Looking at the origin of phenotypic variation from pattern formation gene networks

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This article critically reviews some widespread views about the overall functioning of development. Special attention is devoted to views in developmental genetics about the superstructure of developmental gene networks. According to these views gene networks are hierarchic and multilayered. The highest layers partition the embryo in large coarse areas and control downstream genes that subsequently subdivide the embryo into smaller and smaller areas. These views are criticized on the bases of developmental and evolutionary arguments. First, these views, although detailed at the level of gene identities, do not incorporate morphogenetic mechanisms nor do they try to explain how morphology changes during development. Often, they assume that morphogenetic mechanisms are subordinate to cell signaling events. This is in contradiction to the evidence reviewed herein. Experimental evidence on pattern formation also contradicts the view that developmental gene networks are hierarchically multilayered and that their functioning is decodable from promoter analysis. Simple evolutionary arguments suggest that, indeed, developmental gene networks tend to be non-hierarchic. Re-use leads to extensive modularity in gene networks while developmental drift blurs this modularity. Evolutionary opportunism makes developmental gene networks very dependent on epigenetic factors.

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1. Introduction

Development and evolution involve change: development involves changes in the phenotype over an individual's life cycle and evolution involves changes between generations. Adult phenotypes in one generation do not lead directly to phenotypes in the next generation: changes in between two generations necessarily imply changes in development.

The study of the relationship between development and evolution poses several conceptual problems. There is a widespread view in developmental biology that that since the study of the mechanisms of development in model species is already difficult, the study of how these came to be during evolution should be even more difficult. Then development would need to be understood in detail before its evolution can be studied and that, more simply, it is not necessary to understand the evolution of development to understand how development works (Wolpert 1975).

Development as a process is also a product of evolution. In that sense, how development works depends on many different selective and historical constraints. Even if development could, in principle, be understood without evolutionary considerations, a large number of researchers, and to some extent the field of evo-devo as such, nonetheless use evolutionary approaches to understand many aspects of development. There is a substantial variety among these approaches. Much research in evo-devo has shown that homologous genes and gene interactions are often involved in the production of similar or dissimilar organs in distantly related species (Manak and Scott 1994; Gilbert *et al.* 1996; Raff 1996; Tautz 2000). This finding suggests that much can be learned about genes involved in one species by looking at what is known in other species even when these are

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phylogenetically distant. This has enhanced comparative studies in developmental genetics.

Although these studies probably form the bulk of the research in evo-devo, most of them restrict evolutionary discussions to some comparative statements in the articles' discussion sections. The proposal of general principles, or inferences, about the relationship between development and evolution are rare in this kind of research. Indeed, some argue that the dependence on past selective pressures and historical accidents make the existence of such principles unlikely (Akam 1989; Sander and Schmidt-Ott 2004).

Other researchers argue that some general principles or insights about the influence of development on evolution and about the evolution of development are possible. Some argue, for example, that development would change, over time and by mutation, to produce variation in which functionally linked traits do co-vary (Wagner and Altenberg 1996; Cheverud 2007). This implies that, in principle, mutation can change G matrices in any direction (this is a common implicit assumption in populational and quantitative genetics (Salazar-Ciudad 2006a, 2008)) and that ultimately natural selection can by itself explain how development evolves. Some other authors argue that some aspects of development can be explained on the basis of selective conflicts between cell lineages (Buss 1998).

Research in a non-selectionist paradigm is also common. In fact, the dynamics of development, by determining which phenotypic variations arises from specific genetic and environmental variations, has been proposed as a factor that, together with natural selection, determines the direction of evolutionary change (Alberch 1982; Wake *et al.* 1983; Goodwin 1994; Arthur 2004; Müller 2007; Salazar-Ciudad 2006a, 2008). These kinds of arguments were at the origins of the early evo-devo research before the rise of comparative developmental genetics (Ho and Saunders 1979; Alberch 1982; Wake *et al.* 1983; Goodwin 1994). Along those lines, it has been proposed that general principles about the relation between developmental dynamics and evolution can be extracted from the physical properties of cells, extracellular matrix and cell aggregates (Newman and Comper 1990; Newman and Müller 2000) or from the logic of some pattern formation mechanisms (Oster and Alberch 1982; Goodwin 1994). In a similar way it has been proposed that there are a limited number of ways by which genes can be wired in gene networks and by which they can regulate cell behaviours' (like proliferation, apoptosis, adhesion, etc…) to give rise to pattern formation and morphogenesis in development. Thus, a great deal about the evolution and functioning of development can be understood by understanding these basic mechanisms and how they combine in evolution and development (Salazar-Ciudad 2008).

There is also a group of researchers that consider that understanding development is not crucial to understand evolution (Haldane 1932; Charlesworth and Lande 1982). Development is still considered as a factor to take into account in special cases (the frequency of these being an open question) but of relatively little importance compared with selection or populational genetic structure. Indeed, most evolutionary biology during last century proceeded without considering development. In fact, selection acts on variation irrespectively of how that variation is generated (Mayr 1982). The mechanisms by which genetic information leads to phenotypes, i.e., developmental mechanisms, determine, however, which phenotypic variation is produced by genetic and environmental variation. In other words, development, by producing specific kinds of phenotypic variation in each generation, determines in which ways the phenotype can change in each generation. Natural selection, then, chooses, in each generation, among these disposed directions of change.

Currently, there are only few evolutionary biologists who do not consider that development is an important factor in phenotypic evolution. Differences arise on how development works and exerts its influence in evolution, and on how explicitly this influence needs to be considered. In general, geneticists (populational and quantitative) are more concerned with the kinetics of replacement between variants and thus take patterns of phenotypic variation as given. Evolutionary developmental biologists, instead, are more concerned about how intrinsic patterns of phenotypic variation arise in development and on how these, and thus development, change over evolutionary time.

This article presents a categorization of multiple possible ways by which development could work to produce phenotypic variation from environmental and genetic variation. Some of these represent widespread views among the researchers above mentioned. This comparative discussion is then considered in the light of some simple evolutionary arguments to determine if any of these multiple possible ways can be discarded as implausible. Special attention is directed to the overall superstructure of gene networks in development because general inferences about development are focused mostly on that issue. However, in this article these structural issues are discussed in connection to more mechanistic and predictive approaches in pattern formation.

2. Cutting development into pieces

At a coarse level the questions of development are: how to produce different types of cells and extracellular components and how to put them in the right places. This article considers only the second question. The question of evolution would then be how these spatial distributions

of cells and extracellular components types (from now on called developmental patterns or simply patterns) can change over evolutionary time. Correspondingly, the question of the evolution of development would be how (and why) the logic (or mechanisms) by which these patterns are formed during development changes over evolutionary time.

The generation of an organism during development can be described as a sequence of pattern transformations from the initial zygote to the reproductively mature adult. For practical reasons development can be cut into arbitrary spatio-temporal segments. Pattern transformation can then be studied from an arbitrary initial or input pattern (taken as the spatial distribution of cell types and extracellular matrix in a part of the embryo) to a later resulting or output pattern (taken as the spatial distribution of cell types and extracellular matrix arising from the cells and extracellular components of the initial pattern).

In essence development can be seen as a process in which genetic information stored at the DNA level, and epigenetic information (in the phenotype of the embryo and ultimately in the egg cell) is used to transform one spatial distribution of cell types into another, often more complex, spatial distribution. Thus, development at this level is a process of generation of spatial information from genetic information, epigenetic spatial information already present in the embryo (or initially in the egg cell) and the interaction between these two (and the environment).

Epigenetics are all the aspects of the embryo's phenotype other than its genotype. It often has a causal role in development. Thus, for example, the spatial distribution of a diffusible extracellular signal depends on the spatial distribution of the cells secreting it, its diffusion constant and multiple other geometric factors such as asymmetries in the distribution of other extracellular components that may physically interfere with the signal. All these spatial distributions may depend on previous interactions between genes but the spatial location of those also depends on previous epigenetic factors.

The causal interdependencies can, iteratively, be traced back to the zygote. In fact, inheritance includes both genetic and epigenetic factors (for example the spatial distribution and nature of the molecules in the egg cell and, in some species, the spermatozoan entry point) and both are absolutely required for later development (Jablonka and Lamb 2005). In addition both depend on previous generations' genomes and epigenetic factors. Thus, such cycles of interactions between genetics and epigenetics are not reducible to either genetic or epigenetic factors. Epigenetic factors need to be considered both for a global understanding of the whole developmental sequence and for studies attempting to understand developmental changes between one stage and another. The genotype, however, can have heritable changes while epigenetic factors rarely can.

2.1 *Cell behaviours*

It is currently well established that the task of arranging cells and extracellular components into specific spatial distributions is accomplished by the regulation of a limited number of cell behaviours. Thus, during development early patterns are transformed into later patterns because cells divide, die, change their adhesive properties (this can lead to cell shape changes and cell migration) towards others cells or towards the extracellular matrix, receive extracellular signals or secrete extracellular signals and extracellular matrix components (Larsen and McLaughlin 1987; Salazar-Ciudad *et al.* 2003).

Ultimately, any change in development is the result of the activation or regulation of some of these behaviours in a specific spatio-temporal sequence. Although these behaviours are defined at the cell level they influence and are influenced by supracellular properties. For example, many of these cell behaviours directly or indirectly produce mechanical stresses or strains on cells other than themselves. This can often lead to changes in the spatial location of those cells. The outcome of these forces depends on the mechanical properties of the cells generating these forces and the cells receiving them and on the relative orientation and spatial arrangements of both groups of cells, which effectively changes mechanical responses to incoming forces (Beloussov 1998; Keller 2000, 2006; Forgacs and Newman 2005).

At the same time, the potential outcome of changing cell behaviors such as adhesion, proliferation or extracellular matrix secretion in specific cells can be affected by forces arising in other cells, or by other epigenetic factors (such as the spatial distribution of extracellular matrix and adhesion points in near surroundings). The regulation or activation itself of specific behaviors can also depend on existing forces and other epigenetic forces. Thus, for example, mechanical stress per se has been shown to affect proliferation rates (Nelson *et al.* 2005), apoptosis (Hsieh and Nguyen 2005) and adhesivity (Zemel and Safran 2007).

2.2 *Intracellular computing networks*

Most of development involves transforming a non-spatially homogeneous pattern into another non-spatially homogeneous pattern. To start with, the egg cell is itself spatially heterogeneous in most animal species. Thus, the process of pattern formation is better described as pattern transformation. Spatial differences in the activity or regulation of different cell behaviors are often due to differences in the patterns of gene expression between cells. These differences can arise as a result of the reception of extracellular signals by a cell (from another cell or from the environment) or by some autonomous mechanisms or by mechano-transduction (Tidball 2005; Li and Xu 2007).

Through *autonomous* mechanisms (Salazar-Ciudad *et al.* 2003) a cell acquires a specific pattern of gene expression

(or state) without interacting mechanically or signalling with other cells. Autonomous mechanisms include the division of a spatially heterogeneous cell (so that different daughter cells get different parts of the cytoplasm and cytocortex) and the active polar transport of proteins or mRNAs between two dividing cells (so that these factors become differentially distributed between the two daughter cells).

Most often, however, cells change patterns of gene expression because they receive extracellular signals. These can lead cells to differentiate into specific cell types. Which signal a cell sends and receives depends on which extracellular signals and extracellular signal receptors it expresses. This depends on which transcriptional factors it expresses. But which transcriptional factors a cell is expressing depends on which extracellular signals it receives and on its previous history (Fisher 2002). This previous history is reflected in a cell's metabolic state, previously expressed transcriptional factors and other regulative proteins. These interact with active signal transduction pathways to determine which genes (and consequently which cellular behaviors) are going to be activated in response to incoming extracellular signals. In that sense these transcriptional factors and extracellular signal transduction pathways constitute an intracellular network that integrates and computes, incoming signals to provide a response.

2.3 *Developmental mechanisms*

As noted above, a gene network able to produce pattern transformation must involve changes in at least one cell behaviour. Thus, minimally, any pattern transformation requires at least one of these intracellular networks (that can include gene products but also other molecules involved in signal transduction), incoming extracellular signals and resulting changes in cell behaviours. Here I consider a developmental mechanism (as previously, Salazar-Ciudad *et al.* 2003) as a gene network able to regulate cells' behaviours in such a way that it can transform one pattern into another. The associated cell responses can include the secretion of other extracellular signals, changes in the secretion rates of signals already secreted, the expression of specific receptors of extracellular signals at specific rates or other changes in other cell behaviours such as mitosis, apoptosis, etc… A gene network is defined here as a list of genes and their possible mutual interactions. A gene network reflects genetically encoded interactions only (i.e. in the protein structure and DNA and RNA sequences of regulative sites). These may occur or not depending on which genes in the network are expressed at a specific developmental time point and specific cell type.

Although many genes can be included in a developmental mechanism, the more important ones for understanding how a cell computes its response are those that are affected by two different signal transduction pathways. Those genes or molecules represent nodes of integration between signals and are thus very important for how a developmental mechanism produces pattern transformations. Other molecules that are not part of these intersections can be seen as simple relays of signal transmission (from extracellular signals and integrative nodes to other integrative nodes or cell behaviours) that can quantitatively modulate the signal but do not dramatically affect the computational capacities of the network as such. Indeed, simulation studies have shown that, at least when considering a small number of genes, networks that have the same topology at the integrative nodes and the same input signals and affect the same cellular behaviours tend to produce very similar pattern transformations (Salazar-Ciudad *et al.* 2000).

2.4 *Variational properties*

Developmental mechanisms, as here defined, can be combined to produce other developmental mechanisms (ones providing input to others). In this way, developmental mechanisms can be nested up to encompass the whole of an organism development. Although all pattern transformations in development can be said to be done by developmental mechanisms a specific pattern transformation can be equally realisable by different developmental mechanisms. Indeed, it has been extensively shown that different developmental mechanisms underlie very similar morphologies in different species (Müller 2007; Shook and Keller 2008) or even in the same individual (Catala *et al.* 1995). On the other hand a developmental mechanism does not always produce the same pattern transformation. The range of pattern transformations that a developmental mechanism can produce depends on: the initial pattern over which this developmental mechanism acts, the environment, epigenetic factors originating from regions of the embryo outside the pattern under transformation, genetic variation affecting the intensity by which genes in the network interact (for example how strongly a gene product inhibits or activates another one). The range of pattern transformations possible due to a developmental mechanism constitutes its "variational properties." This is different from phenotypic plasticity that refers to the range of phenotypes that arise in a given genotype under different environmental conditions (so variational properties focuses in a developmental mechanisms and some genetic and environmental changes while phenotypic plasticity refers to a whole organism and environmental changes).

Developmental mechanisms can be categorized in many different ways but three types are especially relevant for this paper: *Autonomous mechanisms*: as already described. *Inductive mechanisms*: In these pattern transformations occur because cell signal to each other.

In morphogenetic mechanisms: In these pattern transformation occurs without cells signalling to each other. Note that a pattern can change simply because the involved cells change their spatial distribution without any change in the cell types present. Many morphogenetic mechanisms may simply include an input (from an extracellular signal or autonomously inherited factor) and no integrative nodes.

3. Constructing an organism

From the previous section it appears that the pattern transformations occurring during development are produced by networks of genetic interactions regulating cell behaviours in response to extracellular signals. This provides some insight into the basic genetic and cellular rules that are used in development. This does not explain, however, how organisms are initially produced nor which variation arises from genetic and environmental variation. The question is then how are these basic interactions organized and spatio-temporally coordinated to produce pattern transformations and variation. As we will see, this is equivalent to asking: what are the structures of the intracellular networks involved in developmental mechanisms and how is their functioning causally affected by the successive input patterns and also by other epigenetic factors in the rest of the embryo.

4.1 *Current metaphors concerning gene networks*

Although not framed in these terms there are some studies that try to extract some few general principles about the organisation of development from the abundant literature on developmental genetics. Early metaphors (Davidson 1971) suggested that development is controlled in an army-like manner in which a limited set of transcriptional factors control the expression of a downstream layer of transcriptional factors and those control lower layers. Each layer controls a successively downstream layer until some effector genes are regulated. These effector genes were supposed to be structural or metabolic genes that implement a cell-type function. This metaphor did not consider pattern transformation as such and at best it could be applied to cell differentiation. However, this metaphor has influenced many later more up-to-date metaphors. These often acknowledge that developmental gene networks need to include extracellular signals. These metaphors (Carroll *et al.* 2005, Davidson and Erwin 2006) retain a highly hierarchic schema in which some very upstream transcriptional factors (normally assignable to specific families like HOX or other homeobox genes) regulate other transcriptional factors and those others, and so on. The more upstream genes are expressed in wider areas of the embryo and successive more downstream genes are expressed in successively more spatially restricted areas of the embryo.

4.2 *Hierarchic metaphors*

Some of these metaphors describe in detail the identity of the genes at different levels of the hierarchy. For example, Sean Carroll's work (Carroll *et al.* 2005) identifies 4 main classes of regulatory genes in development and Davidson and Erwin (2006) identifies 3 similar classes. *Homeotic* genes such as HOX genes are expressed in broad areas of most of the embryo in many different phyla. In *Drosophila* and in other arthropods the wild-type expression of these genes is required for the proper developmental identity of the domains where they are expressed. By identity it is meant, in practice, that the disruption of one of these genes would lead one body part to develop into another body part (for example a leg into an antenna) and not simply the non development of this body part. This later phenomenon occurs in *field-specific selector genes*. These are often homeobox genes. These would act within specific developing areas to regulate the formation and/or the patterning of entire morphological structures. In practice, this claim is based in the inability to develop these morphological structures in the absence of these genes and, at least for the case of *eyeless*, for the development of a complete morphological structure (a *Drosophila* eye) where this gene is ectopically expressed (Halder *et al.* 1995). These genes have been suggested to regulate later development in the areas where they are expressed by activating several signalling molecules in such a way that several coordinate systems are established. These *compartment selector genes* give rise to the expression of a different functional group of selector genes in specific subareas of the field-specific selector genes domains. In their turn, these later genes may regulate extracellular signals that may further partition this compartment in smaller areas where compartment or *cell-type-specific selector genes* may become expressed. In summary, this schema proposes that each successive layers of transcriptional factors regulate extracellular signals to partition their own domains of expression into small areas that express a (or combination of) specific downstream transcriptional factor that in its turn regulates the patterning of the area where it is expressed.

Although appealing, these kinds of schema can be criticized on multiple developmental and evolutionary grounds:

First, developmental gene networks tend to be non hierarchic. Many genes are expressed in multiple developmental stages and in multiple locations in the embryo. Genes in each of the previous categories can be expressed both before and after the expression of genes in allegedly more downstream categories. For example, hunchback is expressed in most anterior nuclei during

Drosophila segmentation and then disappears to be later expressed in some anterior neuroblasts (Brody and Odenwald 2000). Most likely, thus, these later cells are daughters of the anterior cells of the early blastoderm and in them hunchback indirectly leads to its own activation later on in development. In other words, this re-use ensures that a graph or diagram plotting arrows between interacting genes, a gene network, would not contain many obvious layered structures but multiple loops. These loops can be unrolled in space and time into hierarchic or non-hierarchic local interactions (see below).

Obviously, genes expressed early in development are acting (upstream) of genes acting immediately later in development. But, this temporal dependence of later stages on earlier stages is by no means specific development and it is likely a characteristic of most, if not all, physical processes. Thus, this should not be confused with the possible hierarchy of developmental gene networks as such.

Even at small time scales it is often the case that genes expressed at the same time do reciprocally regulate each other (directly or indirectly through some intermediate molecules) forming a non-hierarchic network. This non-hierarchicity is especially apparent among extracellular signals. Thus, for example, BMP4 has been shown to promote, in developing mouse teeth, its own expression (Vainio *et al.* 1993; Bei and Maas 1998) and that of ectodin (Laurikkala *et al.* 2003). Ectodin is an extracellular sequesterer of BMP4 and thus effectively inhibits BMP4.

Most diffusible extracellular signals with a role in pattern formation belong to a few families such as FGFs, BMPs, HHs and Wnts. Although some of these families have many members and not all have the same biochemical functions (for example BMP3 inhibits signal transduction of other BMPs, Bahamonde and Lyons 2001) there is a substantial degree of redundancy within families. Very similar sets of extracellular signals have been found in the development of organs as diverse as teeth (Jernvall and Thesleff 2000), hairs (Botchkarev and Paus 2003), feathers (Lin *et al.* 2006) and jaws (Richman *et al.* 2006) among many others. Since these signals seem to be the same in different pattern transformations it is likely that what makes the difference is not the signals themselves but the input patterns, the spatial distribution of these signals (for example in that input pattern), the surrounding epigenetic context and the specific cell responses (in the form of cell behavior changes) to these signals, which is likely to depend on networks of transcriptional factors expressed by these cells and on their metabolic state.

This schema, as well as much research in developmental genetics, are strongly focused on the genetic identity of developmental genes but directs less attention to the mechanisms by which pattern transformations occur. The claim that development is hardwired (Davidson and Erwin 2006) in the binding sites for transcriptional factors in gene promoters is inadequate for understanding the logic of pattern transformation in development. Promoter regions can be important points for the integration of multiple incoming extracellular signal transduction pathways. As mentioned, these integration points can be important for the structure of a developmental mechanism. However, the integration of multiple incoming extracellular signal transduction pathways can happen at other molecular levels and, indeed, there is substantial experimental evidence for genetic regulation (and variation) at other levels (translational, RNA half life, RNAi, transport to the nucleus, posttranscriptional protein modification, protein degradation, etc…) (Alonso and Wilkins 2005).

More importantly the "hardwired" information needs to be linked to the regulation of some cell behaviours for it to be involved in any pattern transformation. Moreover promoter regions contain no spatial information in themselves (indeed they are the same in all cells). Their possible pattern transformation capacities can only be understood if the spatial distribution of their input transcriptional factors is known. This implies knowing about the gene network in which a gene is embedded and knowing the previous pattern on which it is acting. In practice this leads us back to the concept of developmental mechanisms. In fact, if the dependence of pattern transformation on previous patterns and on epigenetic information is taken into account, development can be better described as "softwired" or "reciprocally dynamic" than as "hardwired". More precisely, then, development would proceed by the interaction of the hardware (the genome) with the software (existing patterns in each stage in the embryo) and the user (the environment if the analogy is extended to natural selection) over the whole biological cycle. In fact, this computer analogy is likely misleading: in development such "software" can barely be described as a program since constant signalling can constantly change, cells behaviours'.

4.3 *Patterning paradigms*

Some work by the proponents of this hierarchic schema presents some relatively explicit models about how pattern transformation can occur. A pattern transformation can arise by one transcriptional factor inhibiting the expression of a target gene and another more widely expressed transcriptional factor activating the target gene. Then, to the extent that the inhibitory gene expression pattern partially overlaps that of the activating gene, the target gene will have a pattern of gene expression that is neither that of the activator nor that of the inhibitor but the subtraction of one into the other. There is experimental evidence for this kind of mechanism (Gompel *et al.* 2005) and simple variations of it can lead to addition, intersection (Abu-Shaar and Mann

1998; Prpic *et al.* 2003) and other simple combinations of previous patterns into resulting patterns (Salazar-Ciudad 2006b). This leads that in practice many "downstream" genes are actually expressed in- between the areas of expression of two upstream genes or in the areas where one or another upstream gene is expressed but not both. Both Carroll and Davidson propose that this subtraction/addition paradigm can be implemented in terms of promoter regions and should be used as a paradigm to understand animal development as a whole (Carroll *et al.* 2005; Davidson and Erwin 2006).

Although this kind of mechanism could be rather common in animal development these authors do not take account of a large body of literature that has experimentally and theoretically identified other kinds of developmental mechanisms. Thus, for example, reaction-diffusion and direct contact lateral inhibition mechanisms do not fit into this schema (Meinhardt 1982; Jung *et al.* 1998; Asai *et al.* 1999; Newman and Müller 2005). They may ultimately rely on gene interactions occurring at the level of promoter binding but an analysis of those would not directly indicate the existence of a reaction-diffusion mechanism. That would require, at least, the identification of two diffusible extracellular signals and their relative rates of diffusion. For other kinds of mechanisms other requirements exist. As it should be clear from previous discussion about developmental mechanisms and pattern transformations, these requirements include, mainly, extracellular signals, and the regulation of a precise subset of cell behaviours, and not just gene network topology at the promoter level.

In fact, the subtraction/addition mechanism is simply the conceptually simplest mechanism that can be conceived by combining gene interactions. This is just a small proportion of the developmental mechanisms that can be theoretically combined by wiring gene interactions and of the developmental mechanisms that have been experimentally suggested (Salazar-Ciudad *et al.* 2003). Thus, the claim that subtraction/addition constitutes a general paradigm to understand development does not arise from a wide understanding of the diversity of developmental mechanisms but from understanding of those mechanisms that seem more amenable to genetic analysis and the assumption that developmental dynamics are linear.

4.4 *Morphogenetic paradigms*

In addition, this perspective considers only inductive mechanisms. Pattern transformation also involves changes in cell positions and, thus, the action of morphogenetic mechanisms. In fact, all known animals make use of them during development. It has been proposed (Wolpert 1994) that inductive mechanisms act prior to morphogenetic mechanisms and determine genetic programs that are followed precisely by morphogenetic mechanisms acting in each cell. From this perspective, morphogenetic mechanisms may seem less fundamental to the understanding of development. In fact, pattern formation is often understood as the phenomenon by which cells in different locations differentiate into different types or acquire different properties (such as different gene expression patterns) without consideration of the actual spatial arrangement of cells themselves. However, even when considering only inductive mechanisms it is not possible to define spatial distributions of cell types (patterning) without considering the physical distributions of the cells themselves. In addition, cell positions can change while signalling is taking place and thus morphogenetic mechanisms can also affect the outcome of inductive mechanisms, as will be later discussed in detail.

There is a relevant difference between the pattern transformation question posed here and questions about patterning underlying much research in developmental biology (Carroll *et al.* 2005). The hierarchic schema posed by Carroll ultimately aims to explain where morphological entities form. But explaining where structures or organs develop does not explain how they form with the particular forms they exhibit. Arguably, organ morphology could be explained by extending down this argument and explaining where in an organ different organ parts form and where in those parts different parts of parts, tissues or cells form (and so on). The placement of cells in space is left behind or conflated with the question of positioning cell types within a field of cells (of unexplained morphology). In effect, the role of morphogenetic mechanism in producing organisms' morphology and affecting the spatio-temporal location of inductions is not considered. Often, morphogenetic mechanisms are considered as subordinate to inductive mechanisms. This bias may correlate with the relatively coarse-grained descriptions of initial patterns and morphology in much of current developmental biology. In contrast, morphological variation after experimental manipulations is often described in reference to ideal coordinate systems that overlooks the actual shape of tissues, organs and whole organisms (thus perpetuating the relative neglect of morphology and morphogenesis).

5. Evolutionary thinking to the rescue

The previous section describes some widespread and influential views in developmental biology and their inadequacy in addressing the question of pattern transformation. That discussion was based, mainly, on inconsistencies with known aspects of morphogenesis. This section puts forward several evolutionary arguments that also indicate the inadequacy of these views.

5.1 *Multilayered hierarchic gene networks*

The multilayered hierarchical view presented above is not unanimously held among developmental biologists. Some researchers have stressed (Akam 1998) that very upstream genes (like Hox genes) can regulate genes one layer down but also genes that are downstream of these downstream genes. Thus, some genes may regulate genes on several of the putative levels and thus produce hierarchic networks with a not clearly layered structure (figure 1). In fact, there is no special reason for which evolution should lead to regular hierarchic multilayered gene networks easily understood by human minds. A hypothetical example can help to clarify this point. Imagine an ancestral hierarchical and regularly layered gene network. Whether a gene can regulate another gene depends on its protein structure (this depends on its sequence and possible conformational changes due to posttranscriptional modifications, allosteric and chemical modifications and microenvironmental factors among others) and that of its target gene or its target gene DNA or RNA. For a transcriptional factor, for example, that depends on the steric structure of its binding sites to DNA, the existence of co-factors and repressors and the promoter sequence of putative target genes. Mutations in these binding sites or in the putative target genes promoters (or other regulatory regions in DNA or RNA) can change the range of target genes that a transcriptional factor can regulate.

These mutations can in principle affect any gene and thus connections can arise between any gene. Thus, the probability of two genes being connected by mutational events is unlikely to critically depend on the positions of these genes in any hierarchic layer per se. At a coarse level, for any hierarchic multilayered gene network there are many non-layered hierarchic networks that can lead to the same logic of activity from the upstream genes to the downstream genes (figure 2). In fact, the hierarchic layered gene network is just one of multiple possible networks. Thus, by simple probability (in a gene network analogous of the second

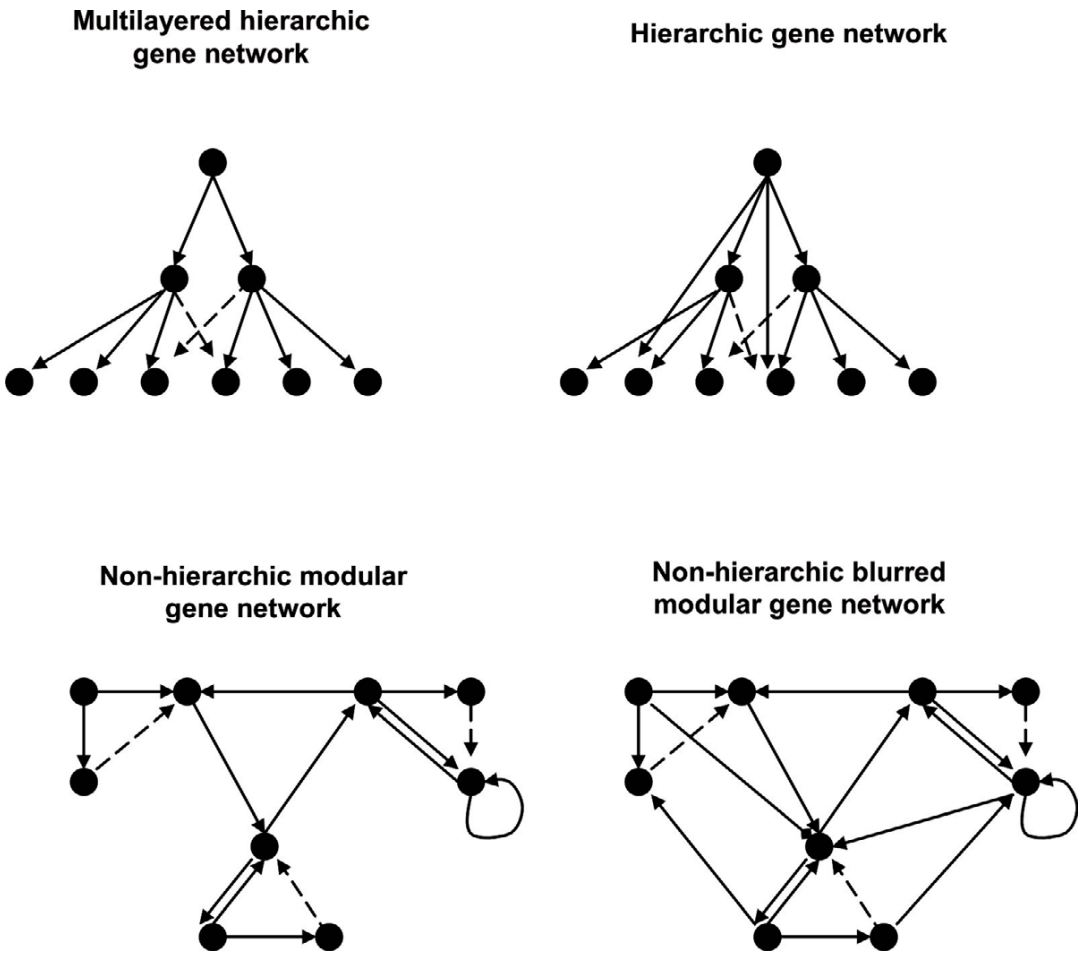

Figure 1. The schema shows four ideal examples of each of the four types of gene network described in the text. Black circles represent genes, full arrows represent activatory interactions between genes and dashed arrows represent inhibitory interactions between genes.

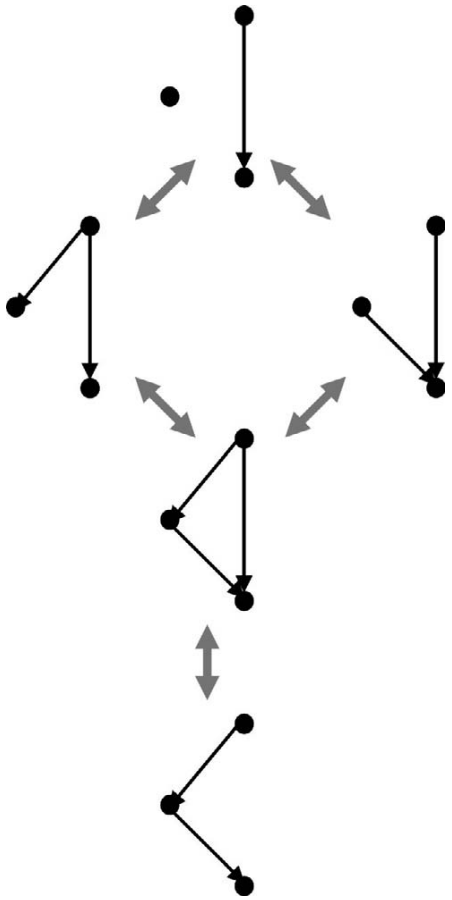

Figure 2. Black circles represent genes, black arrows represent activatory interactions between genes and grey arrows connect gene network that can be transformed into each other by adding or deleting a single gene interaction. Notice that while in the upper gene network the upper gene activates the lower gene directly in the most lower gene network this same activation is produced through an intermediate gene.

principle of thermodynamics) random mutations under conservative selection on produced patterns would lead to non layered gene networks.

5.2 *Hierarchic networks versus non-hierarchic networks*

The same arguments suggest that hierarchic networks, if they ever existed, should not be conserved in evolution. In the first place, only a tiny proportion of randomly wired networks are hierarchic (and this proportion decreases with the number of genes involved). Secondly, as described above, hierarchic networks would disappear under random mutation with selection for the conservation of particular patterns. Moreover, in general, and for the same reasons, it has been suggested that the genetic (and even epigenetic) networks underlying a given morphology can evolve without changes in the produced patterns by "autonomization" or "developmental drift" (Müller and Newman 1999; True and Haag 2001).

There are additional reasons why evolution should not lead to hierarchic gene networks. There are many computations of incoming molecular signals in a cell that can not be performed by hierarchic gene networks or that require many genes if implemented in a hierarchic topology. For example a gene network that activates a target gene only if any of two signals is present, but not both, can easily be constructed by inhibitory connections between the signal transduction pathways of the two signals and self-activatory connections within each signal transduction pathway.

Simulation studies, in which randomly constructed gene networks (that regulate the secretion of diffusible and membrane-attached extracellular signals) were assessed for their capacity to produce pattern transformations in sheets of cells, shows that the networks capable of pattern formation are most often non-hierarchic. Compared with hierarchic networks with the same number of genes, many types of non-hierarchic networks were able to produce more complex and more disparate pattern transformations when small mutations (that did not affect gene network topology) were introduced. These properties ensure that when selection for heterogeneous patterns in *in silico* populations of gene networks with mutation is performed, non-hierarchic networks are the ones found to produce the optimal patterns (Salazar-Ciudad *et al.* 2001a). In other words, non-hierarchic networks are what evolution encounters preferentially because they are the mutationally more likely way to produce more new patterns transformations and, thus, the more likely way to find adaptive variation (in practice that would also depend, however, on which selective pressures are found).

5.3 *Re-use of developmental mechanisms and non-hierarchic networks:*

Non-hierarchic network architecture can also be promoted by "tinkering by re-use." New pattern transformations in development can arise because of small mutations in existing developmental mechanisms, because of mutations that recruit an already existing developmental mechanism in a new context or by the formation of new developmental mechanisms. The second option involves new genetic connections and then it is less likely to occur by random mutations than the first option (which involves only small changes in the interaction between genes in a developmental mechanism). The second option is also more likely to produce more dramatic phenotypic changes since it involves the recruitment of a whole developmental mechanism. The third option is even less likely to occur by random mutation because it may require several mutations in genetic

connections. It can also lead to relatively major phenotypic changes. Thus, the second and the third option may only be selectable in parts of the phenotype that are under relaxed selection, or in cases in which their (comparatively) large phenotypic changes can find an adaptive niche.

Another possibility is that whole developmental mechanism recruitments occur only if they can produce a pattern transformation in a part of the embryo that is already generated by another developmental mechanism. In that situation, two parallel developmental mechanisms would produce the same pattern transformation in the same part of the embryo. Later, mutational or environmental changes in one of them, and the possible disuse (by mutational or environmental changes), of the other would unmask the variational properties of the new developmental mechanism and allow for a more gradual exploration of the variation it can produce.

Recruitment of developmental mechanisms can occur simply because, for example, a gene, A, in a developmental mechanism active in one part and time of the embryo suffers a mutation that causes it to activate the transcription of a gene involved in another developmental mechanism. If this target gene is able to activate the rest of the genes in its developmental mechanism, this developmental mechanism will be recruited (with the input pattern being the pattern of expression of gene A). This may explain the widespread re-use of the same signaling molecules described in previous sections.

Recruitment of developmental mechanisms in new contexts easily leads to overall non-hierarchic networks. This, for example, can occur if a developmental mechanism is active on one pattern, then another developmental mechanism acts on the resulting pattern and then the original developmental mechanism acts, in turn, on the resulting pattern of the resulting pattern. In that case, the overall gene network encompassing the two developmental mechanisms would be non-hierarchic (because genes in the first developmental mechanism lead to their own activation later in time through the activation of the genes in the second developmental mechanism). Since recruitment is more likely than *de novo* creation of a developmental mechanism, re-use of developmental mechanisms is likely to be widespread and leads to a non-hierarchic organization of development. In addition, previously used developmental mechanisms are more likely to produce pattern transformations that, if not necessarily more adaptive, are likely to be more reliable and less subject to developmental errors or dramatic sensitivity to environmental changes, mainly because developmental mechanisms with such likely maladaptive features should have been filtered out, in previous generations, by natural selection.

In the case of the emergence of new developmental mechanisms through mutation, re-use is also likely to arise at the level of single genes or gene network sections. New developmental mechanisms can arise by new connections between existing genes or by the arising of new genes. The first option is the most likely since it involves fewer mutational changes. Thus, in many cases, even the use of new developmental mechanisms would lead to the re-use of existing genes and consequently will lead to non-hierarchic structure in the overall gene network.

5.4 *Evolution of development and the use of epigenetic information*

All known metazoans have distinct morphologies and use morphogenetic mechanisms. They also have different cell types and utilize signaling between cells. The question is, then, how inductive and morphogenetic mechanisms interact and are ordered in time.

As mentioned, some influential metaphors (Wolpert 1994) characterize development as having an initial pattern formation phase in which cells signal to each other to determine their identity and a later morphogenetic phase in which each cell deploys a genetic program of cell movements according to its identity. Thus, the coordination of cell behaviors required for adult morphology is attained because each cell follows its own genetic program of cell behavioral changes. Here, developmental mechanisms composed of inductive mechanisms and later-acting morphogenetic mechanisms are called *morphostatic* mechanisms. *Morphodynamic* mechanisms are those in which inductive and developmental mechanisms act at the same time or in close alternation (Salazar-Ciudad and Jernvall 2002).

Developmental mechanisms are likely to be selected by their capacity to produce adaptive morphologies. As a proxy to that, developmental mechanisms that can produce many different morphologies from few genetic changes are expected to be the more frequent developmental bases of morphological changes in evolution. One of the proposals of this article is that a likely way by which new pattern transformations can be produced without requiring many mutational changes is by increasing the number of epigenetic factors that affect subsequent development. Although epigenetic factors at one stage depend on epigenetic (and genetic) factors at previous stages, they are not equivalent to them and, thus, if fed back into developmental dynamics, they can be considered as new epigenetic information to construct later development. In that sense, complex pattern variation can be produced for a minimum of new mutations by increasing the recursive feed-back between epigenetic information and gene networks at each stage.

Crucial for this argument is the fact that there are several different epigenetic processes that often result in different molecules or cells having different spatial distributions. For example, the spatial distribution of a diffusible extracellular signal is not identical to that of the cells secreting it but ideally,

will be a wider and smoothed version of it. In most cases, the spatial context of the developing embryo contains multiple morphological barriers that channel diffusion in specific directions (figure 3a). Therefore, the spatial distribution of an extracellular signal can differ substantially from that of its source. Moreover, small genetic changes affecting the diffusion constant of the signal or its rate of secretion from the source would produce different variation in the signal's spatial distribution (figure 3b). This spatial distribution can easily be used to produce a new pattern transformation by mutations that lead to the expression of this signal's receptor (which then can be expressed with a homogeneous spatial pattern, for example). Similar effects are likely to occur in signaling by transcytosis (Zhu and Scott 2004). In that case, channeling can result from morphological barriers to transcytosis (such as spatial distributions of cells that do not transcytose, or basal laminas or the like). Similar epigenetic effects have also been described for morphogenetic mechanisms (in the sense described above) (Beloussov 1998; Keller 2000, 2006; Forgacs and Newman 2005). In this case, it is the "diffusion" of cells that can be spatially channeled by the morphology and mechanical properties of involved tissues. It is important to highlight that these epigenetic effects are difficult to grasp from genetic analysis but are a likely source of evolutionary change.

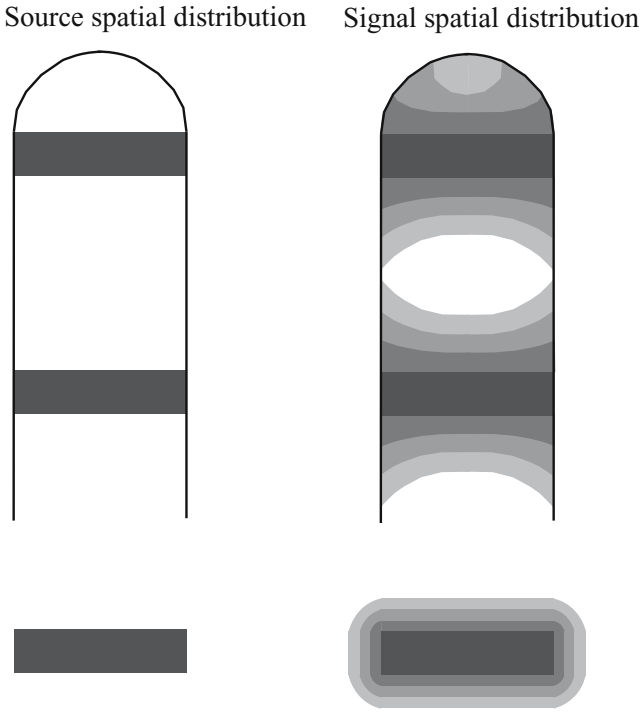

Figure 3. The figure represents two identical hypothetical pieces of mesenchymal tissue surrounded by a finger-shaped epithelium. In one only the distribution of the cells secreting a signal is indicated (in dark grey). In the other the spatial distribution of the signal is indicated as fading shadows of grey. The figure is indicative and does not correspond to any precise calculation. Note, however, that the shapes of these areas of signal distribution are very different due to the limiting effect that the epithelium has on signal distribution. Note also that it is assumed that each signalling centre is producing the same amounts of signal and thus the total area of the grey shadows has approximately the same.

A corollary of these ideas is that, in general, receptors for extracellular signals should have wider and less spatially refined patterns of gene expression than their ligands or that, at least, the spatial distributions of the ligands does not lead to the activation of all their receptors. It is, in fact, these differences that lead to pattern transformation: if the receptor expression domain is totally inside the expression domain of the ligand then no pattern transformation occurs (the spatial pattern of activation of the receptor is the same as its pattern of expression). Thus, for pattern transformation to take place it is required that not all extracellular signal receptors expressed bind to their ligand (or not at the same rate). This leads to the apparent paradox that some receptors are expressed but may never bind to their ligands. This energetic inefficiency leads to greater opportunity for morphological novelty by the processes described above. The reason for such inefficiency, of course, is not because it allows novelty but because the embryo does not typically have a developmental mechanism that avoids it. In some cases, receptors and ligands may have very similar domains of expression, but then they would not be involved in pattern transformation and could be considered a relic of the evolutionary history of development. Up till now there has been no systematic study of the relative areas of expression of receptors and ligands, although some are in preparation.

5.5 *Morphodynamic mechanisms and evolution*

The above arguments indicate that having inductive and morphogenetic mechanisms acting at around the same times allows, for the same amount of genetic variation, the production of a larger diversity of pattern transformations. This is because it allows the different epigenetic dependencies of morphogenetic and inductive mechanisms to be fed-back into development to produce more variation in pattern transformations. Moreover, while morphodynamic mechanisms (similarly to morphostatic mechanisms) permit pattern transformation by morphogenetic mechanisms deforming patterns produced by inductive mechanisms, but they also permit pattern transformation by inductions (extracellular diffusible signal release) arising from these patterns and from the patterns produced by morphogenetic mechanisms (Salazar-Ciudad 2006b). Thus, a substantially wider spectrum of pattern transformations is possible by the action of morphodynamic mechanisms from essentially the same genetic bases than by morphostatic mechanisms.

Simulation experiments with morphodynamic and morphostatic mechanisms support these arguments (Salazar-Ciudad and Jernvall 2004). These same studies suggest, however, than in some situations morphostatic mechanisms can be evolutionarily advantageous. Morphostatic mechanisms do not rely as much on epigenetic information and they can thus exhibit a simpler relationship between genotype and the phenotype, more gradual variation and more independent variation between parts compared to morphodynamic mechanisms. By "gradual" is meant that the different phenotypes embodied in the variational properties of a developmental mechanism are similar to each other, not that any arbitrary morphology is possible by gradual cumulative changes (as in the geometric model of Fisher (Fisher 1930)). In fact, morphostatic mechanisms produce less diverse pattern variation than morphodynamic ones. In other words, they have variation in fewer phenotypic directions.

The study of Salazar-Ciudad and Jernvall (2004) suggests that morphostatic mechanisms may often appear secondarily, once a pattern transformation has arisen by the action of a morphodynamic mechanism, as a more reliable mechanism to produce that pattern transformation. The contrast between morphodynamic and morphostatic mechanisms is similar to the one described between hierarchic and emergent inductive mechanisms (Salazar-Ciudad *et al.* 2001a).

Morphodynamic mechanisms also have the advantage that they more easily ensure the adequate positioning of functionally linked morphological structures. If signaling occurs throughout all development it is easier, for example, to ensure that the mouth forms at the tip of the gut: signaling from the tip of the gut, for example, can induce a specific part of the ectoderm (from a wider area expressing the right receptor) to develop into a mouth. By easier it is meant that environmental fluctuations would have few effects on the coupling between mouth and gut. In contrast, in morphostatic mechanisms environmental fluctuations or small genetic changes affecting the gut or mouth development would likely misalign them and lead to malfunction.

Strictly morphostatic development is also unlikely because the recruitment of existing developmental mechanisms in new contexts by mutation can, in principle, recruit any mechanism at any stage. Thus, some strong selective pressure would be required to restrict morphogenetic mechanisms to late development and inductive mechanisms to early development. It has been argued that early development is, comparatively (Arthur 1997), more conservative since changes in it are more likely to affect many more events in later development than changes in late development. However, since metazoa probably had a specific morphology from early on in their evolution, early development should also have conserved morphogenetic mechanisms (as in gastrulation). It is also possible that because they allow more gradual variation and a simpler relationship between genotype and phenotype, in some early stages of development, morphostatic mechanisms may have replaced morphodynamic mechanisms. To the extent that pattern transformations in early development are evolutionarily older, morphostatic mechanisms should be more common there. However, no comparative study on that has been performed so far).

5.6 *Genetic programs versus response tables*

From the perspective of developmental biology these results and arguments indicate that the most important aspect of a signal is not its chemical or genetic identity nor its interpretation, but its spatial distribution and how that changes over time. It is from this epigenetic spatial information that development is constructed. Signal interpretation is also important, but the metaphor of positional information notwithstanding, this interpretation cannot be complex enough to thoroughly guide all later development. Instead, this intepretation would necessarily be short-lived because cells are receiving different signals constantly and then modifying their behaviors on the fly (so the putative "genetic programs" are better described as scripts or simple response tables). Thus, the previously presented argument that even if morphogenetic mechanisms are important they are finely regulated by a distinct genetic program in each cell (Wolpert 1994) is not tenable except in the case of some secondary morphostatic mechanisms evolved to replace a morphodynamic one. In fact, statements about the genetic determination of morphogenetic mechanisms are often devoid of any kind of evolutionary consideration. Part of the emphasis on tight regulation of morphogenetic mechanisms may reflect the implicit assumption that regular ordered behavior can only occur through tight genetic regulation and cannot occur from the dynamics of physical interactions between collectives of cells themselves (contrary to arguments by morphogenetic developmental biologists (Beloussov 1998; Keller 2000, 2006; Forgacs and Newman 2005). Genetic determinist rhetoric may also reflect a mere stylistic preference in the writing of articles in developmental biology.

5.7 *Neo-Darwinism, developmental constraints and pattern transformations*

Even in hierarchic and morphostatic developmental mechanisms there is no possibility for simple development with a simple relationship between phenotype and genotype

and variation possible in any direction (as in the geometric model of Fisher). A morphological phenotype arises because of interactions between genes and epigenetic factors. By adding genetic interactions, morphological variation can arise in additional directions. Simple developmental processes produce simple morphologies with very few possible directions of change. More complex developmental processes involving more genetic and epigenetic interactions allow more directions of change depending on their structure (morphostatic/morphodynamic).

These ideas indicate that the ideal Fisherian argument by which adaptation can be studied by assuming that small variation is possible in all directions is untenable even as an ideal. The criticism of the Fisherian paradigm according to which development constrains morphological evolution because its highly interactive nature allows only some morphological variation and not other is also untenable because: (i) it is due to genetic interactions that variation is possible in more directions and (ii) development always operates; there is no such thing as a development-free evolution and thus any theory about morphological evolution should explicitly contain hypotheses or information about which phenotypic variations are possible from genetic variation in development. From that it can be explored how natural selection shapes morphological changes. Thus, the alleged null model of variation possible in any direction is a developmental impossibility. A more realistic null model might be a simple developmental process where variation is possible in very few morphological directions (Salazar-Ciudad 2006a)

6. Discussion

The present article has used developmental and evolutionary arguments to highlight that contrary to widespread views among developmental biologists, gene networks are neither hierarchic globally nor locally, morphogenetic mechanisms are an integral part of development, development and its evolution make extensive use of epigenetic factors and morphogenetic and inductive mechanisms frequently act in close interdependence. Thus the overall structure of developmental gene networks is not hierarchic, but employs frequent re-use of developmental mechanisms that leads to some apparent modularity. This modularity is blurred by the re-use of individual genes (a gene can be in different developmental mechanisms) and extensive developmental drift (as suggested figure 1). The developmental and evolutionary arguments are different, although interrelated, and provide an example of how evolutionary considerations help to discriminate between developmental hypotheses, and how developmental arguments are necessary to understand morphological evolution.

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