regulatory processes using a three-tier framework (Oates et al., 2012):  
273 (1) gene expression dynamics within cells; (2) signalling interactions between cells; and (3) the  
274 changing regulatory context along the SAZ.

275

#### 276 *Gene expression dynamics within cells*

277 In vertebrates such as zebrafish, (auto)repressive interactions between Her/Hes transcription factors  
278 (homologs of the *Drosophila* pair-rule gene *hairy*) are thought to form the core of the segmentation  
279 clock, driving oscillations by time-delayed negative feedback (Lewis, 2003; Schröter et al., 2012).  
280 Analogously, it is possible that the arthropod segmentation clock is driven by an intracellular  
281 negative feedback loop formed by some or all of the oscillating pair-rule genes.

282 The main evidence for this is that knocking down primary pair-rule genes can block segmentation  
283 and truncate the body axis, as has been found in *Tribolium* (Choe et al., 2006), the silkworm *Bombyx*  
284 (Nakao, 2015), a second beetle species *Dermestes* (Xiang et al., 2017), and the hemipteran bug  
285 *Oncopeltus* (Auman and Chipman, 2018; Liu and Kaufman, 2005). It can also cause the expression of

286 other primary pair-rule genes to become aperiodic (Choe et al., 2006; Nakao, 2015), suggesting that  
287 at least some of the oscillations are mutually interdependent. This observation distinguishes these  
288 knockdowns from those of downstream patterning genes, which may also yield asegmental  
289 phenotypes but do not perturb expression dynamics in the SAZ (Choe and Brown, 2007; Farzana and  
290 Brown, 2008).

291 The topology for a pair-rule gene segmentation clock is not clear. An early RNAi study in *Tribolium*  
292 found that *eve*, *runt*, or *odd* knockdown resulted in truncation, while *hairy* knockdown resulted only  
293 in head defects (Choe et al., 2006). This led to the hypothesis that *eve*, *runt*, and *odd* are linked into  
294 a three-gene ring circuit, and that even though *hairy* oscillates in the SAZ, it is not required for  
295 segmentation. Specifically, it was proposed that Eve activates *runt*, Runt activates *odd*, and Odd in  
296 turn represses *eve*, returning the sequence to the beginning (**Fig. 2A**). However, more recent  
297 evidence has raised issues with this proposal.

298 First, whether *hairy* is involved in the *Tribolium* segmentation clock or not remains unclear. A later  
299 study found that *hairy* knockdown gave a pair-rule phenotype for gnathal and thoracic segments  
300 (Aranda et al., 2008), and the iBeetle screen (Dönitz et al., 2015) additionally recovered posterior  
301 truncations. *hairy* also has a paralog, *deadpan*, expressed with similar dynamics in the SAZ (Aranda  
302 et al., 2008), and so its role might be masked by functional redundancy. Finally, *hairy* knockdown  
303 was recently found to produce truncations in *Dermestes* (Xiang et al., 2017), and *hairy* is also known  
304 to regulate segment patterning in the cockroach *Periplaneta* (Pueyo et al., 2008), the parasitic wasp  
305 *Nasonia* (Rosenberg et al., 2014), and of course *Drosophila*, indicating that a role in segmentation is  
306 widely conserved.

307 Second, whether *eve* and *odd* are part of the primary oscillator is also not certain. *eve* expression  
308 may be necessary for establishing and/or maintaining the SAZ (Cruz et al., 2010; Liu and Kaufman,  
309 2005; Mito et al., 2007; Xiang et al., 2017), and therefore its severe truncation phenotype may be  
310 independent of its potential role in the segmentation clock. *odd*, on the other hand, has been found  
311 to cause pair-rule defects rather than truncations in *Dermestes* (Xiang et al., 2017) and *Oncopeltus*  
312 (Auman and Chipman, 2018), although the interpretation of these phenotypes is complicated by the  
313 existence of *odd* paralogs, such as *sob*. Notably, neither *eve* nor *odd* shows dynamic expression in  
314 the posterior SAZ of *Oncopeltus* (Auman and Chipman, 2018; Liu and Kaufman, 2005), indicating that  
315 periodicity is likely to be generated by other genes in this species.

316 Finally, the specific regulatory interactions proposed for the circuit seem unlikely. In holometabolous  
317 insects (and also *Strigamia*), *eve*, *runt*, and *odd* are expressed sequentially within each pattern  
318 repeat (Choe et al., 2006; Clark, 2017; Green and Akam, 2013; Nakao, 2015; Rosenberg et al., 2014).

319 In both *Tribolium* and *Bombyx*, Eve is necessary for *runt* expression, and Runt is necessary for *odd*  
320 expression (Choe et al., 2006; Nakao, 2015). However, it is probably not the case that Eve directly  
321 activates *runt* and Runt directly activates *odd*, as was proposed for *Tribolium*. Instead, genetic  
322 evidence from *Bombyx* and *Drosophila* (and wild-type expression dynamics from *Tribolium*) suggest  
323 something closer to a “repressilator” scenario (Elowitz and Leibler, 2000), where each gene in the  
324 sequence represses the one before it (**Fig. 2A**).

325 In summary, while it is likely that cross-regulation plays a considerable role in shaping dynamic pair-  
326 rule gene expression, it is not yet clear whether the oscillating genes are linked into a single circuit,  
327 whether this circuit is sufficient to generate oscillations, what the topology of this circuit is likely to  
328 be, nor indeed the extent to which it may have diverged in different lineages (Krol et al., 2011).

329

### 330 *Signalling interactions between cells*

331 Regardless of whether the pair-rule gene network is capable of producing intracellular oscillations  
332 autonomously, the segmentation clock must also involve intercellular communication to keep  
333 oscillations synchronised across the SAZ. Notch signalling, known to synchronise oscillations during  
334 vertebrate somitogenesis (Liao and Oates, 2017), is the key candidate for this role. Indeed, Notch  
335 signalling components appear to oscillate along with the pair-rule genes in chelicerates  
336 (Schoppmeier and Damen, 2005b; Stollewerk et al., 2003), myriapods (Chipman and Akam, 2008;  
337 Kadner and Stollewerk, 2004), crustaceans (Eriksson et al., 2013), and some insects (Pueyo et al.,  
338 2008), suggesting that arthropod segmentation ancestrally involved Notch.

339 Experiments in *Cupiennius*, *Periplaneta*, and the branchiopod crustacean *Daphnia* have found that  
340 segment boundaries and the expression of segmentation genes become disorganised when Notch  
341 signalling is perturbed (Eriksson et al., 2013; Pueyo et al., 2008; Schoppmeier and Damen, 2005b;  
342 Stollewerk et al., 2003). Inhibiting Notch signalling also blocks segmentation (but not axial  
343 elongation) in anostracan crustaceans (Williams et al., 2012). These findings indicate that Notch may  
344 play an explicit role in generating and/or coordinating pair-rule gene oscillations, perhaps via  
345 regulation of *hairy* (**Fig. 2B**).

346 However, the pleiotropy of the Notch pathway means that characterising this potential  
347 segmentation function may be difficult. During development, Notch signalling also regulates cell  
348 proliferation (Go et al., 1998), SAZ establishment (Chesebro et al., 2013; Oda et al., 2007; Schönauer  
349 et al., 2016), and fertility (Xu and Gridley, 2012). Accordingly, strong Notch perturbations in

350 sequentially-segmenting arthropods often result in uninterpretable axial truncations, or simply a  
351 failure to lay many eggs (Kux et al., 2013; Mito et al., 2011; Stahi and Chipman, 2016).

352 Surprisingly, in the insects *Gryllus*, *Oncopeltus*, and *Tribolium*, the Notch ligand *Delta* is not  
353 expressed in the posterior SAZ (Aranda et al., 2008; Auman et al., 2017; Kainz et al., 2011). Either  
354 Notch signalling acts through a different ligand in these species, or it does not directly regulate the  
355 clock. *Delta* also seems not to play a segmentation role in the honeybee *Apis* (a simultaneously-  
356 segmenting species), even though it is expressed in stripes at an appropriate time (Wilson et al.,  
357 2010).

358 If a role for Notch signalling in sequential segmentation has indeed been lost in some insect lineages,  
359 it is not clear what mechanism(s) might synchronise cells instead. One possibility is the Toll genes,  
360 which are thought to influence intercellular affinity and are expressed dynamically in the SAZ across  
361 arthropods (Benton et al., 2016; Paré et al., 2014). However, they seem only to affect  
362 morphogenetic processes downstream of segment establishment, rather than segment patterning.  
363 Another possibility that has been raised is intercellular communication via Tenascin major (Ten-m)  
364 (Hunding and Baumgartner, 2017), a transmembrane protein that was erroneously identified as a  
365 *Drosophila* pair-rule factor owing to an *opa* mutation present on the balancer chromosome of its  
366 stock (Zheng et al., 2011). However, mutation/knockdown of *Ten-m* does not affect segmentation in  
367 either *Drosophila* or *Tribolium* (Choe et al., 2006; Zheng et al., 2011), and Ten-m is expressed  
368 periodically only after segment-polarity stripes have formed (Baumgartner et al., 1994; Jin et al.,  
369 2019).

370

### 371 *The changing regulatory context along the SAZ*

372 The segmentation clock oscillates in the posterior SAZ and its phase is read out in the anterior SAZ.  
373 Therefore, the “wavefront” can be loosely identified with the boundary between these regions,  
374 which retracts posteriorly across the embryo over time. The posterior SAZ and the anterior SAZ are  
375 apparently defined by the differential expression of specific regulatory factors (“timing factors” in  
376 our terminology), which are expressed dynamically over the course of axial elongation, determining  
377 where and when segment patterning takes place (Clark and Peel, 2018). Understanding the  
378 mechanistic basis for the wavefront therefore entails characterising (1) the identities of these  
379 factors, (2) how they regulate segmentation gene expression, and (3) how they themselves are  
380 regulated in the embryo.

381 Many genes are specifically expressed in a subregion of the SAZ (Oberhofer et al., 2014). However,  
382 most studies to date have focused on *Wnt* and *caudal*, supplemented recently by *Dichaete/Sox21b*  
383 and *odd-paired (opa)/zic*. The expression patterns of these genes are relatively consistent across  
384 species (**Fig. 2C**). *Wnt* is expressed in a small zone around the proctodaeum (Janssen et al., 2010).  
385 (We note that this population of cells appears to be distinct from the SAZ proper, and may not  
386 contribute to segmental tissue). In *Tribolium*, two of its receptors are expressed ubiquitously in the  
387 embryo, and one is expressed in the anterior SAZ and in segmental stripes (Beermann et al., 2011).  
388 *caudal* is expressed in the posterior SAZ (Copf et al., 2004; Schulz et al., 1998), and *Dichaete* is  
389 expressed in a similar zone to *caudal*, but does not overlap with posterior *Wnt* (Clark and Peel, 2018;  
390 Janssen et al., 2018; Paese et al., 2018). In contrast, *opa* is expressed in the anterior SAZ, i.e. anterior  
391 to or slightly overlapping *caudal* and *Dichaete*, and also in segmental stripes (Clark and Peel, 2018;  
392 Green and Akam, 2013; Janssen et al., 2011). Across arthropods, *Wnt*, *caudal* and *Dichaete* are  
393 required to establish and maintain the SAZ (Angelini and Kaufman, 2005b; Bolognesi et al., 2008;  
394 Chesebro et al., 2013; Copf et al., 2004; McGregor et al., 2008; Miyawaki et al., 2004; Nakao, 2018;  
395 Paese et al., 2018; Schönauer et al., 2016; Shinmyo et al., 2005). In *Tribolium*, *opa* is required for  
396 segmentation, following earlier roles in blastoderm formation and head specification (Clark and Peel,  
397 2018).

398 Caudal and Dichaete are strong candidates for activating the segmentation clock, since their  
399 expression domains roughly correlate with the extent of its oscillations, and they positively regulate  
400 pair-rule gene expression in *Drosophila*. Caudal has also been shown to be necessary for *eve* and  
401 *runt* expression in *Parasteatoda* (Schönauer et al., 2016). *Opa*, on the other hand, may be important  
402 for reading out the phase of the clock, since it activates segment polarity genes and regulates late  
403 pair-rule gene expression in *Drosophila* (Clark and Akam, 2016). Given that all three are transcription  
404 factors, they might regulate segmentation by activating or repressing specific genes, modulating the  
405 regulatory effects of other transcription factors, or switching expression control between different  
406 enhancers. However, the severity of their knockdown phenotypes in sequentially-segmenting  
407 species means that uncovering the details may require precisely targeted functional perturbations,  
408 and probably transgenic reporters.

409 In sequentially-segmenting species, the relative expression patterns of different timing factors  
410 remain consistent across development, suggesting that they regulate each other's expression. *Wnt* is  
411 thought to act as a posterior organiser (Chesebro et al., 2013; Oberhofer et al., 2014), and we have  
412 hypothesised that regulatory interactions between *caudal*, *Dichaete* and *opa* drive their sequential  
413 expression over time (Clark and Peel, 2018). In addition, *caudal* has been found to be activated by  
414 *Wnt* in diverse arthropods (Beermann et al., 2011; Chesebro et al., 2013; McGregor et al., 2008;

415 Miyawaki et al., 2004), while Opa, as a Zic factor, might physically bind the Wnt effector TCF and  
416 modulate its effects on downstream genes (Murgan et al., 2015; Pourebrahim et al., 2011).  
417 Therefore, while details are currently sketchy, it seems probable that the timing factors are  
418 integrated into a regulatory network that ensures the maintenance of the SAZ over time, and also  
419 governs its gradual posterior retraction. Given the numerous parallels between posterior  
420 development in arthropods and posterior development in other bilaterian phyla, a similar network  
421 might have ancestrally coordinated cell differentiation during axial extension, and only later been  
422 exploited to regulate segmentation.

423 In the basic clock-and-wavefront model, the clock stops abruptly when it is hit by the wavefront.  
424 However, in both arthropod segmentation and vertebrate somitogenesis, segmentation clock  
425 oscillations may resolve into narrowing travelling waves before they stabilise, indicating that the  
426 clock winds down relatively gradually. The way in which the oscillation period varies along the SAZ is  
427 described phenomenologically by a “frequency profile” (Morelli et al., 2009), and this can vary over  
428 developmental time, as well as between species. While the shape of the frequency profile is not  
429 predicted to affect segmentation rate or segment size, models suggest that a graded profile might  
430 make patterning more robust (El-Sherif et al., 2014; Vroomans et al., 2018).

431 Wnt signalling perturbations distort the size and proportions of the SAZ (as judged by the expression  
432 of *caudal*), and cause equivalent distortions to the frequency profile (as judged by the expression of  
433 *eve*) (El-Sherif et al., 2014). This indicates that Wnt signalling affects the dynamics of the  
434 segmentation clock, and that its effects might be mediated by SAZ timing factors. However, the  
435 mechanism for modulating the oscillation period is not clear. One hypothesis proposes that the clock  
436 is quantitatively regulated by a morphogen gradient of Caudal (El-Sherif et al., 2014; Zhu et al.,  
437 2017), but the effects of specific timing factors are yet to be disentangled and assessed. Currently, it  
438 is unknown whether the period of the clock is indeed explicitly determined by the concentrations of  
439 particular timing factors (i.e. given control of these levels one could produce sustained oscillations of  
440 arbitrary period), or whether the slowing of the segmentation clock is an inherently transient  
441 phenomenon inseparable from its temporal transition from an oscillating to a non-oscillating state  
442 (Verd et al., 2014).

443

#### 444 **Segment patterning by the pair-rule network**

445 *Reading out the pattern*

446 In the anterior SAZ, each segmentation clock cycle resolves into an anterior-to-posterior array of  
447 partially overlapping stripes of pair-rule gene expression. Because the pair-rule genes are expressed  
448 in a strict sequence across a clock repeat (e.g. first *eve*, then *runt*, then *odd*), they convey  
449 unambiguous phase information to the cells they are expressed in, which provides significant  
450 patterning benefits over a single-gene oscillator (**Fig. 3A**). The internal organisation of a parasegment  
451 consists of at minimum three distinct segment-polarity states (Jaynes and Fujioka, 2004; Meinhardt,  
452 1982). Therefore, each pair-rule gene expression repeat must specify at least three output domains  
453 in species with single-segment periodicity, and at least six output domains in species with double-  
454 segment periodicity (**Fig. 3B**).

455 In *Drosophila*, the relative expression patterns of pair-rule genes and segment-polarity genes have  
456 been characterised in a variety of genetic backgrounds, allowing us to infer the regulatory  
457 interactions involved in specifying and resolving the segment pattern (reviewed in Clark and Akam,  
458 2016; Jaynes and Fujioka, 2004). Equivalent data is generally lacking from other arthropod species.  
459 However, as far as we can tell from what does exist (mainly single or double stains in wild-type  
460 embryos) the overall process appears to be fairly conserved, at least in its broad outline (Auman and  
461 Chipman, 2018; Damen et al., 2005; Green and Akam, 2013; Xiang et al., 2017).

462 First, the primary pair-rule genes pattern the secondary pair-rule genes. Across arthropods, *prd* and  
463 *slp* are expressed in a conserved, partially overlapping arrangement, which aligns with prospective  
464 parasegment boundaries (Choe and Brown, 2007; Green and Akam, 2013). In both *Drosophila* and  
465 other arthropods, *prd* turns on earlier than *slp*, at a time when upstream pair-rule gene expression is  
466 still dynamic. In *Drosophila*, both genes are patterned by Eve, and we have proposed that the  
467 dynamic nature of the Eve stripes (see below) helps differentially position the two domains (Clark,  
468 2017) (**Fig. 3C**).

469 Next, the segment polarity-genes are activated. Each segment-polarity gene is activated or repressed  
470 by particular pair-rule factors, which combinatorially define where it is expressed within the pattern  
471 repeat (Bouchard et al., 2000; Choe and Brown, 2009; DiNardo and O'Farrell, 1987). In species with  
472 double-segment periodicity, odd-numbered and even-numbered segment-polarity stripes may be  
473 driven by different regulatory logic (**Fig. 3D**).

474 At the same time, some of the pair-rule genes also start being expressed in segment-polarity  
475 patterns. In pair-rule species, this involves the splitting of existing stripes or the intercalation of new  
476 ones. The new patterns are explained by a new network of regulatory interactions between the pair-  
477 rule genes (Clark and Akam, 2016). In contrast to the earlier network, which drives dynamic  
478 expression, this later one behaves like a multistable switch, "locking in" specific segment-polarity

479 fates (Clark, 2017). Interestingly, different primary pair-rule genes undergo frequency doubling in  
480 each of *Drosophila*, *Bombyx*, *Tribolium*, and *Nasonia* (Choe et al., 2006; Clark and Akam, 2016;  
481 Nakao, 2015; Rosenberg et al., 2014), contrasting with the conserved expression of the segment-  
482 polarity and secondary pair-rule genes.

483 The resulting segmental patterns go on to regulate morphological segmentation. Note that the pair-  
484 rule genes are therefore pleiotropic: they are involved in generating the segment pattern, but some  
485 additionally play roles in maintaining segment-polarity, and they also regulate the development of  
486 other structures, such as the nervous system. In some cases, these functions have become  
487 distributed between multiple paralogs, e.g. *prd/gooseberry/pox-neuro* in *Drosophila* (He and Noll,  
488 2013), or the three copies of *eve* in *Strigamia* (Green and Akam, 2013). Across species, there can be  
489 considerable variation in both the number of paralogs present in the genome and the degree of  
490 subfunctionalization between them, complicating the interpretation of genetic perturbations.

491

#### 492 *The evolution of pair-rule patterning*

493 In several insect species, and also the centipede *Strigamia* (Chipman et al., 2004), segmentation  
494 gene expression undergoes a striking transition from double-segment periodicity to single-segment  
495 periodicity as the segment pattern is resolved. However, there is no indication of an initial double-  
496 segment periodicity during sequential segmentation in the spiders *Cupiennius* (Davis et al., 2005;  
497 Schoppmeier and Damen, 2005a) and *Parasteatoda* (Schwager, 2008), the millipede *Glomeris*  
498 (Janssen et al., 2011), or the crustacean *Daphnia* (Eriksson et al., 2013) (**Fig. 1A**). This suggests that  
499 the ancestral arthropod segmentation clock had a single-segment periodicity, and that pair-rule  
500 patterning in insects and centipedes originated independently.

501 Beyond this, it is not clear exactly when or how many times pair-rule patterning evolved in either of  
502 the centipede or insect lineages. *eve* is expressed segmentally rather than in pair-rule stripes in a  
503 different centipede species, *Lithobius* (Hughes and Kaufman, 2002b), which could indicate that pair-  
504 rule patterning evolved relatively recently within the centipede clade, possibly correlating with the  
505 origin of longer bodied forms. However, the dynamics of the *Lithobius* segmentation clock will need  
506 be investigated to rule out a transient or cryptic double-segment periodicity.

507 In insects, most of the available data come from holometabolan or orthopteran species, as well as  
508 the cockroach *Periplaneta* and hemipteran bug *Oncopeltus* (**Fig. 1A**). Holometabolans (Binner and  
509 Sander, 1997; Nakao, 2010; Patel et al., 1994; Rosenberg et al., 2014) and orthopterans (Davis et al.,  
510 2001; Mito et al., 2007) both show obvious transitions from double-segment to single-segment



511 periodicity, but the mapping between the pair-rule pattern and the segmental pattern is different in  
512 the two groups, suggesting that their respective pair-rule mechanisms might have evolved  
513 independently. Consistent with this possibility, gene expression in *Periplaneta* (more closely related  
514 to orthopterans than to holometabolans) appears to be single-segmental (Pueyo et al., 2008),  
515 although, as with *Lithobius*, the dynamics of its segmentation clock have not been explicitly  
516 investigated. Finally, *Oncopeltus* is a rather strange case: based on the expression and function of  
517 *eve*, it appears to lack pair-rule patterning, but pair-rule expression and/or function of certain other  
518 genes hints at an underlying double-segment periodicity (Auman and Chipman, 2018; Benton et al.,  
519 2016; Erezylmaz et al., 2009; Liu and Kaufman, 2005).

520 Thus, while the evidence from some of these species is ambiguous, the current picture suggests that  
521 pair-rule patterning may have evolved within crown-group insects, possibly multiple times. This is  
522 puzzling, because the specialised and relatively invariant body plan of insects presents a  
523 morphological constraint that is hard to reconcile with a saltational doubling of segmentation rate.  
524 (Instead, it is much easier to imagine pair-rule patterning evolving in remipedes, which are thought  
525 to be the sister group of hexapods (Schwentner et al 2017), and have homonomous, centipede-like  
526 bodies.) How was the evolution of double-segment periodicity coordinated with compensatory  
527 changes to Hox dynamics and the duration of axial extension, so as to keep segment number (**Box 2**)  
528 and segment identity constant? Given that *Strigamia* seems to switch to a single segment periodicity  
529 when adding its most posterior segments (Brena and Akam, 2013), and that pair-rule patterns are  
530 seen during the anterior patterning of otherwise segmental species (Dearden et al., 2002; Janssen et  
531 al., 2012), one possibility is that pair-rule patterning was introduced gradually along the AP axis,  
532 allowing other developmental parameters the chance to adapt.

533 Since pair-rule patterning requires half the number of clock cycles to generate a given number of  
534 segment-polarity stripes, its evolution may have been driven by selection for faster development (in  
535 holometabolans) or a longer body (in centipedes). However, it is currently not obvious how the  
536 ancestral segment patterning mechanism was modified to become pair-rule. Segmental frequency  
537 could have been doubled by changing the “readout” of a conserved clock, i.e. by evolving new  
538 enhancers to drive additional segment-polarity stripes in between the originals, or altering the  
539 control logic of existing enhancers to drive a pair of stripes instead of just one. Alternatively, the  
540 clock itself could have been modified, e.g. by recruiting new genes into the original cyclic repeat and  
541 thereby expanding its patterning potential. To reconstruct the specific regulatory changes that  
542 occurred, it will be informative to find out how the gene expression and enhancer logic of pair-rule  
543 species compares to their closest segmental relatives.

544

545 **The evolution of simultaneous segmentation**

546 *Reconciling sequential and simultaneous segmentation*

547 A segmentation clock is one strategy for generating periodicity, but another is simply to regulate  
548 each stripe individually, exploiting whatever positional information is locally available (François et  
549 al., 2007; Salazar-Ciudad et al., 2001; Vroomans et al., 2016). This latter method is used in the  
550 *Drosophila* blastoderm, where over 20 “stripe-specific elements” (SSEs) regulate the expression of  
551 the five primary pair-rule genes (Schroeder et al., 2011). These elements receive spatial information  
552 from gap factors, and each drives expression at a different AP position along the blastoderm,  
553 contributing just one or two stripes to a gene’s overall 7-stripe pattern. Sepsid flies (which diverged  
554 from drosophilids about 100 million years ago) are also known to use this kind of element (Hare et  
555 al., 2008), and it is likely that similarly ad hoc regulatory mechanisms are used wherever periodicity  
556 emerges simultaneously, e.g. in the blastoderms of *Nasonia* (Rosenberg et al., 2014) and *Oncopeltus*  
557 (Stahi and Chipman, 2016), or in the chelicerate prosoma (Pechmann et al., 2011; Schwager et al.,  
558 2009). While less “elegant” than using temporal oscillations, this explicitly spatial mode of  
559 segmentation can—in principle—occur much faster, since a number of different pattern repeats can  
560 be initialised at once.

561 Simultaneous segmentation, typified by *Drosophila*, is traditionally thought of as mechanistically  
562 distinct from sequential segmentation, typified by e.g. *Tribolium* or *Gryllus*. The textbook model of  
563 the hierarchical “subdivision” of a syncytial blastoderm by morphogen gradients seems a world away  
564 from waves of gene expression within a cellularised, elongating germband. However, the *Drosophila*  
565 blastoderm is now known to be more dynamic than was previously imagined, and the basic structure  
566 of its segment patterning network seems remarkably similar to that of other arthropods (**Fig. 4A**).

567 As the *Drosophila* blastoderm stage is so short, the effects of dynamic gene expression are subtle,  
568 and for years were overlooked. However, quantitative expression atlases suggest that expression  
569 domains in the posterior half of the blastoderm travel anteriorly across cells over time (Jaeger et al.,  
570 2004; Keränen et al., 2006; Surkova et al., 2008), and this has recently been demonstrated through  
571 live imaging (El-Sherif and Levine, 2016; Lim et al., 2018). The shifts reflect sequential patterns of  
572 transcriptional states within cells, and trace back to asymmetric repressive interactions in the gap  
573 gene network (Jaeger, 2011; Verd et al., 2018) (**Fig. 4B1**) – perhaps similar to the ones driving their  
574 temporal expression in the SAZs of sequentially-segmenting species.

575 In the *Drosophila* blastoderm, the expression dynamics of the gap genes are directly transferred to  
576 pair-rule genes via their SSEs (**Fig. 4B2**). In addition, the pair-rule genes cross-regulate each other  
577 through “zebra elements”: enhancers that drive expression in all of the trunk stripes simultaneously  
578 (Schroeder et al., 2011). (Some primary pair-rule genes, and both secondary pair-rule genes, possess  
579 zebra elements.) These regulatory interactions are also dynamic, and they combine with the stripe  
580 shifts driven by the gap genes to generate a staggered sequence of pair-rule gene expression within  
581 each double-segment repeat (Clark, 2017) (**Fig. 4B3**). This spatiotemporal sequence is the same as  
582 that driven by the segmentation clock in sequentially-segmenting species such as *Tribolium* and  
583 *Strigamia* (Choe et al., 2006; Green and Akam, 2013), suggesting that zebra enhancers and “clock”  
584 enhancers may be homologous.

585 Once primary pair-rule gene expression is properly phased within each double-segment repeat,  
586 *Drosophila* segment patterning proceeds just as it would in the anterior SAZ of a sequentially-  
587 segmenting species, beginning with the activation of *prd* and *slp*, and moving on to segment-polarity  
588 gene expression and stripe doubling. This conserved process of pattern resolution is apparently  
589 regulated by a conserved sequence of timing factor expression: posterior SAZ factors Caudal and  
590 Dichaete are expressed throughout the trunk during the early, dynamic stages of pair-rule gene  
591 expression in *Drosophila*, and are replaced by anterior SAZ factor Opa as the segment-polarity  
592 pattern is being resolved (Clark and Peel, 2018).

593 The *Drosophila* blastoderm therefore seems effectively equivalent to a SAZ, except that rather than  
594 maturing gradually from anterior to posterior, it does so all at once (**Fig. 4C**). We suspect that much  
595 of the ancestral segmentation machinery remains intact. However, since spatial information is no  
596 longer conveyed by the delayed maturation of posterior tissue, gap genes and SSEs preload it into  
597 the system instead (**Fig. 4A**). Importantly, while genetic perturbations tend to result in different  
598 phenotypes in the two modes of segmentation (e.g. primary pair-rule genes cause pair-rule  
599 phenotypes in *Drosophila* rather than truncations), this might often be explained by the divergent  
600 deployment of the genes in the embryo, rather than divergent function.

601

#### 602 *The evolution of stripe-specific elements*

603 Simultaneous segmentation differs from sequential segmentation in two key respects: its temporal  
604 regulation (determined by the expression profiles of the timing factors), and the spatial pre-  
605 patterning of the pair-rule genes by gap genes (**Fig. 4C**). Simultaneous segmentation is also  
606 associated with an anterior shift of the blastoderm fate map and an increase in the number of

607 segments patterned prior to gastrulation. (Note, however, that although segment patterning in the  
608 blastoderm is often simultaneous and regulated by gap genes, this need not be the case: *Tribolium*  
609 patterns its blastoderm segments sequentially, using retracting timing factors and a clock (El-Sherif  
610 et al., 2014, 2012).)

611 The evolution of simultaneous segmentation appears to be constrained by early embryogenesis  
612 (French, 1988). Some insects, such as orthopterans, have “panoistic” ovaries, in which all germline  
613 cells become oocytes, and the eggs contain little but yolk (Büning, 1994). These species pattern their  
614 segments sequentially. Other insects, such as hemipterans and holometabolans, have “meroistic”  
615 ovaries, in which germline-derived “nurse” cells load oocytes with maternal mRNA. These species  
616 frequently have a biphasic mode of segmentation, in which anterior segments are patterned  
617 simultaneously. Meroistic ovaries (which facilitate pre-patterning of the egg), may therefore be a  
618 pre-adaptation for simultaneous segmentation.

619 Extreme examples of simultaneous segmentation (e.g. *Drosophila*) have evolved independently  
620 within each of the major holometabolan orders (Davis and Patel, 2002). (Intriguingly, there has also  
621 been at least one reversion to sequential segmentation, within braconid wasps (Sucena et al.,  
622 2014)). A *Drosophila*-like mode of segmentation likely requires far-reaching changes to early  
623 embryogenesis, such as a novel anterior patterning centre to help spatially pattern gap genes along  
624 the entire AP axis of the egg (Lynch et al., 2006) (**Fig. 4A**). Here, we focus on understanding how SSEs  
625 and gap genes are together able to take over stripe patterning from the clock. It seems likely that  
626 this transition to intricate spatial regulation involves a series of selectively favourable regulatory  
627 changes, which incrementally increase the speed or robustness of segmentation, while strictly  
628 preserving its output (**Fig. 5**).

629 First, new SSEs seem to be easy to evolve, because they tend to be short, with simple regulatory  
630 logic and high sequence turnover between closely related species (Hare et al., 2008; Ludwig et al.,  
631 1998). Some of them may have been selected simply to increase the robustness of segmentation  
632 clock expression; this might have occurred in either a blastoderm or a SAZ context. (There is one  
633 report from *Tribolium* suggesting the existence of SSEs that drive expression in the germband (Eckert  
634 et al., 2004)). Importantly, because gap gene expression is inherently dynamic (whether in the  
635 blastoderm or the SAZ), SSE-regulated stripes are predicted to “shadow” stripes driven by the clock,  
636 allowing them to take over downstream functions quite gradually (Verd et al., 2018) (**Fig. 5A**).

637 Second, only a single new SSE need evolve at one time. Simultaneous patterning seems likely to have  
638 evolved progressively, from anterior to posterior, with each new SSE-driven stripe reducing the  
639 number of cycles needed from the clock (Peel and Akam, 2003) (**Fig. 5B**). Furthermore, cross-

640 regulation between the pair-rule genes means that a SSE for one gene could in principle go on to  
641 organise a whole pattern repeat, with the remaining genes evolving their own SSEs afterwards, to  
642 make patterning faster or more robust (Clark, 2017) (**Fig. 5C**). This process might be highly  
643 contingent: in *Drosophila*, *eve* and *runt* have full sets of SSEs and *odd* is patterned largely through  
644 cross-regulation (Schroeder et al., 2011), but RNAi evidence from *Bombyx* suggests precisely the  
645 opposite (Nakao, 2015).

646 Finally, SSEs can be reused. In *Drosophila* there are several SSEs that drive a pair of stripes, typically  
647 arranged symmetrically around a particular gap domain (Schroeder et al., 2011). This suggests that  
648 posterior gap gene expression evolved to duplicate the regulatory environments of anterior stripes,  
649 initialising additional pair-rule gene stripes without the need to evolve additional SSEs (**Fig. 5D**).

650 Interestingly, *Drosophila eve* stripes 3 and 7, which are co-driven by a single SSE, are regulated by  
651 the same gap genes as are *eve* stripes 3 and 6 in *Anopheles* (Goltsev et al., 2004), which has led to a  
652 proposal that certain stripes have been lost or gained from these lineages over time (Rothschild et  
653 al., 2016). This hypothesis is hard to reconcile with the gradualist scenario we favour, since the  
654 transitional states would have severely compromised fitness. We think it more likely that the  
655 posterior gap gene domains were recruited in a different order in the *Drosophila* and *Anopheles*  
656 lineages, resulting in a homologous “stripe 3” element additionally driving non-homologous  
657 posterior stripes. In support of this alternative, a midge species more closely related to *Drosophila*  
658 than to *Anopheles* patterns only five *eve* stripes before gastrulation (Rohr et al., 1999), indicating  
659 that the two lineages probably evolved fully simultaneous segmentation independently (Jaeger,  
660 2011).

661

## 662 **Conclusion**

663 Our current understanding is that arthropod segment patterning is an inherently dynamic and a  
664 significantly conserved process, ancestrally taking the form of a clock-and-wavefront system. Note,  
665 however, that many of the conclusions in this review extrapolate from fragmentary data gathered  
666 from a small number of model species, with functional data available from an even smaller number.  
667 This is certainly not the last word on arthropod segmentation, but we hope to have provided a  
668 coherent framework for further thought and experiment.

669 We anticipate that future investigation will centre on two contrasting but interrelated tasks. First,  
670 better resolving the nature of the ancestral arthropod clock-and-wavefront system: the topology of  
671 the gene regulatory networks comprising the clock, the production of timing factor wavefronts by a

672 retracting SAZ, and the mechanistic basis for the interactions between them. Second, reconstructing  
673 how arthropod segmentation networks have diversified over time, giving rise to such remarkable  
674 novelties as simultaneous patterning and double-segment periodicity. In addition, we believe that  
675 sequentially-segmenting arthropod models are well placed to complement and inform the study of  
676 vertebrate axial patterning, especially given their benefits of cost-efficiency, short generation times,  
677 experimental tractability, and relatively simple genomes.

678 The most pressing next step is to collect good-quality multiplexed expression data from a variety of  
679 arthropod species (Choi et al., 2018, 2016) and cross-reference this with information about tissue  
680 dynamics (Wolff et al., 2018), to better characterise how segmentation gene expression changes  
681 over space and time. Building on a solid descriptive foundation, there are numerous exciting  
682 directions to pursue: genome editing to generate mutants, misexpression constructs, and live  
683 reporters (Gilles et al., 2015; Lai et al., 2018); construction and analysis of data-informed dynamical  
684 models (Sharpe, 2017); single-cell sequencing of segmenting tissues (Griffiths et al., 2018); *ex vivo*  
685 culturing of SAZ cells (Lauschke et al., 2013). Over the past four decades, arthropod segmentation  
686 has contributed enormously to our understanding of developmental gene networks and their  
687 evolution. As we enter a new “golden age” of developmental biology, we see great promise for this  
688 legacy to continue.

689

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**Fig. 1. Overview of arthropod segmentation.** (A) Phylogenetic tree of notable arthropod model species (based on Misof et al., 2014; Schwentner et al., 2017). Red text indicates species known to use pair-rule patterning; the status of *Oncopeltus* is currently unclear. Branch lengths not to scale. (B) Diagram showing the relationship between parasegments and segments. Pink=*engrailed* expression; 'A'=anterior; 'P'=posterior. (C) Schematic time series of an arthropod embryo undergoing sequential segmentation. *engrailed* stripes (pink) emerge sequentially from a retracting segment addition zone (SAZ, blue) as the germband extends posteriorly. Green dots mark the progress of a specific individual cell that starts in the posterior SAZ (dark blue), passes through the anterior SAZ (light blue), and ends up in the segmented germband.

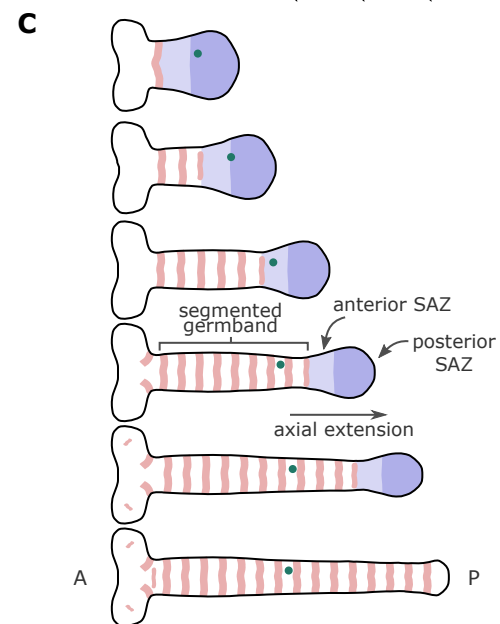
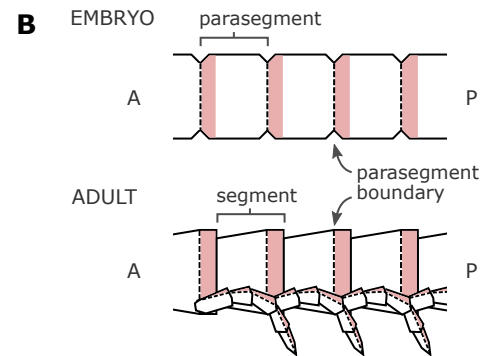
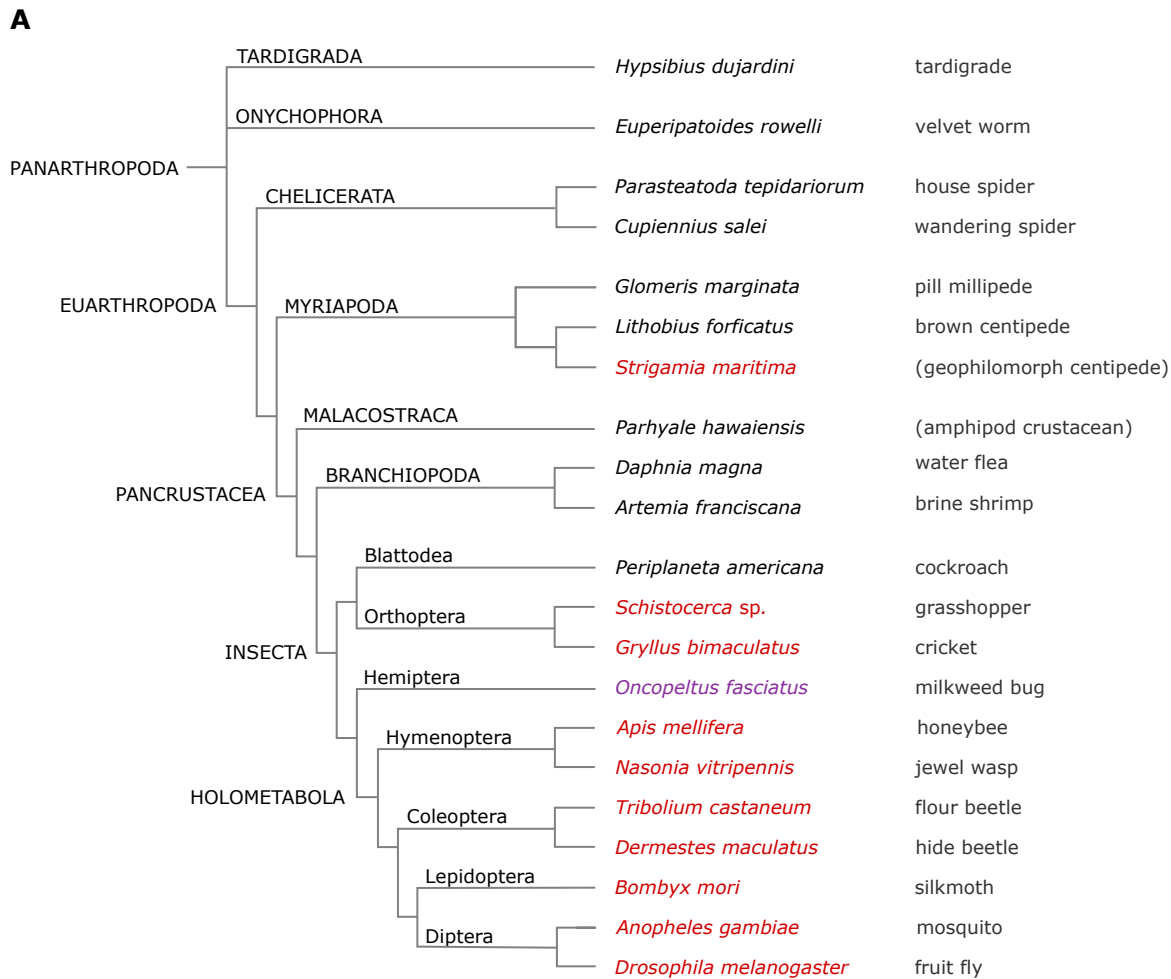
**Fig. 2. Within cell, between-cell, and tissue-level aspects of the arthropod segmentation clock.** (A) Pair-rule gene oscillations may be driven by a cross-regulatory feedback loop within cells. The two hypothetical topologies shown (left) would be capable of driving similar, although not identical, cycles of *eve*, *runt*, and *odd* expression within cells (right). In *Tribolium*, the relative expression patterns of *Eve* protein, *runt* transcript and *odd* transcript resemble the predicted expression of model 2, rather than model 1 (see Supporting Information from Choe et al., 2006). Expression predictions assume Boolean regulatory logic and equal time delays for protein synthesis and protein decay (Clark, 2017). (B) Notch signalling might indirectly synchronise intracellular oscillations of *eve*, *runt*, and *odd* across cells, by acting through *hairy*. This figure shows a hypothetical regulatory network, which synthesises genetic interactions documented from various different arthropod species (Clark, 2017; Eriksson et al., 2013; Nakao, 2015; Pueyo et al., 2008; Stollewerk et al., 2003). The left half of the network ("oscillator 1") would synchronise oscillations of *hairy* across neighbouring cells, by coupling *hairy* expression to Notch signalling. The oscillations of *hairy* would then influence the phase of the genetic ring oscillator that forms the right hand of the network ("oscillator 2"), by repressing some of its component genes. (C) Genes such as *Wnt*, *caudal*, *Dichaete*, and *opa* have distinct expression patterns within the SAZ, which correlate with different phases of segment patterning. 'A'=anterior; 'P'=posterior. (Based on *Tribolium* data from Clark and Peel, 2018.) Note that *Wnt* and *opa* have segment-polarity patterns in the segmented germband. *caudal* and/or *Dichaete* stripes (not shown) are seen in the anterior SAZ of some species, indicating that the clock feeds back on their expression (Chipman et al., 2004; Clark and Peel, 2018).

**Fig 3. Resolving the segment pattern: from oscillations to stable stripes.** (A) Comparison of patterning using a single-gene oscillator versus patterning using a three-gene oscillator. With a single-gene oscillator, different cell fates are determined by different expression levels of the oscillator. The output is sensitive to noise in the amplitude of, or measuring of, the signal, and must be palindromic, because the input signal is symmetrical. With a three-gene oscillator, different cell fates can be determined by different combinations of input factors. The output is more robust to noise, and has an inherent polarity. (B) Comparison of the segment-polarity fate readout for clocks with single-segment or double-segment periodicity. Parasegment boundaries (red lines) form wherever a cell with an anterior segment-polarity fate ('A'; i.e. expressing *engrailed*) abuts a cell with a posterior segment-polarity fate ('P'; i.e. expressing *slp* and *wg*). A third cell fate (light grey; e.g. *odd* in *Drosophila*) prevents ectopic boundaries. Note that species with double-segment periodicity have a different, more complex mapping between the input pattern (pair-rule gene expression) and the output pattern (segment-polarity gene expression). (C) Dynamic model for the patterning of *prd* and *slp* in *Drosophila*: the staggered expression boundaries of *prd* and *slp* are caused by the *Eve* stripes shifting anteriorly across the tissue over time. The posterior border of the *prd* stripe is patterned at timepoint  $t_1$  (*Eve* expression shown by dotted line), while the posterior border of the *slp* stripe is patterned a short while later, at timepoint  $t_2$  (*Eve* expression shown by solid line). (Based on Clark, 2017). (D) The staggered pattern of pair-rule gene expression comprises a positional code, which specifies narrow stripes of segment-polarity gene expression. The regulatory logic (top) and resulting expression pattern (bottom) of *Drosophila engrailed* (*en*) is shown as an example. Note that odd-numbered and even-numbered *en* stripes are regulated differently. (Based on Jaynes and Fujioka, 2004).



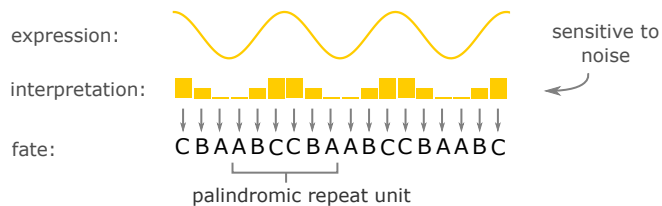
**Fig. 4. Reconciling sequential and simultaneous segmentation.** (A) Structural overview of arthropod segmentation gene networks. The core of the system (yellow box) is relatively conserved across species. In sequential segmentation, spatial information is provided by the timing factor network, which generates a wavefront. Gap genes do not play a major role in segment patterning, although late gap gene expression may be important for “shutting down” the SAZ, by repressing timing factors that maintain it (dashed blue arrow). In simultaneous segmentation, timing factors only provide temporal information. Spatial information is usually provided by a novel anterior patterning centre (i.e. a morphogen gradient such as Bicoid (Liu et al., 2018; McGregor, 2005)), which regulates gap gene expression. Gap genes pass this information to the primary pair-rule genes, through newly-evolved regulatory elements (SSEs). (B) Spatial patterning in *Drosophila* is inherently dynamic. (1) Regulatory interactions between gap genes cause gap domains to shift anteriorly across the blastoderm over time. (2) Stripes of pair-rule gene expression regulated by gap inputs also shift anteriorly. (3) Regulatory interactions between the pair-rule genes convert these shifts into a staggered pattern of expression overlaps across the pair-rule repeat. Note that each panel zooms in on a smaller region of the AP axis. (C) Schematic kymographs (i.e., plots of how gene expression along the AP axis changes over time) comparing the key spatiotemporal features of sequential and simultaneous segmentation. In sequential segmentation, timing factor expression (blue) matures from anterior to posterior across the tissue, producing a wavefront (diagonal line). Periodicity is generated by sustained oscillations (note how *even-skipped* turns on and off over time within the blue zone). The wavefront converts the oscillations into a stable segment-polarity pattern. In simultaneous segmentation, there is little spatial regulation of timing factor expression across the tissue, and pair-rule stripes are present from the start. Embryo diagrams depict the specific timepoints they line up with on the kymographs (*eve* expression is not shown). Patterning has double-segment periodicity. Note the different scales of the two time axes.

**Fig 5. The evolution of simultaneous segmentation involves a gradual replacement of the segmentation clock by SSEs.** (A) Clock enhancers (potentially homologous to zebra elements) and SSEs both drive stripes that shift anteriorly over time. SSEs can therefore gradually assume regulatory control over particular clock-driven stripes, without disrupting downstream patterning. (B) (1) Simultaneous patterning is likely to evolve stepwise along the AP axis, via the acquisition over evolutionary time of new SSEs that control expression in increasingly posterior stripes. Embryo diagrams assume a segmentation clock with double-segment periodicity. (2) Simultaneous patterning is likely to evolve stepwise within each pair-rule gene expression repeat, as more of the primary pair-rule genes evolve their own SSEs. Additional SSEs reduce the time required to organise pair-rule gene expression across the repeat. (D) Changes in gap gene expression can be sufficient to generate additional SSE-driven stripes, without accompanying changes in cis-regulatory logic. In *Drosophila* (right panel), SSEs such as *eve 3+7* and *eve 4+6* each drive a pair of stripes. The current situation likely evolved from a simpler scenario (left panel), in which the same enhancers drive expression in only one stripe each. Hb=Hunchback; Kr=Krüppel; Kni=Knirps; Gt=Giant. Note that *eve 3+7* and *eve 4+6* are both repressed by Kni and Hb, but with different relative strengths, represented by different arrow thicknesses (Samee et al., 2017). Diagrams are colour-coded such that transcription factor names (top) have the same colour as their corresponding expression domain(s) (below).

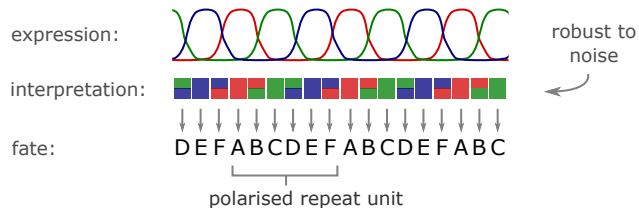




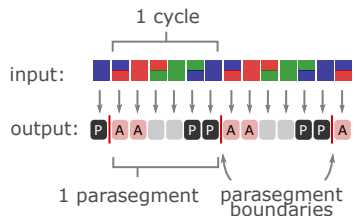
### A single-gene oscillator:



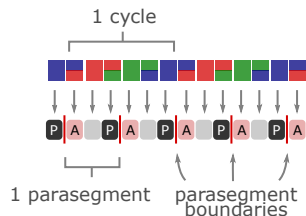
### three-gene oscillator:



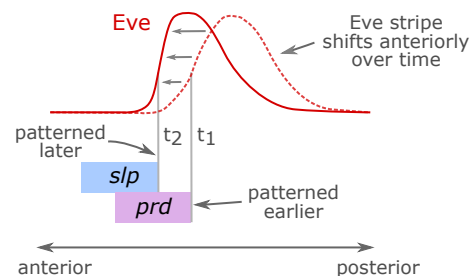
### B single-segment periodicity:



### double-segment periodicity:

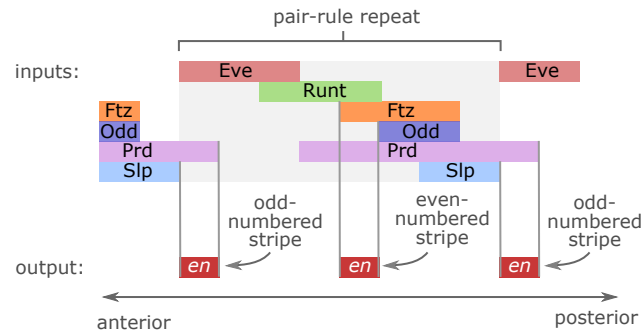
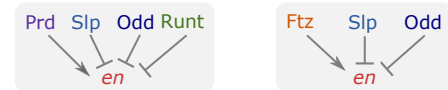


### C dynamic model for the patterning of *prd* and *slp*

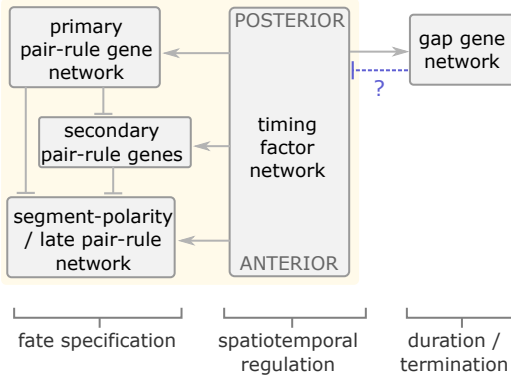


### D segment-polarity genes are patterned by a "combinatorial code" of pair-rule gene expression

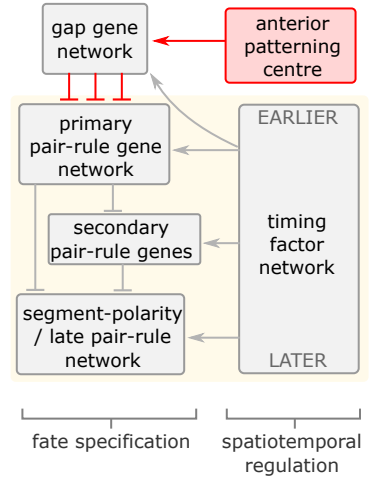
control logic: odd-numbered stripes    even-numbered stripes



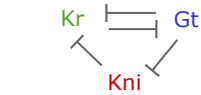
### A sequential segmentation



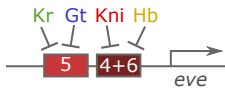
### simultaneous segmentation



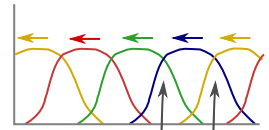
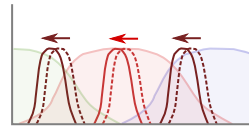
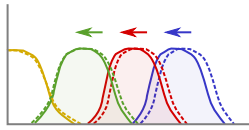
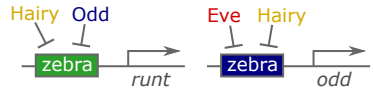
### B 1) cross-regulation causes gap expression shifts



### 2) gap shifts cause SSE-driven stripes to shift

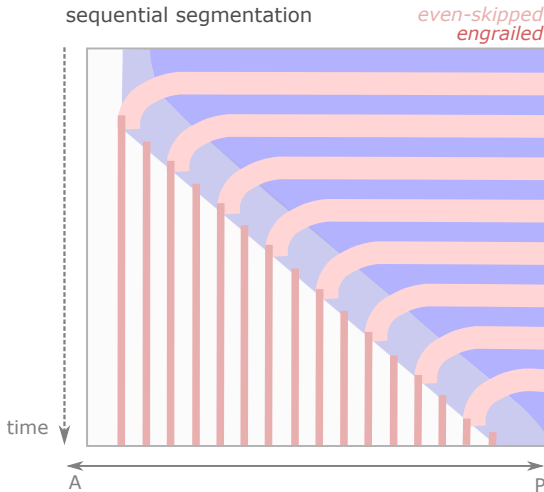


### 3) shifts plus cross-repression organise pair-rule pattern



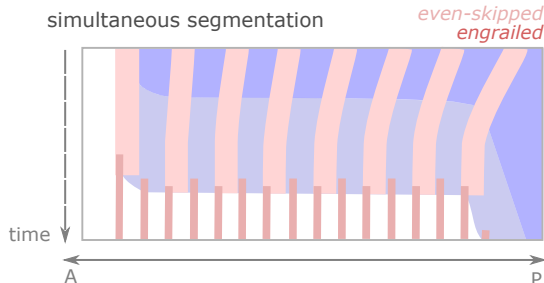
unstable but functionally important overlaps

### C sequential segmentation



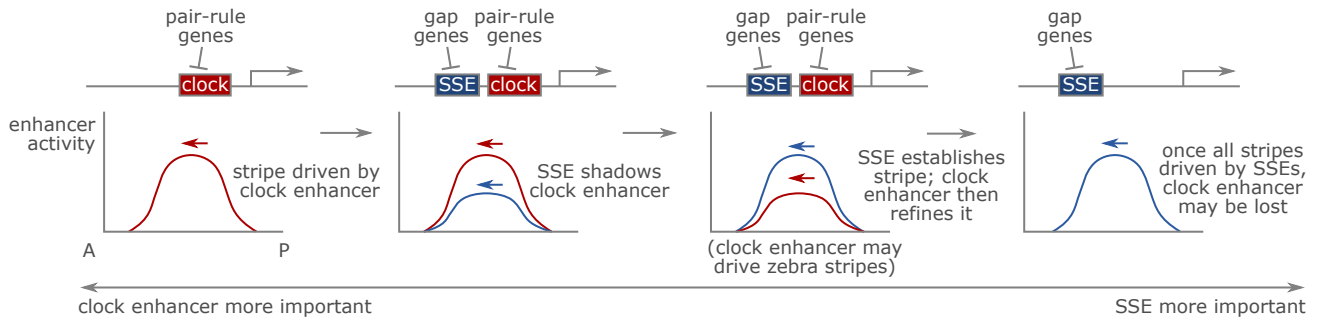
*even-skipped*  
*engrailed*

### simultaneous segmentation



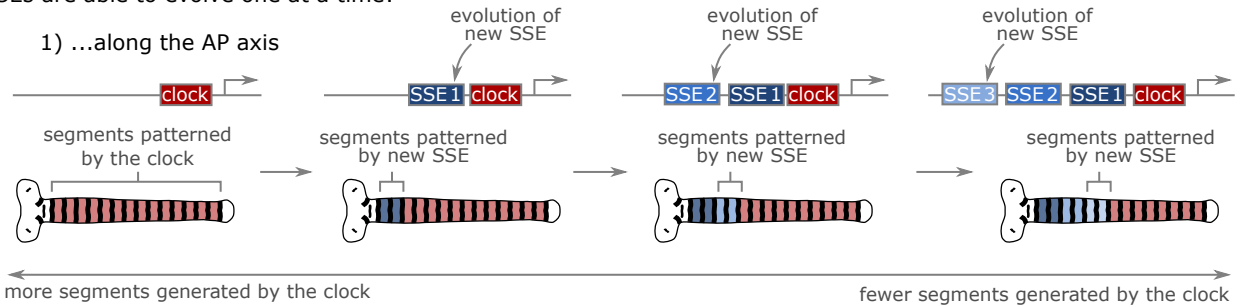
*even-skipped*  
*engrailed*

**A** each SSE can take over from the clock gradually

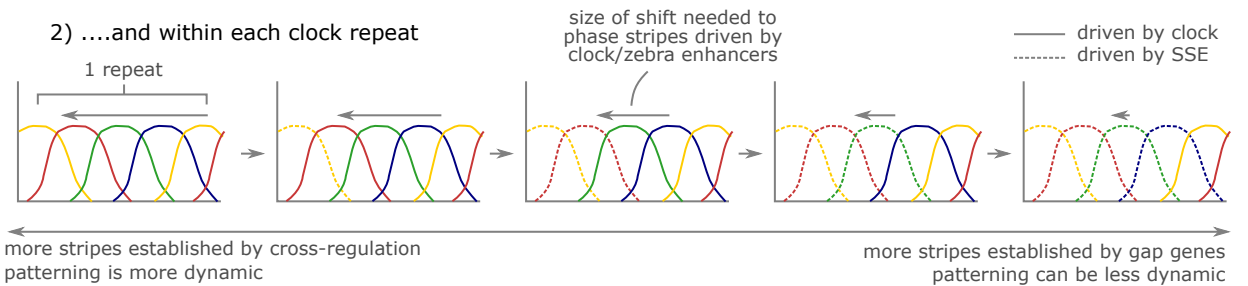


**B** SSEs are able to evolve one at a time:

1) ...along the AP axis



2) ....and within each clock repeat



**C** existing SSEs can be recruited to drive additional stripes

