

Mechanisms and constraints shaping the evolution of body plan segmentation^{*}

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Abstract. Segmentation of the major body axis into repeating units is arguably one of the major inventions in the evolution of animal body plan patterning. It is found in current day vertebrates, annelids and arthropods. Most segmented animals seem to use a clock-and-wavefront type mechanism in which oscillations emanating from a posterior growth zone become transformed into an anterior posterior sequence of segments. In contrast, few animals such as *Drosophila* use a complex gene regulatory hierarchy to simultaneously subdivide their entire body axis into segments. Here I discuss how *in silico* models simulating the evolution of developmental patterning can be used to investigate the forces and constraints that helped shape these two developmental modes. I perform an analysis of a series of previous simulation studies, exploiting the similarities and differences in their outcomes in relation to model characteristics to elucidate the circumstances and constraints likely to have been important for the evolution of sequential and simultaneous segmentation modes. The analysis suggests that constraints arising from the involved growth process and spatial patterning signal—posterior elongation producing a propagating wavefront *versus* a tissue wide morphogen gradient—and the evolutionary history—ancestral *versus* derived segmentation mode—strongly shaped both segmentation mechanisms. Furthermore, this implies that these patterning types are to be expected rather than random evolutionary outcomes and supports the likelihood of multiple parallel evolutionary origins.

1 Introduction

Body plan segmentation, the subdivision of the body plan into similar, repeating units, is one of the major evolutionary innovations that arose in bilaterian animals [1]. In modern animals overt body plan segmentation is found in the vertebrate, arthropod and annelid clades, with the large majority of animals being seemingly unsegmented (fig. 1A). However, recent experimental advances have shown some remarkable similarities between mechanisms and genes involved in segmentation in these three animal groups [2–5]. As a consequence it is currently heavily debated whether body plan segmentation evolved once at the root of the bilaterians and was subsequently lost in most animal groups, or whether the segmentation mechanisms of vertebrates, arthropods and annelids are an example of parallel, convergent evolution (fig. 1A) [6–10,1].

Although most segmented animals display a very similar mode of body plan segmentation, in which a clock-like mechanism periodically produces new segments from

a posterior growth zone in an anterior to posterior fashion [22,23,24,24], some insects—most notably the well studied *Drosophila*—display a distinct mode of segmentation (fig. 2). Here, body segments are formed simultaneously rather than sequentially very early during development. While sequential segmentation is characterised by a regulatory network producing clock-like oscillations, the simultaneous segmentation of *Drosophila* is characterised by a complex hierarchy of so-called maternal, gap, pair rule and segment polarity genes [25–28] (fig. 2). It has been suggested that the *Drosophila* mode of segmentation arose through selection for developmental speed and that replacing sequential with simultaneous segmentation may have come at the cost of decreased developmental robustness. However, such ideas are hard to test experimentally. It is unclear why such a complicated hierarchical system evolved to dictate simultaneous segmentation [28]. That is, we do not know whether this hierarchy arose as a mere evolutionary accident, or that particular evolutionary mechanisms and developmental constraints made it likely for such a network topology to evolve. Furthermore, given that spread out over the insect tree other species show simultaneous or partially simultaneous segmentation it appears that this complex mechanism may have evolved multiple times (fig. 1B).

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A Overt body segmentation in the Bilaterian tree

B Long germ segmentation in the Insect tree

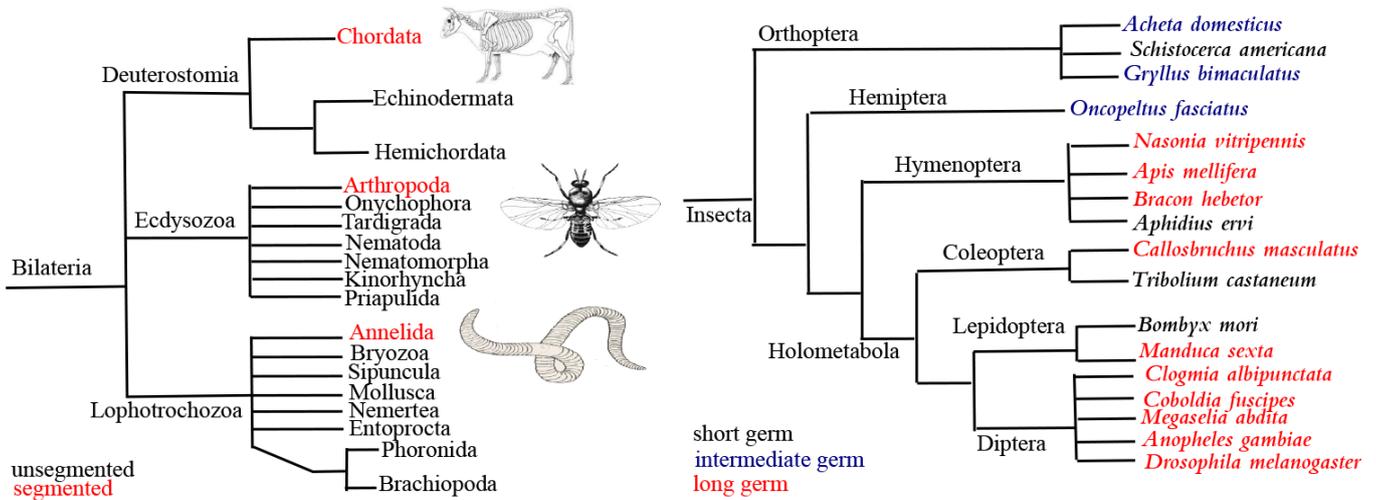


Fig. 1. A) Spread of segmentation in the Bilaterian tree. B) Spread of long germ band segmentation in the Insect tree. In A) a schematised overview of the bilaterian phylogenetic tree is shown, with indicated in red the bilaterian groups displaying overt body plan segmentation. The tree and spread of segmentation is based on [9,10,1] and tolweb.org. In B) an overview of the insect phylogenetic tree is shown, with at the leaves of the tree example species found in in each clade. In blue and red are indicated example species showing intermediate or short germ band segmentation. Tree and segmentation modes based on [11–21] and tolweb.org.

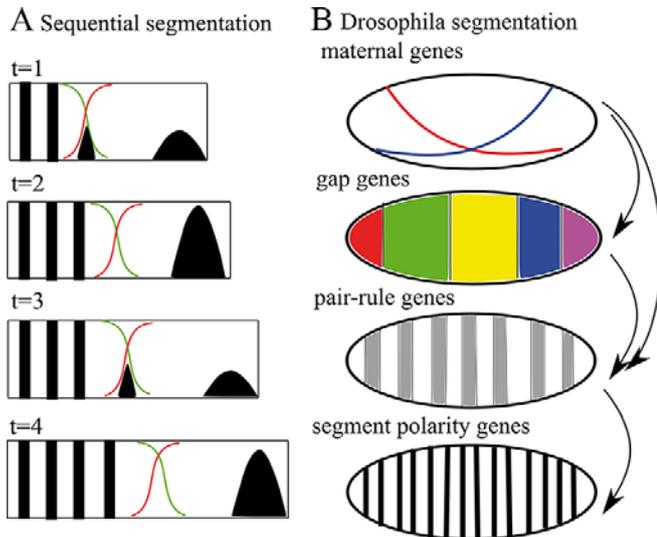


Fig. 2. Schematic depiction of sequential, clock driven (A) and simultaneous Drosophila-type (B) segmentation. In A) a schematised overview of clock-and-wavefront type segmentation is shown. The increasing and decreasing black curve in the back represents the posterior growth zone oscillator, the intersection between the red and green curve represents the wavefront that determines where the transition from temporal oscillations into spatial stripes occurs. Over time, sequentially more segments are laid down, resulting in growth of the segmented domain. In B) a schematised overview of the Drosophila segmentation hierarchy is shown, depicting the information transfer from maternal, to gap, to pair rule to segment polarity genes that results in a subdivision of the axis into smaller and smaller domains.

While detailed comparative experimental studies play a crucial role in answering historical questions of how different segmentation modes may have evolved, they can make only a limited contribution to answering mechanistic questions on why these particular developmental processes evolved. In this paper I will demonstrate the role of evolutionary developmental (evo-devo) simulation studies in answering such questions. First, I will discuss the general ideas and approaches behind these simulation studies, showing how the repeated simulation of evolutionary processes under both identical and different conditions allows us to establish what are likely evolutionary outcomes and on what assumptions and conditions this depends.

Next, I will perform a new, in-depth comparison of simulation studies performed in this area [29–33], analysing what their combined results —both their commonalities and differences— can tell us about the mechanisms and constraints shaping the evolution of segmentation mechanisms. This comparative analysis suggests that both sequential, oscillation-driven segmentation and simultaneous, hierarchic segmentation are to be expected rather than random evolutionary outcomes once we take the constraints arising from their evolutionary history and available patterning signals into consideration. This lends credibility to the hypothesis of parallel evolution of sequential segmentation in vertebrates, annelids and arthropods and also explains why similar simultaneous segmentation modes have evolved multiple times within the insects.

I will end with suggestions for future work needed to further test these ideas, and to investigate the potential influence of additional constraints such as those arising from the characteristics of gene regulation.

2 Materials and methods

2.1 Construction and use of *in silico* evo-devo models

There are three major ingredients for an *in silico* evo-devo model, a procedure simulating a Darwinian evolutionary process, a genotype phenotype mapping (GP map) dictating how the genome encodes the developmental process and resulting phenotype, and a fitness criterium.

Darwinian evolution. An algorithm simulating Darwinian evolution simply emulates the processes of inheritance of parental characters by offspring, mutations producing variation of characters in offspring relative to parents, and fitness-based competition for limited resources, together resulting in the biased survival of better adapted organisms.

In the models discussed here, the genotype of the *in silico* organisms consists either of a genome encoding a gene regulatory network (GRN) or directly of a regulatory network. Offspring inherits the genotype from a single parent. Mutations can build new or remove existing regulatory connections between genes, change their nature (activating or repressing gene expression) and strength, and in addition may affect maximum gene expression and protein decay rates. In the models discussed here, organisms have a fitness-dependent chance to produce offspring, thus resulting in fitness-based competition.

Genotype-phenotype mapping. The architecture of the gene regulatory network determines the direction and nature of regulatory interactions among genes. Together with a set of rules or equations that specify how regulatory inputs impinging on a gene are integrated to define its expression level, this dictates the dynamics of gene expression. In a subset of models these rules also govern how cell-cell signalling influences gene expression. In order for the gene expression to be able to produce spatial patterning, the simulated organisms should have a multicellular body. For reasons of computational efficiency and simplicity, the models assume a preformed one-dimensional embryo consisting of a row of cells, thus ignoring morphogenetic processes of cell division and movement as well as patterning in higher dimensions. Finally, given that all cells in the body are genetically identical, the models use a symmetry breaking signal to allow for spatial patterning.

Fitness criterium. The final important model ingredient is the fitness criterium. Generally, when biologists think about the fitness of an organism and hence about the number of offspring it may produce this is in functional terms, *i.e.* whether an organism is well capable of locating food, escaping predators, or finding mates. If this type of fitness criterium is used in an evolutionary simulation model, what phenotype evolves is left free, as long as it produces an organism “that works”. *I.e.*, a final evolved organism may find mates through its courtship behaviour, through its looks, or by killing all competition, depending on both chance and what works best in the particular simulation. In contrast, here we are specifically interested in

the evolution of a particular phenotype, *i.e.* a segmented body plan. Thus, to ensure the evolution of these phenotypes we select for them, making the fitness of our *in silico* organisms depend on the number and pattern of body segments generated. As a consequence, the fact that segmentation evolves in these simulations is to be expected rather than an interesting result.

Instead, the interesting results are the type of segmental patterning, the type of developmental program producing that patterning, the genetic encoding of this process, and the evolutionary trajectory leading to it. Put differently, what general type of phenotype evolves is now strongly constrained by the fitness criterium, but its details, and how it is developmentally, genetically and evolutionarily achieved are left completely free. It is these issues then that are the subject of interest. They will allow us to answer how developmental programs underlying segmentation evolve and why particular development programs evolve. Figure 3 shows an overview of how *in silico* evo-devo models typically work.

The single most important advantage of these models is that they allow us to rerun *in silico* life’s tape again and again under both the same and different conditions. Rerunning the tape under similar conditions allows us to determine the number and variety of possible evolutionary outcomes and how these differ in frequency of occurrence and hence in likelihood of evolving. By subsequently varying for example developmental and gene expression noise, or fitness criterium we can also determine how these outcomes differ in robustness or future evolutionary potential. Finally, we can change conditions to determine how evolutionary outcome depends on factors such as population size, selection pressure, mutation rate, and mutational operators, allowing us to for example establish the relative importance of coding *versus* regulatory mutations or of selection *versus* drift. Together, these experiments enable us to answer whether a given evolutionary outcome is merely a chance outcome in a sea of many other possible outcomes, one of a few possible outcomes, or the evolutionary most accessible or most robust outcome of a set of outcomes. By comparing simulation outcomes with natural developmental mechanisms we can thus get an idea of whether these natural mechanisms arose accidentally or are a to be expected outcome and what were the mechanisms and constraints responsible for this.

An additional advantage is that a perfect *in silico* fossil record of genotypes, phenotypes, mutations and ancestry relations can be preserved. This allows for an exact reconstruction of which mutations and network structures led to major phenotypic innovations.

2.2 Analysed body segmentation evo-devo studies

We focus our analysis on a total of 5 simulation studies on the evolution of body plan segmentation. The first studies in this area were performed by Salazar-Ciudad and colleagues [29,30], and were followed by studies by Francois and co-workers [31], Fujimoto *et al.* [32], and Ten Tusscher and Hogeweg [33]. While these studies all applied the

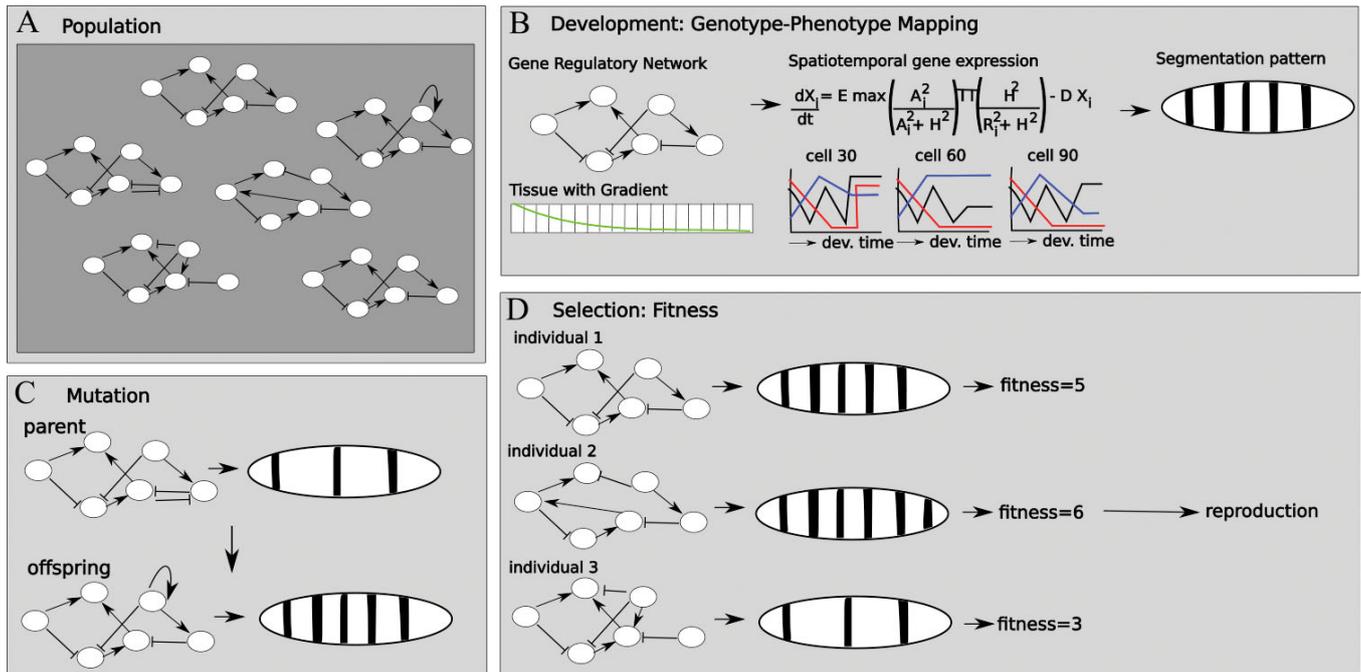


Fig. 3. Schematic overview of the ingredients of a typical *in silico* evo-devo model. The model contains an evolving population of individuals, with each individual having a gene regulatory network as its genotype (A). The GRN topology, together with an initial gradient over the one-dimensional body axis, and equations describing how gene expression depends on regulatory input, together dictate the spatiotemporal developmental gene expression pattern and resulting phenotype (number of stripes of the segmentation gene in the final expression pattern) (B). Mutations occur during reproduction on the GRN topology, changing network wiring, and hence gene expression dynamics and final phenotype (C). The reproduction chances of individuals are fitness-based, with fitness increasing with segment number (D).

general approach described in the previous section, they differ in some of the more detailed assumptions and model settings, as described in table 1.

Differences related to evolutionary trajectories. Except for the study by Ten Tusscher and Hogeweg [33] that used a genome encoding the regulatory network, studies used the GRN directly as the genotype. As a consequence, mutations occur on the network rather than on the genome sequence. Table 1 furthermore shows that studies differ in whether a small but evolutionary expanding developmental gene toolkit [29–31] or a larger but constant toolkit size [32,33] is modelled. In network-based models, only connections between currently present genes may evolve, while in genome-based models transcription factor binding sites and genes encoding transcription factors (TFs) may evolve independently. When network-based models are combined with an initially small number of genes, this introduces a bias towards evolving interactions between genes present early during evolution.

Differences related to the GP mapping. As can be seen in table 1, the studies also differ in details influencing how the genotype is mapped to a phenotype. First, the models differ in the rules used to integrate the impact of multiple TFs into a single gene expression level, ranging from simple additive integration [29,30], OR-gate-like integration for both activators and repressors [31,33], or even

multiple, alternative enhancers (see [32], personal communications). In addition, models differ in whether cells behave in a fully autonomous matter, or whether cell-cell signalling is taken into account. Finally, studies differ in the symmetry breaking input pattern used to enable spatial pattern formation, using either a distinct initial gene expression pattern in a single cell [29,30], a morphogen concentration gradient mimicking the Bicoid gradient in *Drosophila* [31,32] or a travelling wavefront resembling the RA/FGF/Wnt wavefront system implicated in vertebrate clock and wavefront somitogenesis [31,33]. Together these differences influence the type of developmental processes and segmentation patterns (phenotypes) that can possibly occur.

Differences related to fitness criteria. Even though all studies select for segmentation, used fitness criteria differ in how generic they are, that is whether selection is for a maximum number of segments (generic) or for a particular number of segments at particular locations (specific). While a general selection pressure for increasing segment numbers allows for gradual evolutionary hill climbing in a smooth fitness landscape, selection for a very specific segmentation pattern results in a random search in a rugged fitness landscape with a high possibility of getting stuck in local optima [31,34]. This significantly influences both the ease with which patterns evolve as the type of patterns that may evolve. In addition, while most studies selected

Table 1. Comparison of model settings used in the different studies.

Model characteristic	Salazar-Ciudad	Francois <i>et al.</i>	Fujimoto <i>et al.</i>	Ten Tusscher and Hogeweg
Genetic architecture	network	network	network	genome encoding network
Developmental toolkit size and evolutionary dynamics	few genes, duplication and divergence	few genes, duplication and divergence	32 gene types, no duplication or divergence	16 gene types, duplication, no divergence
Regulatory integration	all additive	activators: OR, repressors: OR, multiply activation with repression	activators: AND, repressors: OR, enhancers: additive	activators: OR, repressors: OR, multiply activation with repression
Signalling mode	diffusive	none, cell autonomous	diffusive	none, cell autonomous
Symmetry-breaking input pattern	single cell different	gradient or wavefront	gradient	wavefront
Body size	25 cells	100 cells	100 cells	100 cells
Fitness criterium	specific number of segments at regular positions, temporally stable	maximum number of segments, temporally stable	10 segments	maximum number of segments, temporally stable
Population size	100	100	100	500
Number of generations	600	400	2000	5000

for a segmentation pattern that was stable over time, this was not the case in the study by Fujimoto *et al.* [32]. As we will discuss later this also has important implications for the type of developmental processes that may evolve.

A final difference not discussed in table 1, is that while most studies investigated the evolution of segmentation in isolation, Ten Tusscher and Hogeweg studied the combined evolution of segmentation and spatial differentiation [33]. The need for segmentation to be coordinated with differentiation may affect the type of segmentation mechanisms likely to evolve.

In our analysis, these model differences, combined with both the similarities and differences in model outcomes, are used as a bootstrapping means to help us elucidate the mechanisms and constraints shaping the evolution of particular segmentation mechanisms.

Note that apart from the discussed studies, several related studies have been performed. We refrained from discussing these in detail as these were either non-evolutionary but rather took an ensemble approach studying all possible networks topologies and their pattern forming potential [35,36], limited their study to very small networks [37,36], or studied the evolution of differentiation without segmentation [38,39].

3 Results

3.1 General results

Despite the discussed differences between the models, these simulation studies produce a range of remarkably similar results demonstrating the generality of their findings.

A first general finding of the models is that whether the spatial heterogeneity is in the form of a gradient, moving wavefront or small initial perturbation of gene expression, segmentation patterns frequently evolve. Thus, although axial segmentation is a seemingly complex property, the complex, non-linear genotype-phenotype mapping from GRN to developmental process allows for it to reasonably easy evolve through a gradual, incremental process.

Another general finding of these model studies is that independent of whether simulations started with a constant number of 16-32 gene types [33,32] or started with a small but expanding genome [29,31], a limited number of final genes combined with regulatory rewiring was found sufficient for complex developmental patterning to evolve. This is consistent with the finding that animals ranging in body plan complexity from cnidarians to arthropods and vertebrates share a largely similar developmental toolkit [40–42]. It supports the hypothesis that an increased regulatory complexity in higher more complex animals explains the resulting paradoxical non-correlation between body plan complexity and genome size [43,40,44,45,41,46–48].

Finally, all studies show that evolution produces only a few discrete developmental patterning modes (table 2). This clearly demonstrates that developmental programs do not arise from a random mutational process producing all possible kinds of programs and patterns with selection subsequently selecting a subset of them. Rather, developmental programs are the result of a complex interplay between the multi-level, non-linear genotype-phenotype map and selection that together constrain the number of possible, evolutionary accessible and developmentally robust phenotypes [49].

Table 2. Comparison of evolved segmentation modes. Indicated are whether segments are generated sequentially (Seq) or simultaneously (Sim) through development. For sequential segmentation it is indicated whether it is oscillation-driven (Osc) or occurs in a segment specific manner (Segm Spec), and whether it becomes temporally stable (St) or not (-). For simultaneous segmentation it is indicated whether the underlying network architecture is hierarchic (Hier), whether segments are generated in a segment specific manner (Segm Spec) and whether mutual repression and gap-like non-overlapping domains (Mut Repr) are formed, or not (-). For Francois *et al.* we indicate under which conditions (W: wavefront; G: gradient) a developmental mode evolves (table 1).

Study	Evolved developmental segmentation modes
Salazar-Ciudad	Sim: -; -; - Sim: Hier; -; -;
Francois <i>et al.</i>	Seq: Osc; St (W) Sim: -; Segm Spec; Mut Repr (G)
Fujimoto <i>et al.</i>	Seq: Osc; - Sim: Hier; Segm Spec; -
Ten Tusscher and Hogeweg	Seq: Osc; St Seq: Segm Spec; St

3.2 Sequential segmentation

Sequential segmentation is best studied in vertebrates, with gradually more knowledge accumulating for arthropods and annelids [22, 23, 2–4, 24, 50]. During sequential segmentation, new segments are formed with clock-like regularity from a posterior growth zone, thus producing segments in an anterior to posterior sequence. In 1976 Cooke and Zeeman postulated the so-called clock-and-wavefront model [51], proposing that regular, sequential segment production results from the interaction between an intercellular oscillator clock and an anterior to posterior propagating determination wavefront. In the original formulation the wavefront confers the potential to differentiate into a somitic cell, while the oscillator phase at the time of wavefront passage speeds up or slows down this differentiation process, ensuring synchronous transformation of blocks of cells into somites (see also [52–54]). Thus, the original focus was on the final phase of differentiation, in which changes in cell motility and adhesion lead to somite formation [51, 55].

Meinhardt was the first to propose an alternative interpretation, in which oscillation phase, rather than serving to synchronise differentiation, itself confers differentiation, by becoming stably memorised into a spatial stripe pattern [56]. Note that in this case an earlier phase of differentiation is considered, namely the prepatterning of segment boundaries rather than final somite morphogenesis. This segment prepatterning interpretation of the clock-and-wavefront model has considerable experimental support, and is the type of segment patterning we are concerned with in this study.

In vertebrates, oscillations start in the posterior growth zone, propagate through the presomitic mesoderm and stop and freeze in their current phase upon reaching the previously formed segments. The oscillator clock arises from a complex network of interactions involving the Wnt, FGF and notch signalling pathways. Synchronisation of these cellular oscillations is achieved via the notch signalling pathway [57, 58]. The determination wavefront is thought to be formed by the mutually repressive, opposed gradients of retinoic acid and Wnt/FGF that move along the body as it extends posteriorly [59–64], slowing down oscillation speed. Thus, the wavefront is thought to stop the oscillations, transforming them into a stable stripe pattern (see, for example, [65]).

Recently, convincing evidence was reported for the presence of a segmentation clock in the beetle *Tribolium castaneum* [16, 24, 50]. However, while oscillations, wave propagation, and transformation to stable stripe patterns were observed, candidate signals for a wavefront are still missing. Thus, rather than a wavefront, an alternative mechanism may be responsible for transforming oscillations into stripes.

Indeed, alternative mechanisms for oscillation to stripe transformations have been proposed. Meinhardt showed that a Turing-Hopf bifurcation sequentially transforms oscillations into stripes provided that a gradient induces the first pair of stripes [56]. Similarly, the presence of an oscillator phase gradient can transform oscillations to stripes [66]. In both these cases the wavefront arises as an emergent property of the time-space transformation rather than causing it. Recent studies suggest that a phase gradient rather than wavefront may also be responsible for freezing oscillations in vertebrates [67].

In the Francois and Ten Tusscher studies the incorporated propagating wavefront (table 1) allowed for the evolution of a clock-and-wavefront segmentation mechanism in which cell-autonomous oscillations become transformed into stable stripes due to the passage of the wavefront (table 2, fig. 4A). In the other studies, the absence of a propagating wavefront (table 1) prevented this developmental mechanism from evolving. Theoretically, in the Fujimoto and Salazar-Ciudad studies the input or formation of a gradient combined with diffusive cell-cell signalling could allow both for a Turing-Hopf or a frequency-gradient type mechanism to sequentially transform oscillations into segments. However, neither authors reported such a segmentation mechanism. Instead, in the Fujimoto study the absence of selection for pattern stability allowed for the evolution of a developmental program in which oscillations starting at the high end of the morphogen gradient propagate across the tissue through cell-cell signalling but never become transformed into a stable stripe pattern (table 2, fig. 4B) in a subset of cases. This patterning mode has limited resemblance to real-life developmental mechanisms. In the Salazar-Ciudad studies no oscillation-driven segmentation mechanisms were reported to evolve.

A possible interpretation of the above is that whether or not realistic, oscillatory segmentation evolves depends on whether or not a propagating wavefront is present. In the absence of a wavefront it is hard to evolve other,

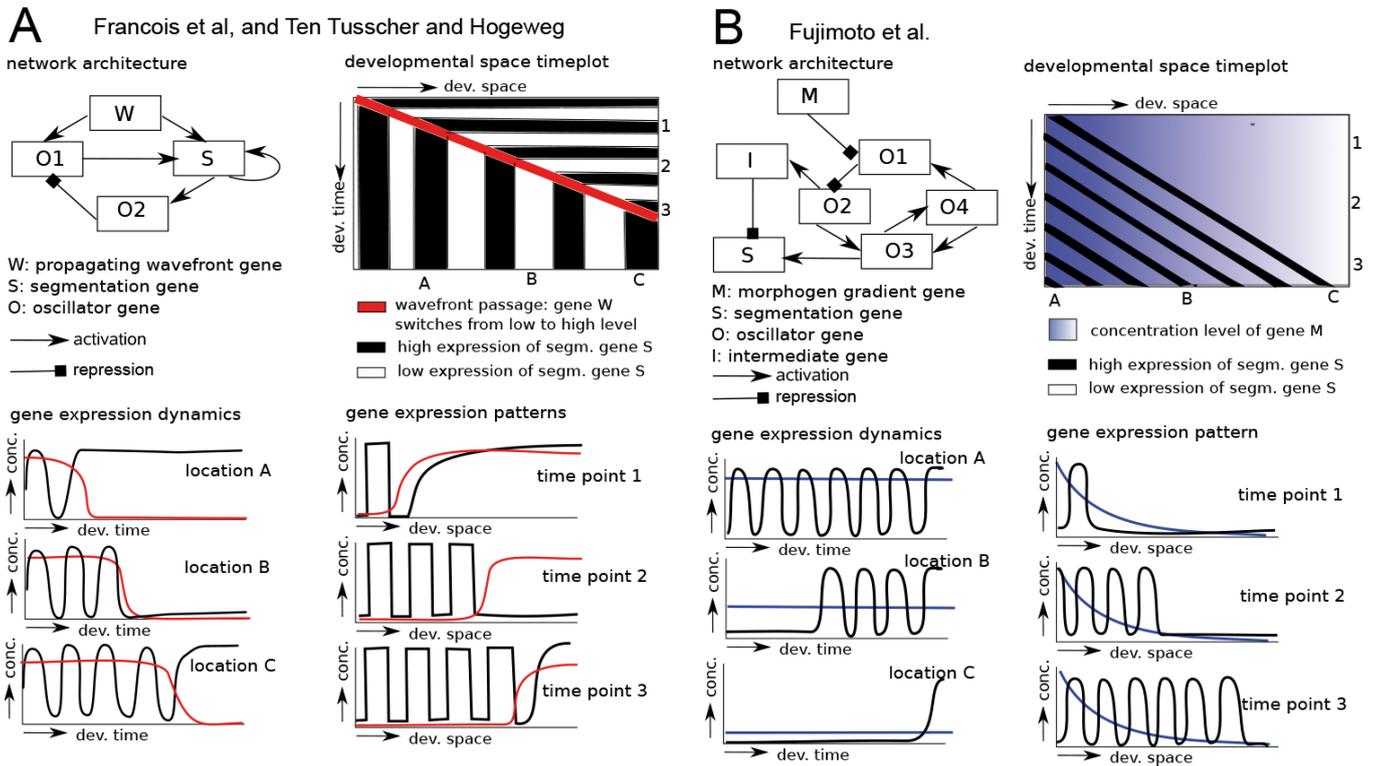


Fig. 4. Comparison of evolved oscillation-driven sequential segmentation mechanisms. Shown are GRN architecture, the resulting developmental space timeplot, gene expression dynamics at different positions along the body axis and gene expression patterns at different time points of development illustrating the spatiotemporal unfolding of development into a segmentation pattern. A) Clock-and-wavefront segmentation in the Francois *et al.*, and Ten Tusscher and Hogeweg studies. The GRN combines an oscillator motif (the negative feedback loop between genes O1, S and O2) with a bistability motif (the positive feedback of gene S onto itself), both dependent on the wavefront gene W. Upon passage of the wavefront the expression of gene W switches from a high to low level, causing oscillations to stop and the system to stabilise in either the low or the high S expression equilibrium state dictated by the bistability motif. B) Oscillation waves in the Fujimoto study. The GRN combines an oscillator motif (the negative feedback loop between genes O1, O2, O3, O4) with a bistability motif (the mutual positive feedback between genes O2 and O3) and a feed forward motif (FFL, genes O1, O2, I and S), all downstream of morphogen gradient gene M. Oscillations can only arise at the high end of the gradient, with diffusive cell-cell signalling causing propagation of oscillation waves across the tissue. The FFL links the oscillations to the segmentation gene. This figure is based on figs. 4, 5 and 6 from [31], figs. 3 and 6 from [33] (A), and figs. 1, 2, S2 and S9 from [32] (B).

gradient-dependent, oscillatory segmentation mechanisms and therefore alternative mechanisms evolve. When a wavefront is present, oscillations present the simplest segmentation mechanism and therefore evolve the easiest. However, it is important to bear in mind that evolutionary processes tend to generate complex, redundant rather than parsimonious mechanisms [68]. Often, such more complex mechanisms are more easily found by evolution (see *e.g.* [69]). Indeed, both the Fujimoto and Ten Tusscher studies show that besides the evolution of oscillatory segmentation mechanisms, alternative, more complex segmentation mechanisms also frequently evolve (table 2). It was shown that, rather than oscillatory segmentation simply being the easiest evolutionary findable mechanism, it is more robust [32,33], and evolvable [31,33] compared to alternative patterning mechanisms, and can be easily coordinated with regional differentiation [33]. This indicates that fitness aspects also play an important role in the evolution of oscillation driven segmentation.

When comparing used fitness criteria (table 1), we see that while the Francois and Ten Tusscher studies used a general fitness criterium maximising segment numbers, Fujimoto *et al.* selected for 10 segments and Salazar-Ciudad and coworkers even selected for a specific segmentation pattern. This difference in generality of used fitness criteria may also influence the likelihood for oscillatory segmentation mechanisms to evolve. Indeed, in the Francois and Ten Tusscher studies we see the gradual evolution from networks generating bistability, oscillations, to faster oscillations, thus gradually increasing segment numbers while shifting segment locations. Such an evolutionary trajectory would be significantly less likely under the more specific fitness requirements used in the Salazar-Ciudad and Fujimoto studies. Considering that sequential segmentation is an ancestral mode of segmentation, it is likely to have evolved gradually and hence been governed by a general fitness criterium.

Concluding, both posterior elongation producing a propagating wavefront as well as gradual evolution from an unsegmented ancestral state likely contributed to the chances for oscillation driven sequential segmentation to evolve. The relative importance of these two factors remains to be investigated.

3.3 Simultaneous segmentation

Like vertebrates, annelids and most other arthropods, short germ insects generate all their body segments in a sequential manner from a cellularized blastoderm. In contrast, intermediate germband insects generate their more anterior body segments simultaneously during the syncytial phase, later on generating the more posterior body segments sequentially from a cellular blastoderm. Finally, long germ insects such as *Drosophila* and *Anopheles* generate all their body segments simultaneously from a syncytial blastoderm. Based on its wider phylogenetic spread sequential short germband segmentation is considered the ancestral developmental mode and intermediate and long germband segmentation are considered evolutionary derived [6, 14, 7, 70, 71, 18]. The intermediate and long germband segmentation modes are thought to have arisen through the gradual recruitment of segments from the posterior, sequentially patterned region to the anterior, simultaneously patterned region.

Drosophila body axis segmentation is among the best studied processes in development. The *Drosophila* segmentation process is generally represented as a regulatory cascade, running from maternal morphogen gradients, to gap genes, pair rule genes and finally segment polarity genes, that is responsible for dividing the body axis into sequentially smaller regions until the individual segments are formed [25–28] (fig. 2B). First, the morphogen gradients of the so-called Hunchback, Bicoid and Caudal genes dictate the initial expression domains of the gap domains, which then get repositioned, refined and stably maintained through the mostly negative cross regulatory interactions between the gap genes [72, 73]. Next, the morphogen gradient and gap gene expression patterns together generate an initial expression pattern for the pair rule genes, thus generating the transition to a repetitive pattern. Segment specific enhancer elements controlling pair rule gene expression are crucial for allowing the repetitive expression of the same pair rule genes to arise from different morphogen levels and gap gene expression conditions [74]. Cross-regulatory interactions subsequently tune and maintain the pattern [75]. Finally, the pair rule genes initiate segment polarity gene expression, thereby doubling the number of repetitive segments (fig. 2B). At this time in development cellularization occurs, and cross regulation of segment polarity genes hence involves cell-cell signalling, but again serves to refine and stably maintain the patterns [76–79]. Thus, the major directions of information transfer in the segmentation network are down along the cascade and within a single hierarchical level.

In all of the model studies discussed here, the evolution of a developmental patterning mode containing one or

a few of the characteristics of *Drosophila*-type segmentation was reported. However, none of them fully resembled *Drosophila*-type patterning.

First, in the studies by Salazar-Ciudad, a single cell with distinct initial gene expression allows for spatial patterning. In their studies, two distinct developmental modes and network types evolved, termed emergent and hierarchic (table 2). The hierarchic networks generate a splitting up of expression domains into sequentially smaller and more numerous ones. These networks have a hierarchical architecture and cause unidirectional information flow (fig. 5A), thus resembling *Drosophila* segmentation. However, all segments are generated in a similar rather than segment specific manner. Also, no cross-regulatory interactions between genes occupying the same hierarchical network level are observed.

Both the later study by Francois *et al.* and that by Fujimoto and colleagues [31, 32] considered the evolution of simultaneous segmentation in the presence of a morphogen gradient. Still, these studies produced rather different results. In the Francois *et al.* study developmental programs evolved that produce a limited number of segments, generated by a few mutually repressive genes directly downstream of the morphogen gradient gene (fig. 5, middle panel). The network thus resembles the gap-gene layer of the *Drosophila* hierarchy (fig. 5B), but rather than feeding into intermediate control layers that amplify the number of segments formed, these directly controlled segment formation. In contrast, in the Fujimoto *et al.* experiments, a full blown hierarchy of gap-like, pair rule-like and segment polarity-like genes evolved in a subset of cases (11 out of 70, personal communications), with pair rule-like genes translating the gradient and gap patterns into a repetitive pattern and segment polarity genes causing a period doubling of the repetitive pattern (fig. 5C). However, here the gap-like genes displayed more combinatorial, overlapping expression and neither gap-like, pair rule-like nor segment polarity-like genes show the mutual repression observed in *Drosophila*.

Finally, the study by Ten Tusscher and Hogeweg used a wavefront as input pattern, hence only producing sequential segmentation. Still, frequently a developmental mode evolved in which segments were generated in a segment specific manner, downstream of spatial differentiation genes, rather than through oscillations (table 2).

Hierarchy. What can these similarities and differences in model setup and outcomes tell us about the shaping forces behind *Drosophila*-type segmentation? First, a complex regulatory hierarchy was only observed in the Salazar-Ciudad and Fujimoto studies, but not in the Francois study. A principle difference between these studies is in the generality of the used fitness criterium. While the Salazar-Ciudad study selects for a certain number of regularly spaced segments and the Fujimoto study selects for 10 segments, the Francois study uses a general fitness criterium maximising segment numbers. Interestingly, this suggests that fitness criteria that are more specific and involve significant segment numbers not only affect the ease

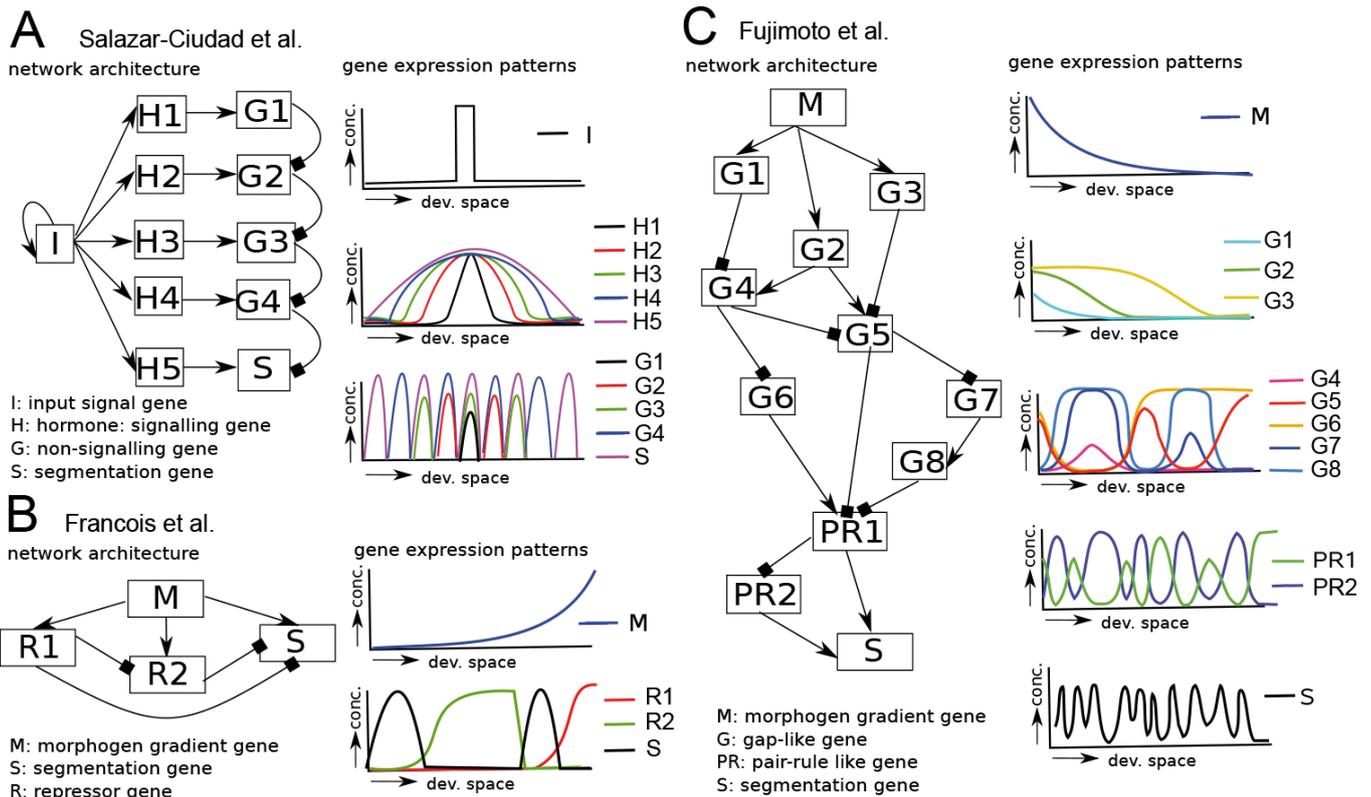


Fig. 5. Comparison of evolved simultaneous segmentation mechanisms. Shown are GRN architecture and spatial expression patterns of both the input gene (I in A, M in B and C) and final spatial expression patterns of the downstream genes. Note that it is non-trivial to derive the gene expression patterns from GRN architecture alone without knowing parameter values and, for the Fujimoto study, how regulatory inputs are organised into distinct enhancers (table 1). A) Hierarchy of repressors (R) controlled by signalling genes (H) in the Salazar-Ciudad study B) Gap-like segmentation system displaying mutual repression and non-overlapping expression domains in the Francois *et al.* study. C) Hierarchy of gap-like genes (G) pair-rule-like genes (PR) and a single segment-polarity-like segmentation gene (S) in the Fujimoto *et al.* study. This figure is based on fig. 2 from [29] (A) and fig. 3 from [31] and figs. 1, 2, S2 and S7 from [32].

with which developmental patterning programs evolve but also influence the type of developmental program evolving. A more specific fitness criterium involving a significant number of segments follows logically from the evolutionary history of *Drosophila* segmentation. As it is an evolutionary derived mode, it had to maintain the number and locations of segments previously formed by an ancestral sequential segmentation mechanism. Thus, the fact that *Drosophila* segmentation replaced an earlier segmentation mechanism rather than evolving from an unsegmented ancestral state has likely contributed to its complex hierarchic architecture. In addition, the Salazar-Ciudad studies showed that an increased mutational robustness and fine tuning capacity of hierarchic networks may have contributed to the likelihood of their evolution.

Note that findings in *Tribolium*, *Schistocerca*, *Gryllus*, *Tetranychus*, *Strigamia* and *Apis* indicate that the period doubling system of pair rule and segment polarity genes preceded the evolution of long germband segmentation [16, 24, 50, 80, 17, 81, 82, 20]. Thus, the lowermost tiers of the *Drosophila* hierarchy are not the result of the evolutionary transition to simultaneous patterning, but are

most likely caused by an earlier phase of selection for faster development and/or larger segment numbers.

Segment-specific regulation. Segment-specific regulation evolved in the simultaneous patterning modes of the Francois and Fujimoto studies but not in the Salazar-Ciudad study (table 2). This suggests the importance of a tissue-wide morphogen gradient for the evolution of segment specific control. In addition, segment-specific sequential segmentation was observed in the study by Ten Tusscher and Hogeweg that considered the combined evolution of segmentation and regional differentiation (table 2). This suggests a relation between the coordination of segmentation with spatial differentiation and the evolution of segment specific regulation.

Indeed, during the transition from sequential to simultaneous patterning, the control of segment and Hox gene patterning would have had to change in a coordinated manner. In vertebrates Hox genes experience an initial cluster level colinear activation [83–93], while their later more individual regulation is linked to the somitogenesis oscillator to ensure fine tuning between segment and Hox domain boundaries [94, 95, 92, 52, 53]. In contrast,

the simultaneous patterning in *Drosophila* involves region specific regulation of Hox genes by maternal, gap, pair rule and segment polarity genes [96,97]. The need for region specific Hox gene control most likely increased the chances for segment specific patterning to evolve.

Mutual repression and non-overlapping gap domains. Mutually repressive gap-like domains only evolved in the Francois *et al.* study. A likely reason for the evolution of mutual repression between gap-like genes in the Francois but not Fujimoto study lies in the fact that the Francois study starts with only a few genes while the Fujimoto study uses a constant number of 32 genes. This results in a positive bias towards mutual gene regulation in the Francois study. Furthermore, in the Fujimoto study selection is for 10 segments, while in the Francois study selection is simply for an increasing segment number, thus also allowing for lower segment numbers. This may introduce a bias in the Fujimoto study for a combinatorial rather than gap-like initial subdivision of the body axis as this more easily produces larger numbers of segments with a given number of genes. Together, this points to the potential importance of two more historical factors in influencing the evolution of *Drosophila*-type segmentation. First, the fact that *Drosophila* segmentation evolved gradually from a sequential segmentation mode, evolving simultaneous segmentation of only one or a few segments at a time may have made the evolution of gap-like rather than combinatorial domains more likely. Second, possible pre-existing regulatory interactions between gap genes may have increased the likelihood of mutual interactions.

The absence of gap-like domains in the Salazar-Ciudad studies can be explained by the absence of a global morphogen gradient and segment specific patterning in these studies.

4 Discussion

Segmentation of the major body axis into repeating units is thought to be a major evolutionary innovation that contributed significantly to the evolutionary success and diversity of the vertebrates, annelids and arthropods. Most of these animals use a sequential segmentation mechanism, in which oscillations combined with growth produce a regular anterior posterior sequence of segments [22, 23, 24, 24]. Only a small subset of long germ insects such as *Drosophila* and *Anopheles* [13] produce their segments simultaneously, through a complex regulatory cascade partitioning the body axis into successively smaller regions [25–28]. Given that overt body segmentation is observed only in the vertebrate, annelid and arthropod clades, yet recent experimental evidence points to remarkable similarities in the deployed sequential segmentation mechanisms, an important question is whether sequential segmentation evolved once or multiple times [6–10, 1]. Another important issue is how and why the complex hierarchic structure of *Drosophila*-type segmentation evolved [28]. In order to answer these questions insight into the specific mechanisms and constraints that have

been involved in shaping the evolution of these segmentation modes is needed.

In recent years experimental research has substantially increased our knowledge of the details of segmentation mechanisms deployed in various vertebrates, annelids and arthropods. Still, mechanistic questions as to why the particular naturally occurring segmentation mechanisms evolved are hard to answer based on experimental research. In this article we first explained how *in silico* evo-devo simulation studies can be used to answer these kinds of questions. Next, we performed an in depth analysis of *in silico* evo-devo studies on body plan segmentation. We exploit both the similarities and differences in model approaches and simulation outcomes to elucidate the mechanisms and constraints involved in shaping the evolution of segmentation mechanisms.

This comparative analysis indicated that sequential clock-and-wavefront type segmentation is a likely evolutionary outcome when considering that it evolved gradually from an ancestral, unsegmented state and assuming posterior body elongation producing a propagating wavefront. The easy and reproducible evolution of this patterning mode “from scratch” furthermore suggests that triple, parallel evolution of a sequential segmentation mechanism in the vertebrate, annelid and arthropod lineages may not be that unlikely.

Still, to better understand the evolution of sequential segmentation, additional questions need to be answered. First, it remains to be investigated whether oscillatory sequential segmentation is still a likely evolutionary outcome if posterior growth and wavefront formation have to evolve rather than being used as an input to the simulations. In addition, the evolutionary and developmental role of the propagating wave patterns observed in the posterior region of sequentially segmenting animals remains poorly understood [67].

Regarding *Drosophila*-type simultaneous segmentation, the comparative analysis showed how different, largely historical constraints are likely to have shaped distinct aspects of *Drosophila*-type segmentation. First, the fact that *Drosophila* segmentation replaced an ancestral segmentation mechanism and hence needed to reproduce a specific pre-existing pattern has likely contributed to the evolution of a complex, hierarchical regulatory architecture and the resulting subdivision of the body axis into progressively smaller domains. Furthermore, both a morphogen gradient as the signal for spatial patterning and the need to coordinately pattern segments and differentiation domains appear to favour the evolution of segment specific regulation. Finally, the gradual, segment by segment, replacement of sequential by simultaneous patterning and possibly the pre-existing interactions between gap genes contributed to the non-overlapping gap domains typical of the second tier of the *Drosophila* regulatory hierarchy.

In addition to the factors suggested from this comparative study, we suggest an additional historical factor that may have shaped the regulatory architecture of *Drosophila* segmentation. During the evolutionary transition from sequential to simultaneous patterning both systems had to

be functional at all times. *I.e.* while pair rule and segment polarity genes functioned in a clock-and-wavefront type mechanism in the posterior still sequentially patterned segments, they also had to function in a region specific, maternal and gap-gene-dependent manner in the newly formed anterior, simultaneously patterned segments. This need for a dual deployment of the genes in two different patterning modes may have been an important driving force in the early duplication and divergence of distinct pair rule gene enhancers. Future simulation studies incorporating an increasing number of these historical factors are needed to determine whether these are indeed sufficient to recapitulate the evolution of a *Drosophila*-type simultaneous segmentation mechanism.

In addition, simulation models should be made more realistic —incorporating for example gene expression and signalling noise, the observation that most transcription factors have only activating or repressive effects, and more complex regulation incorporating alternative enhancers— to investigate the role of these factors in shaping regulatory network architecture and resulting developmental patterning. It is for example highly likely that noise increases the necessity of mutually repressive interactions to form robust domains and hence enhances the likelihood of their evolution, thus possibly also explaining characteristics of *Drosophila* segmentation.

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