# ON THE ORIGINS AND EVOLUTION OF MORPHOLOGICAL COMPLEXITY:

# A DEVELOPMENTAL PERSPECTIVE

**Pascal Felix Hagolani**

Institute of Biothechnology
Helsinki Institute of Life Science (HiLife)

Department of Biosciences,
Faculty of Biological and Environmental Sciences

Doctoral Programme in Integrative and Life Sciences (ILS)

University of Helsinki

DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Sali 1041, Biokeskus 2, on the 10th of June 2021 at 12:00.

Helsinki 2021

**Supervisor**

Isaac Salazar Ciudad, University of Helsinki

**Thesis advisory Committee**

Maria Vartiainen, University of Helsinki
Mikael Fortelius, University of Helsinki
Jukka Jernvall, University of Helsinki

**Pre-examiners**

Roeland Merks, Instituut Biologie Leiden
Kaandorp Jaap, University of Amsterdam

**Opponent**

Nicolas Goudemand, Institut de génomique fonctionnelle de Lyon

**Custos**

Ville Mustonen, University of Helsinki

ISBN 978-951-51-7193-1 (PRINT)
ISBN 978-951-51-7194-8 (ONLINE)
ISSN 2342-3161 (PRINT)
ISSN 2342-317X (ONLINE)
http://ethesis.helsinki.fi

Unigrafia
Helsinki 2021

# ABSTRACT

Organismal complexity has astonished biologists for centuries. How complexity has evolved, has given rise to much debate. Many have claimed that natural selection is the main factor that made it possible to achieve high degrees of complexity. On the contrary, others argue that this is far from the truth, as complexity can increase passively, without the need of natural selection. Either way, the exact mechanisms by which complexity has increased in some groups of organisms remains largely unknown. For morphology, to understand which mechanisms have enabled an increase in complexity, requires to study development. As development is the process that establishes morphology, any evolutionary change in morphology is preceded by a change in its development. Additionally, to understand how morphological complexity evolves, it is necessary to comprehend the phenotypic variation that different developmental mechanisms can produce

These are the two main questions that I am interested to answer in this dissertation:

1. Are there some logical requirements that developmental mechanisms should fulfill in order to lead to complex morphologies?
2. How does morphological complexity affect evolution?

To tackle these questions, I used a general computational model of development, EmbryoMaker. This model can accommodate any gene network and model different types of tissues. Thus far, this model has been used to model teeth, spiralian development, and random organoids. EmbryoMaker includes many animal cell behaviors, such as cell division, cell polarization, cell contraction, and includes realistic biomechanical interaction between cells. Therefore, EmbryoMaker is a general model that allows to simulate the development of 3D morphologies of any type. It is precisely this generality of EmbryoMaker which was vital for this dissertation, since it allowed an unconstrained exploration of developmental mechanisms, without being limited to certain organisms or systems. This allowed me to tackle the questions I posed in a general way.

For the first question, are there some logical requirements that developmental mechanisms should fulfill in order to lead to complex morphologies? I built five different ensembles of random developmental mechanisms. That is, I built random gene networks regulating random cell behaviors (i.e. random developmental mechanisms) using a different set of rules for each ensemble. Each ensemble was built to study the effects of different developmental factors, such as: cell signaling, gene networks, gene expression patterns (gradients and homogeneous cell gene expression) and

cell polarization. In each of these ensembles I run thousands of simulations in order to find statistical differences between the groups and then, under careful study of the simulations find the causal factors responsible for these differences.

For the second question, how does complexity itself affect evolutionary dynamics? Instead of performing evolutionary simulations, which are computationally demanding and for the purpose of answering this question less effective, I performed two different types of parameter explorations. These allowed me to study the variational properties of different developmental mechanisms, that is, what kind of phenotypes result from mutating different developmental mechanisms. The first method consisted in exploring the 1-mutant neighborhood of each developmental mechanism studied this way. This means that each gene (or parameter in our model) was mutated once, while the rest of the genes remained unchanged. The second method consisted in an iso-morphological random walk. With this method I explored the size of the parameter space that kept the morphology the same, that is, the number of neutral mutations that can accumulate before the morphology changes.

The general results obtained are:

1. The development of complex morphologies does not require cell signaling or complex gene networks.
2. Extracellular signaling enhances robustness through the compartmentalization of the embryo into different regions of gene expression
3. Complex morphologies are rare
4. The more complex a morphology is, the more finely tuned its developmental parameters need to be
5. The more complex the morphology, the larger the mutational asymmetry towards simplicity
6. The more complex morphology, the more complex the GPM

These results indicate that there are qualitative differences in the way complex and simpler morphologies evolve. Complex morphologies evolve under a complex GPM and higher developmental instability. Additionally, complex morphologies produce a higher morphological diversity than simpler morphologies for the same amount of genetic variation, therefore offspring of complex individuals spread across large regions of the morphospace. Finally, these results also indicate that the evolution of morphological complexity becomes progressively slower as complexity increases, until possibly arriving at a complexity trap, where it cannot effectively increase.

Luonnosta löytyvien muotojen moninaisuus on ällistyttänyt tutkijoita vuosisatoja ja muotojen kompleksisuuden synnystä on väitelty paljon. Yhtäältä on väitetty luonnonvalinnan olevan ensisijainen eliöiden muodon kompleksisuutta ajava tekijä ja toisaalta on ehdotettu kompleksisuuden voivan kehittyä passiivisesti luonnonvalinnasta riippumatta. Muodon monimutkaistumisen taustalla vaikuttavat mekanismit ovat pitkälti tuntemattomia. Kompleksisuuden lisääntymisen ymmärtäminen edellyttää ymmärrystä yksilönkehityksestä; muodot syntyvät yksilönkehityksen aikana, joten evolutiivinen muutos muodossa edellyttää muutoksia yksilönkehityksessä. Ymmärtääksemme, miten kompleksisuus lisääntyy evoluution myötä, on ymmärrettävä millaista ilmiasujen muuntelua  yksilönkehitys voi tuottaa.

Kaksi väitöskirjassani käsiteltävää pääkysymystä ovat:

Vaaditaanko kehitysmekanismeilta tiettyjä ominaisuuksia, jotta ne voivat tuottaa kompleksisia muotoja?
Miten muotojen kompleksisuus vaikuttaa evoluutioon?

Käytän työssäni tietokonemallia, EmbryoMakeria, joka mahdollistaa minkä tahansa muodon kehityksen mallinnuksen kolmiuloitteisesti. EmbryoMakerin simulaatiot eivät rajoitu tiettyihin mallieliöihin tai järjestelmiin, mikä on olennaista tutkimukselleni.

Tutkimukseni päätulokset ovat:

1. Kompleksisten muotojen kehitys ei edellytä soluviestintää tai monimutkaisia geeniverkostoja.
2. Solujen välinen viestintä lisää kehitysmekanismin vakautta jakamalla alkion rajattuihin geenien ilmentymisalueisiin.
3. Kompleksiset muodot ovat harvinaisia.
4. Mitä kompleksisempi muoto on, sitä tarkemmin sen kehitystä on säädeltävä.
5. Mitä kompleksisempi muoto on, sitä todennäköisemmin mutaatiot johtavat muodon yksinkertaistumiseen.
6. Mitä kompleksisempi muoto on, sitä kompleksisempi on sen taustalla toimiva geeni-ilmiasu-kartta.

Tulokseni viittaavat laadullisiin eroihin kompleksisten ja yksinkertaisten muotojen evoluution välillä. Kompleksisten muotojen evoluution taustalla vaikuttava geeni-ilmiasukartta on monimutkaisempi kuin yksinkertaisilla muodoilla. Lisäksi kompleksisten muotojen kehitys on epävakaampaa kuin yksinkertaisten muotojen kehitys. Yksinkertaisiin muotoihin verrattuna kompleksiset muodot johtavat suurempaan monimuotoisuuteen vaikka geneettinen muuntelu taustalla olisi yhtä suurta; tämän vuoksi kompleksisten yksilöiden jälkeläiset levittäytyvät laajoille alueille muotoavaruudessa. Tulokseni osoittavat myös, että kompleksisuuden lisääntyessä kompleksisuuden evoluutio hidastuu, kunnes saavutetaan ’kompleksisuusansa' jossa kompleksisuus ei voi enää lisääntyä.

# ACKNOWLEDGEMENTS

The journey for obtaining my PhD has not been a lonely one. I have had the support of amazing people along the way, from the start to the very end. It is difficult to imagine a better ensemble of people to be with me on this long, but ultimately successful journey.

The first of this amazing group of people that I must acknowledge is my supervisor, Isaac Salazar Ciudad, whose innovative perspectives and knowledge has helped me to bring this PhD to its conclusion. He always found time to discuss and share ideas, giving me his honest opinion and expertise, which I really appreciate. He has helped me to develop into a mature and independent scientist.

My thesis committee could not have been any better, and they have played a large role in aiding me on this journey. Maria Vartiainen was always really helpful in bringing some of my crazy theoretical questions back to "reality" and in giving great advice for my career development. Mikael Fortelius has a deep understanding of evolutionary theory and always kept me on my toes and motivated me greatly. Jukka Jernvall, who showed me how to approach scientific problems in original ways and inspired me by how he finds the most interesting questions to research. All of these committee members took great care to ensure that my PhD was being properly done, from a scientific and a personal point of view.

I am truly honoured to have Nicolas Goudemand as my opponent. His expertise in evolution and development will ensure that I am fit to become a PhD.

The practice of science is at its best when embedded in an active community, and I had the best one I could have wished for. When I first arrived in Helsinki Irepan Salvador-Martin, Roland Zimm, and Mona Christensen immediately made me feel at home. They helped me in so many different capacities, from having a beer after work to deep scientific discussion, sometimes even at the same time. Special thanks to Mona for lending me her ipad when I most needed one. Miguel Brun-Usan and Miquel Marin-Riera were always the best when I needed help with EmbryoMaker or any kind of crazy idea or methodology I wanted to implement. Yoland Savriama and Johann Muszynski provided some amazing movie nights, and between buckets of coffee and bananas helped me to improve my methodology. Jhon Alves was a great influence with his positivity and sharp discussions. Lisandro Milocco, whom I will leave behind in an empty Helsinki lab, has been an exceptional coworker and friend. He was and is always ready to provide great advice in all

fronts, from recognizing flaws in my methods and useful discussions on how to correct them, to being the best cheerleader and peer-support when things were not easy. Renske Vroomans, always ready to help and share her insightful ideas. Hugo Cano Fernandez was an incredible asset to discuss complexity, a topic that not everyone enjoys as much as him. Carlos Mora Martinez always brought a fun cynicism to our discussion and I always enjoyed my discussion with him.

The Helsinki Evo-Devo community has been extremely welcoming and kind, providing a platform to openly discuss science and socialize. I will like to show my gratitude to the members of this community: Rishi Das Roy, Outi Hallikas, Aida Kaffash Hoshiar, Otto Stenberg, Vilma Väänänen, Ian Corfe, Ona Saarnisalo, Jacqueline Moustakas-Verho, Susanna Sova, and Mia Valtonen. Special thanks to Susanna Sova for her patience and support in many of my bureaucratic endeavours.

I grateful to all the other members of the Helsinki University who have shared this experience with me, making it a more fulfilling and fun experience, at the risk of forgetting some names I want to thank: Fabien, Ewelina, Julia, Maria, Vinod, Solja, Otto, Isabel, Ibrahim, Ana-Maria, Irma Theselef, Marja Mikkola, Laura Ahtiainen, Osamu Shimmi and Nicolas Dipoi.

Finally, I would like to thank my parents and siblings for their support and love. They have helped me beyond what I could have possibly asked of them, which has made me the person I am today. Last, but certainly not least, none of this would have been possible without my wife, who incidentally I met during the first week of my PhD. She has been a constant through this journey, always there for me, in the hard times, and in the most amazing moments of my life. She has also helped me with countless hours of editing and been an open ear and support while we worked on our respective doctorates. Thank you for everything, it has not always been easy, but you have helped me to find joy even in the hardest stretches. I also want to make a special mention to Elizabeth Albov and the late Michael Albov for having such a wonderful daughter.

# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Cell signaling stabilizes morphogenesis against noise

II On the evolution and development of morphological complexity: A view from gene regulatory networks

The publications are referred to in the text by their roman numerals.

**Author contributions**

In study I, PFH and ISC.did the conceptualization; PFH, RZ, MMR and ISC work on the methodology; PFH, RZ, MMR and ISC did the software; PFH and RZ did the validation; PFH the analysis; PFH, RZ and ISC the investigation; PFH and RZ provided the resources; PFH and RZ did the data curation; ISC wrote the original draft; PFH and ISC reviewed and edited the manuscript; PFH, RZ and ISC did the visualization.

In study II, PFH and ISC.did the conceptualization; PFH and ISC work on the methodology; PFH, RZ, RV and ISC did the software; PFH and RZ did the validation; PFH the analysis; PFH and ISC the investigation; PFH provided the resources; PFH and RZ did the data curation; PFH and ISC wrote the original draft; PFH, RZ, RV and ISC reviewed and edited the manuscript; PFH, and ISC did the visualization.

# 1 REVIEW OF THE LITERATURE

In this section I will introduce some ideas and concepts that I consider have had a great influence in the approach I have taken for this dissertation. Before getting into the gist of the dissertation, and to prepare the reader for what is to come, I will briefly summarize some of the major ideas I will cover.

Over the last century, the study of evolution has focused mainly on how natural selection is able to mold organisms to achieve high levels of adaptation and complexity. Most of these modern studies on evolution use the Neo-Darwinian framework, which is based on a gene centered view of evolution. Under this framework, how organisms evolve can be fully explained by natural selection and changes in gene frequencies [Fisher 1930, Dobzhansky 1937, Mayr 1963]. However, it has been argued that this is an over-simplification of evolutionary dynamics that fails to capture phenotypic variation, since phenotypic variation cannot be simply reduced to genotypic variation [Alberch 1980, Salazar-Ciudad 2005, Uller 2018]. Phenotypic variation, together with natural selection, is fundamental to understand evolution. Therefore, misunderstanding variation constitutes a major caveat in current evolutionary theory.

In the case of morphological evolution, what is needed to understand phenotypic variation is development, since any change in morphology is due to a change in development. Development can be viewed as a complex system which transforms an embryo into an adult morphology. Therefore, for morphologies to evolve, changes at the developmental level are necessary. This means that if we want to improve our understanding of how complex morphologies have evolved, we need to consider how development has evolved in order to make complex morphologies possible in the first place. This is precisely one the questions that I tackle in the first study: at the level of development, what is needed to build a complex and robust morphology?

Gaining knowledge about how development builds complex and robust morphologies allowed me to study some aspects of the evolution of morphological complexity. This is the approach I took in the second study. For this, I examined the morphological variation that can be obtained from different developmental mechanisms that can produce morphologies of different complexity. Since natural selection can only select from what is readily available, analyzing the morphological variation that different developmental mechanisms yield, allows me to make some general statements about how, in light of development, the evolution of morphological complexity should be. Regarding the evolution of complexity many hypotheses have been proposed. On one hand there are hypotheses which propose that complexity

itself is adaptive and is being selected for, which explains the continuous trend of increasing complexity. On the other hand, there are researchers that negate such a complexity trend. They argue that only a few groups of organisms show an increase in complexity, which can be explained without invoking natural selection.

In the following sections I will introduce some of the previously mentioned concepts, such as complex systems and development, complexity and complexity trends. Additionally, I will introduce the methodology I used in this dissertation, mainly computational modeling, in order to offer an overview of how they work. Before I introduce these concepts, I will start by establishing a historical background to clarify the approach I use in this dissertation, which underpins the importance of phenotypic variation in evolution and why development is fundamental to understand morphological variation.

## 1.1 HISTORICAL BACKGROUND

### 1.1.1 GOLDEN AGE OF EMBRYOLOGY

We are surrounded by fascinating complexity, from miniscule molecular complexes to colossal cosmological superclusters. However, for over 300 years, science's main approach to study complex systems has been to decompose them into their smallest parts, in order to be able to study simpler systems, which are easier to discern [Sarkar 1998]. This decomposition of complex systems works under the assumption that once the parts are studied, the whole can be understood by putting the pieces back together. This reductionist approach was popularized by René Descartes [Cottingham 1988], who described the world as a clockwork mechanism which can be understood by studying its individual pieces. This view was further strengthened by Isaac Newton in his famous Principia Mathematica 1687, where he describes the clockwork universe [Manuel 1975]. He stated for example that “Truth is ever to be found in simplicity, and not in the multiplicity and confusion of things” [Manuel 1975].

However, during the 1800s holism was the predominant approach in biology [Gilbert 2000]. Holistic approaches, as opposed to reductionistic ones, aim to consider whole systems, since the properties of the whole cannot be deduced from the properties of the parts. This is especially germane for complex systems. In fact, even the properties of individual parts change depending on the specific context, for example the electrical charge of a molecule depends on the pH of the environment. In order to have a context, a more holistic approach is necessary. Holism has been an intrinsic part of

embryology, since it was considered that the different parts of an organism develop in relation to other parts the organism, so that the development of one part depends on the development of the whole [Hertwig 1892].

Developmental biology was in vogue during the 1800´s due to a series of scientific events. First, evolutionary theories used comparative embryology as part of their argumentation [Lamarck 1809, Darwin 1859]. Next, spontaneous generation was finally disproved by Pasteur in 1864, which reinforced the need to explain where organisms come from. Finally, preformationism was also negated in light of the cell theory, which was born by the findings of by M. Schleiden in 1838 and T. Schwann in 1839, which also highlighted the need to understand how complex organisms develop from a single egg cell. Thus, embryology was essential not only to understand the ontology of organisms but also their phylogeny. Scientists like Muller, von Baer or Haeckel [Baer 1828; Müller, 1864; Haeckel 1866] used development to understand the relationship between species by looking for homologies in their embryonic developmental stages. For example, Darwin based many of his arguments on these embryonic comparative studies, which can be specially seen in chapter 13 [Darwin 1859].

Darwin's explanations on his theory of "descent with modification" from a common ancestor, acknowledged two of the major laws accepted in his time, the "conditions of existence" and the "unity of type", both of these laws were generally attributed to God´s plan, and although Darwin and other naturalist of his time rejected the involvement of God in these laws, they kept using these laws in their scientific arguments. The conditions of existence focused on how different animals were perfectly adapted to their environment. The unity of type focused on the similarities or homologies between organisms, arguing that variation should be understood as deviations from a basic plan (God's ideal plan). Darwin introduced the concept of natural selection to explain the conditions of existence, since natural selection "slowly and beautifully adapts each form to the most complex relations of life" [Darwin 1859]. The unity of type, Darwin argues, highlights the existence of a common ancestor, as many features of organisms are maintained between lineages.

Lewontin [Lewontin 1974] reminds us that Darwin's main contribution was not only the concept of natural selection (which explains the conditions of existence), but also the distancing from Plato's eidos, the idea that all physical shapes are merely deviations of the Forms. In Plato's philosophy [Alican 2013] all physical forms we experience are merely deviations of some conceptual Form, which themselves are immutable. Many naturalists had implicitly or explicitly accepted this idea, and generally explained the diversity of Life (at least in the Western World) as creations of God. Some naturalists took this idea to an extreme, considering that every single variation in different local species was to be considered an "original creation" [Agassiz 1857]. On the

other extreme, we can find naturalists such as George-Louis Buffon [Mayr 1981], who considered for example that all felines were part of the same initial "type", but showing high degrees of morphological variation. However, he believed that there could not be transition between "types" or creation of new ones. One of the first scientists to reject the idea of "fixed types" was Lamarck, he accepted that organisms could change indefinitely, to the point that even new types could be created. However, Lamarck did not consider the variation within populations to be an important factor in this process. For Lamarck [Gillispie 1960], the mechanism that allowed species to evolve were external, as environmental forces would induce new characters that would then be inherited and accumulate over time. This way, all individuals in a population would change in the same way, as imposed by the environment. However, in Darwin's theory, it is precisely the variation between individuals in the same population what makes evolution possible in the first place. The importance of this variation between individuals was, at least partially, brough to his attention by breeders.

Breeders had recognized that individuals from the same species showed random variation in some of their characters. According to Darwin, breeders thought that this variation was random in the sense that it did not confer any natural advantage to the individual and, therefore, the environment was not responsible for them [Darwin 1859]. Environment was believed could make direct modifications on organisms to confer them natural advantages, which could then be passed along to their offspring. This way, adaptive changes were not considered random, but induced by the environment. Breeders used these random differences between individuals to breed specific individuals and improve some characteristics that the breeder deemed useful. However, it was widely accepted that artificial selection could not break the boundaries between types.

Darwin was greatly inspired by breeders, and by using Malthus theories, he found a way to justify that organisms in nature struggle to survive and reproduce [Ruse 1975]. Through this struggle, nature could select which individuals would contribute to the next generation, based on some advantage that the randomly occurring variation could confer to the different individuals. For Darwin, individuals would not change in a specific direction as Lamarck had previously suggested. Variation between individuals arises from random perturbations in the "gemmules", the particles responsible for inheritance. These random perturbations would distort the normal development and introduce changes in the normal morphology. As the "gemmules" are copied from generation to generation, including the perturbations, changes would accumulate over time, allowing species to transform even beyond the "type" boundaries. This way, although some naturalists had previously recognized that organisms can change over many generations, Darwin argues that these changes are the result of the variation between individuals in a population, and

natural selection, which selects between the more fit variants. This contrast with Lamarck views for example, as Lamarck argued that the environment pushes individuals in a specific direction, e.g. to have longer necks, but the actual variation in the population is irrelevant.

The study of variation and homologies was a fundamental aspect to prove Darwin's theory. These studies included embryonic development, as it reinforced the idea that species came from common ancestors and showed how they diverged. Two of the main researchers on embryonic development in the 1800´s were Haeckel and Von Baer.

Haeckel believed that development was the motor of evolution and in 1866 he developed his Biogenetic Law [Haeckel 1866], which is famously summarized as: ontogeny recapitulates phylogeny. According to this "law", changes happen in the adult or last phase of development, while all the previous developmental and adult stages are conserved (although he also considered possible changes in speed or simplification of some developmental stages). This way, development is a chronological replay of all the previous forms. Therefore, by observing the development of an embryo, we could have an idea of how the ancestors of that species were. Currently it is widely accepted that ontogeny does not recapitulate phylogeny. But in Haeckel`s time, it was a very popular hypothesis which helped to establish links between evolution and development, for example it helped to establish taxonomic relationships between different organisms.

In contrast, Von Baer had a different view of how development plays a role in evolution. He saw phylogeny as the separation of embryonic forms that came from a common origin, a view, which according to Gould was more in line with Darwin's thoughts [Gould 2002]. This approach was later supported by Walter Garstang work [Garstang 1922], who showed how new features were based on changes in the developing embryo, and not in the adult and stated that "ontogeny does not recapitulate phylogeny, it creates it".

Although Von Baer and Darwin agreed on some points, they also had some disagreements. Some of these disagreements are clearly stated in some of his correspondence with Anton Dohrn in 1875 [Baer 1993]. In these letters von Baer´s expressed some disagreement with Darwin's hypothesis, as he did not think that natural selection could be enough to explain all of the evolutionary processes: "I cannot help but find transmutation probable to a high degree; but I cannot declare Darwin's hypothesis of selection to be sufficient and believe therefore that transmutation should be explained as a developmental phenomenon." In these letters von Baer also underpins the importance of development in order to understand how new organs or features appear in organisms: "... many Darwinist seem to believe, that an animal cannot have a component that was not previously owned, because of this, arms and legs must

have been present in a roundworm, I am on the contrary convinced that, … , if an animal needs legs they will develop according to the laws of mechanics". He also mentions how under Darwinian theory there is no "reason" or objectives in nature: "It [natural selection] assumes there is no reason or objectives in it, either immanent or transcendent. When drawing a circle by hand it will never turn out as good as if drawing the circle by keeping always the same distance from a point. When drawing freely, it would take many more attempts to get a near perfect circle". With this analogy, von Baer defends that evolutionary changes occur on top of preexisting developmental processes, and therefore adding new circles (changes in development which result in phenotypic changes) must happen by considering the previously existing circles. This way, von Baer believed that development guided evolution [Rensh 1954, Riedl 1975]. Although in his thinking evolution was driven by internal forces (orthogenesis) towards specific goals (teleology). Nevertheless, the idea that development is an integral part of evolution as it determines morphological variation and therefore what selection can act upon (although not towards specific goals) is a fundamental part of the current evolutionary and developmental (evo-devo) framework [Raff and Kaufman 1983; Gilbert et al. 1996].

### 1.1.2 DOWNFALL OF EMBRYOLOGY

In the late 1800s and early 1900s, embryologists became interested in the mechanisms by which an egg develops into an adult. There were two main hypotheses on which part of the cell predominantly directs development and inheritance. On one hand, Edmund Beecher Wilson and Theodor Boveri defended the nucleus as the responsible for directing development, already pointing out chromatin as the main responsible biochemical agent in charge of development and inheritance [Wilson 1896]. On the other hand, Thomas Hunt Morgan defended that the cytoplasm was responsible for directing development. However, data accumulated in favor of the nucleus as the responsible agent for the inheritance of characters, which was finally proven by Morgan himself. This discovery initiated genetics as an independent discipline in the 1930s [Gilbert and Barresi 2019].

Genetics rapidly gained popularity and previous approaches to study evolution became quickly disfavored. For example, Bateson, who initiated his career by using embryology to study phylogeny, claimed that : "Morphology having been explored in its minutest corners, we turned elsewhere … the geneticist is the successor of the morphologist" [Bateson 1922]. Similarly, in 1932 Morgan, already fully converted into a geneticist, claimed that the only valid approach to study evolution was genetics [Morgan 1932].

F.R. Lilie [Lillie 1898] proposed that what needed to be understood were the differences between organisms and not their similarities, putting the focus on natural selection and ultimately forgoing development, which at the time was mostly used to identify the "unity of types", i.e. homologies. Following this new trend, one of Haeckel's students, Roux, started a new group where he explicitly separated the study of development from the evolutionary branch, as he expected the former to advance faster. This way, while embryology was separated from evolutionary studies, genetics gained further popularity and partially merged with evolutionary research, planting the seeds to form the Modern Synthesis (in contrast to the "unmodern synthesis", which was the union between embryology and evolution [Gilbert 2003].

The Modern Synthesis or Neo-Darwinism (I will use these two interchangeably), was born in the 1940s as the combination of Darwin's basic principles of evolution (heredity, natural selection and variation) and genetics [Charlesworth et al. 1982, Corning 2020]. Under the umbrella of Neo-Darwinism, genes can simultaneously explain both the genetic material (genotype) and the properties of the organisms on which natural selection can act on (phenotype). In fact, Neo-Darnism assumes that the genotype directly regulates development, which in turn regulates the phenotype (see Fig. 1A). Under this hierarchical structure, development can be neglected [Alberch 1989]. Therefore, by studying how gene frequencies change in a population (evolution happens at population levels, since individuals cannot evolve), it can be explained how phenotypes evolve, as genetic variation and natural selection are supposed to be the main causes of evolution. This way of thinking eliminated the need of development to study evolution, since development, according to the Modern Synthesis is simply the readout of a genetic program [Alberch 1991, Laubichler and Maienschein 2007, Pigliucci 2010].

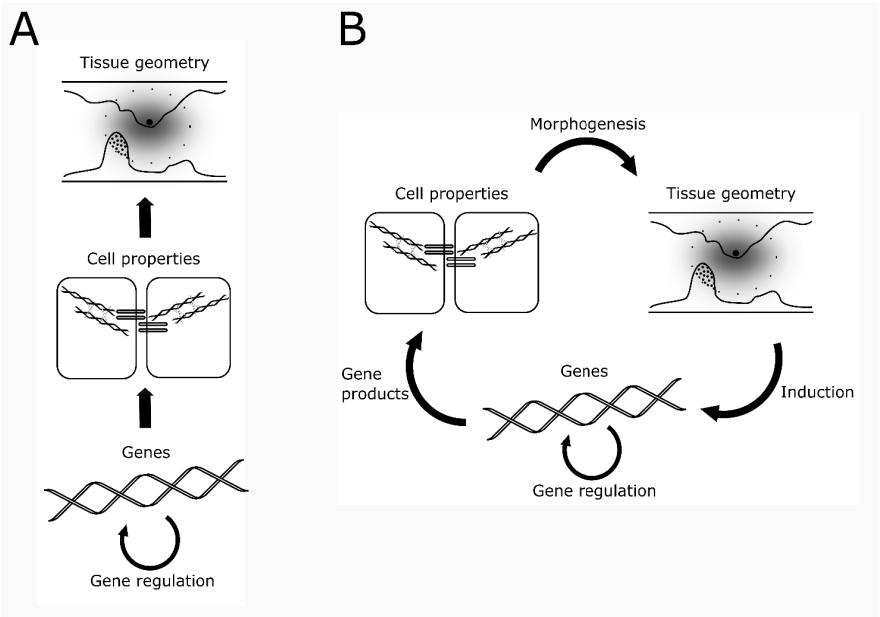



Fig 1 **Two views of development** (Adapted from Alberch 1991). **(A)** Hierarchical view in which morphologies are completely determined by genes. Under this view development can be treated as a black box, as the genes are the only controlling agent needed to understand how morphologies form. **(B)** Under the cyclical view of development, development cannot be understood as a one directional process in which genes are the sole controlling agents of morphology. Genes do not specify morphology nor how development should proceed. Genes are part of the dynamics that occur during development, in which iterative interactions at different biological levels will determine the outcome of development. Genes produce gene products that can regulate other genes and affect cell properties, such as adhesion, cell division, extracellular matrix secretion, etc. These cell properties, together with tissue geometry and generic physical mechanisms, will determine the biomechanical interactions between cells, which will lead to morphogenesis, changing the tissues geometry. The changing positions of cells in a tissue will determine which responding tissue (bottom-dotted tissue in the right of the figure) is in range of the inductive tissue (top tissue in the right of the figure). The inductive signaling will then affect the gene regulation of the target tissue, which will then produce different gene products, and the cycle repeats.

Nevertheless, during the establishment of the Modern Synthesis there were also advocates for the necessity to include development in evolutionary thinking. Notably, Goldschmidt argued that contrary to what the Modern synthesis claimed, an accumulation of small genetic changes was not enough to generate new structures [Goldschmidt 1940]. He argued that in order to

develop new features, changes in genes regulating development had to occur and that “a single mutational step affecting the right process at the right moment can accomplish everything, providing that it is able to set in motion the ever present potentialities of embryonic regulation” [Goldschmidt 1940]. Goldschmidt claimed that thanks to the regulatory process of development, there is no need to modify thousands of genes, and therefore that evolution is not simply a statistical genetic problem, but also a problem of what development can do [Goldschmidt 1951]. Although Goldschmidt point is a valid one, even if we accept that evolution only happens by a myriad of small mutational steps, development is still fundamental to understand what kind of phenotypic variation is possible, since any change in morphology is first a change in development. Development determines how mutations in the genetic material affect the morphology of an organism. Depending on developmental dynamics, some morphologies will be more frequent than others, and some will not be possible at all [Alberch 1980].

Nevertheless, since the Modern Synthesis was established, most of the scientific efforts to understand evolution have revolved around understanding gene dynamics during evolution, e.g. how allele frequencies change or dominance relationships between genes. This way, biology too, like physics and biochemistry, became highly reductionistic (being the genes the smallest piece which needs to be studied). In part this was also the result of the migration of physicists and biochemists into the newly formed field of genetics, coming from fields with a long history of reductionist approaches, they brought their methods with them [Mazzocchi 2008]. For example, Francis Crick famously said that “the ultimate goal of the modern movement in biology is to explain all biology in terms of physics and chemistry” [Crick 1966].

## 1.2 COMPLEX SYSTEMS AND REDUCTIONISM

As genetics was established as an independent field and gained popularity, it cemented the Modern Synthesis as the main evolutionary theory and reductionist approaches spread over different biological disciplines [Mazzocchi 2008]. Paradoxically, the huge amount of information that is being obtained by the different “-omics" (genomic, proteomics, etc.) has highlighted the high level of complexity in these systems, which cannot be studied nor understood by reductionist approaches. There are several aspects of complex systems that reductionist methods fail to capture (Webster and Goodwin 1996):

1. Emergent properties. Aristotle´s classic quote “The whole is more than the sum of its parts” somewhat describes emergent

properties. A complex system has intrinsic properties which cannot be deduced simply by the units that compose it. Therefore, to study such a system we need to place our hypothesis at the appropriate hierarchical level. For example, if we want to understand the chemical properties of nucleotides, we can study just nucleotides, but in order to understand the properties of RNA, we need to study the macromolecule. The macromolecule can have new properties that arise from the way in which the nucleotides assemble and fold. For example, certain RNA structures, such as hairpins, can guide RNA folding, protect RNA from degradation or serve as a substrate for enzymatic reactions. Simply studying the nucleotides that are part of the hairpin, without considering the structure, would not have revealed these properties. [Svoboda and Di Cara 2006]

2. Non-Deterministic. In deterministic systems, there are no stochastic or random effects, i.e., every cause always produces the same effect. Assuming that biological systems are deterministic can be problematic, since stochastic effects are present at many different levels, for example the chromosomal assortment during meiosis or the random protrusions of lamellipodia or filopodia in cells. It is especially problematic when considering that many biological systems are highly complex, and even small perturbations can have huge and non-linear effects in their outcome [Lorenz 2001].

3. Self-organization. Complex systems can spontaneously arrange their components into different structures without the need of a centralized control. This is a special case of emergent property, where the different components of a complex system interact locally and achieve some structure or activity that was not specifically planned by central control centers, like the brain. Some claim that the genome acts as the organizer of organisms, which is why genomes are often referred to as the blueprint of organisms [Adami 2002], although this metaphor has been put into question [Pigliucci 2010, Stanley 2007]. An example of self-organization can be found in multicellular aggregates of cells exhibiting variation in their amount of adhesion molecules. In these aggregates, cells will sort themselves in such a way that the more cohesive cells (with more adhesion molecules) will cluster together while being surrounded by the less cohesive cells. This way, from a mass of cells, a multilayered structure can form automatically, with no previous plan. [Newman and Muller 2000].

Development is the complex system that can include all of these properties and ties together the different components needed for morphologies to form, which includes not only genes, but also interactions between cells, tissues and generic physical mechanisms [Newman and Comper 1990; Newman and

Muller 2000]. However, as with any complex system, some degree of simplification will be necessary to study development. These simplifications need to be done according to the phenotypic level we are researching. In some cases, it will be enough to consider gene networks and diffusing gene products [Salazar-Ciudad et al. 2000, Jaeger et al. 2004] while in others biomechanical interactions might also be necessary [Odell et al. 1980, Salazar-Ciudad and Jernvall 2005]. Simplifications are a fundamental aspect to understand complex systems, but they need to be considered into the resulting theory and oversimplifications need to be avoided, which can turn the results into the physicist joke of the spherical cow [Fig. 2]. With the growing body of literature on how different tissues and organisms develop, from gene interactions to biomechanical interactions, more holistic theories are possible,

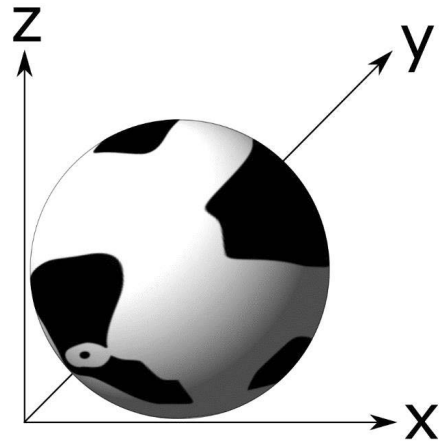



Fig. 2. **The spherical cow is a classical metaphor used to refer to the oversimplification in models** [Harte 1988]. Although any model includes simplifications, these need to be considered carefully when building a model and interpreting the results. Depending on the question at hand, different levels of simplification can be useful. For example, if we want to model a reaction-diffusion system able to recreate the spots in a cow, this spherical cow could be an acceptable simplification. However, if we are interested in the developmental events that lead to an adult cow, a model that results in this kind of spherical cow, will tell us little to nothing about them, unless we are interested in how spherical cows develop.

and even necessary, to understand how these pieces work together during development.

## 1.3 DEVELOPMENT

Development can be defined in different ways, some definitions limit development to the processes that transform eggs into adults [Horder 2010], while others prefer to include the whole life cycle of an organism [Huxley and De Beer 1963, Gilbert 2011]. In this thesis I will mostly refer to development as the process that results from the interactions of all developmental mechanisms, which transform a given input (for example an egg) into an adult or until the next egg is formed, if the whole life cycle is to be taken into account. A developmental mechanism as defined in [Salazar-Ciudad et al. 2003] is composed of a genetic network with a given topology that regulates some concrete cell behaviors (such as cell division or cell adhesion). Different developmental mechanisms will have different network topologies and/or regulate different cell behaviors. Given an input pattern, a developmental mechanism will transform it into an output pattern (see Fig. 3). A pattern encompasses gene expression patterns as well as the distribution of cells in space and their biomechanical properties. Although a developmental mechanism is defined only by a gene network topology and the cell behaviors it regulates, it is important to consider that there are other aspects which are vital to understand how organisms are formed during development. Most importantly, there are a set of generic properties that all living tissues have, such as viscosity, elasticity, cohesivity or surface tension. These generic properties define a specific set of responses from tissues to physical effects which give rise to certain morphological transformations. For example, a cell population that includes cells with two different adhesion molecules, will eventually sort, leaving the more cells that adhere more strongly surrounded by the other, less adhesive, cells. In a similar way, other structures commonly seen in organisms can form without the need of any genetic control. Some of these structures include lumens, gastrulation or segmentation [Newman and Muller 2000]. All these factors, gene networks, cell behaviors, tissue geometry, generic properties and other bio-physical properties, are brought together in development to form morphologies.

As development is responsible for producing morphologies (changing one pattern into another), any morphological change must happen first at the level of development. Voices against this point have been raised [Adami 2002, Williams 2015] arguing that since genes are ultimately responsible for directing development and they are the heritable elements ; it is enough to study the genes. However, this point has been debated at length by several authors [Oyama 1985, Nijhout 1990, Muller and Wagner 1991, Bolker and Ralf 1996, Salazar-Ciudad et al 2003]. As explained before, such a reductionist approach can only explain part of the whole dynamic while missing other critical parts of the system.

## 1.4 GENOTYPE PHENOTYPE MAP

Waddington [Waddington 1953] pointed at the dichotomy existing in evolutionary studies, in which animals are either a genotype (when studied by geneticists) or a phenotype (when studied by taxonomists). Waddington identified a lack of studies focusing on the processes that unite the genotype and the phenotype, which he called the "epigenetics of development" or the epigenotype [Waddington 1942]. Waddington first defined epigenetics as the "branch of biology which studies the casual interactions between genes and their products which bring the phenotype into being" [Waddington 1968]. For Waddington, development was about how genes interact and regulate each other in order to differentiate cells and form different tissues and organs. This can be seen in his now well-known drawings which depict how a ball (representing part of the egg) can follow different paths on its way to differentiation. The epigenetic landscape, made of channels that guide the ball, is determined by pegs (which represent the genes) and guy-ropes (chemical tendencies of the genes) which shape the epigenetic landscape and interact with each other, in such a way that by modifying one guy-rope, others could be affected. Waddington´s metaphor stresses that the relationship between genetic and phenotypic variation is not simple, as the effects of altering one gene on the epigenetic landscape, does not depend on that gene alone, but also on the genes that interact with it [Jablonka and Lamb 2002].

However, the idea that the connection between genotype and phenotype needs to be studied and not simply treated as black box was not generally acknowledged. As Neo-Darwinism explicitly assumes that natural selection is the only force that needs to be considered in order to understand evolution, it implicitly assumes that variation is limitless and that selection can optimize any phenotype, making the role of development irrelevant [Alberch 1980, Salazar-Ciudad 2005]. Despite the Neo-Darwinian assumptions, some scientists over the last century have defended the role of development as critical in determining the variation that is possible, by constraining or facilitating certain morphological variation or simply, by producing certain phenotypic outcomes [Goldschmidt 1940, Bertanlanffy 1952, Alberch 1980, Salazar-Ciudad and Jernvall 2004, Takeuchi and Hogeweg 2008, Salazar-Ciudad and Marin-Riera 2013].

In the 1970s Burns and Lewontin [Burns 1970, Lewontin 1974] created a theoretical framework to consider the epigenome or development when studying how changes in the genotype affect the phenotype (the genotype-phenotype map or GPM). The GPM was to be used to approach the synthetic problem. This problem refers to the disconnect between the increase in the understanding of molecular biology and the lack on the ability to make quantitative predictions via population genetics [Burns 1970]. In order to tackle the synthetic problem, Burns proposed to use computational models to

study how different gene networks behave under a range of environmental conditions and how their gene expression patterns (phenotype) changes. By including developmental dynamics as a set of differential equations, which define how different gene products interact, the effect of the epigenome could be considered. However, the GPM concept was not broadly used until after Alberch [Alberch 1980] studied it in detail.

Alberch reintroduced the concept of the GPM by claiming that in order to have a theory of morphological evolution it is fundamental to include development, since focusing solely on how gene frequencies change during evolution cannot explain how morphologies evolve. Genes are only part of the whole developmental process. In order to study how changes in the genotype affect the phenotype, we need to include not only how gene products regulate each other, but also how genes affect cell properties, which can change the geometry and biomechanical properties of tissues and the physical interaction between different cells and tissues (See Fig. 1B). For example, morphogenetic effects (e.g. changes in the shape of tissues) can play an important role in gene regulation. On one hand, changes in tissue geometry influence which cells are in range of diffusing gene products that cells are signaling. On the other hand, changes in the biomechanical properties of cells can regulate different gene products, for example, depending on how firm a substrate is, cells can regulate the adhesion molecules they express [Evans 2009]. Therefore, there is a fundamental feedback loop between tissue geometry and cell induction (Alberch 1991). These dynamics cannot be predicted just from studying the genes or gene networks.

Different attempts have been made to study how development, or some other complex system, determines phenotypic variation, which is then described by GPMs, see Table 1. Therefore, a GPM tells which of the morphologies that are possible by development are associated with which specific genetic variants. Due to the complexity of studying the GPM in a laboratory, most studies are done with computational models, although some studies exist on molecular systems such as transcription factor [Aguilar-Rodriguez et al 2018]. The computational models include a wide range of systems, but in general they are restricted to relatively simple ones, as studying the properties of GPM´s is a very computational consuming endeavor and we lack the understanding to model other more complex systems. Some of the properties found in the different GPM´s are common to all the systems, which seems to imply that there are some universal properties of the GPM´s. Nevertheless, due to the limited complexity of the systems studied so far, these universal properties should not be assumed unquestionably when studying new systems. The most common properties found in GPM´s are:

1. The GPMs are degenerated, i.e. many genotypes map to the same phenotype. The set of genotypes that give rise to the

same phenotype are generally organized in neutral networks or iso-morphological spaces. In these spaces, all genotypes can be accessed by accumulating small (e.g. single nucleotide changes) neutral mutations. In other words, from any given genotype in an iso-morphological space, any other genotype in the same iso-morphological space can be reached by several single neutral mutations without changing the phenotype [Schuster et al. 1994, Jörg et al. 2008, Aguirre et al 2011, Dingle et al, 2015, Weiß and Ahnert 2020].

2. The size of the different iso-morphological spaces is very disparate, with most genotypes mapping to a few very common phenotypes [Schuster et al. 1994, Jörg et al. 2008, Fortuna et al. 2017, Reidys et al. 1997].

3. Big iso-morphological spaces (i.e. common phenotypes) are accessible from other neutral networks by few mutational steps. It is widely accepted that these big iso-morphological spaces percolate the genotypic space and are adjacent to many other iso-morphological spaces. This way, it is more likely that from a given iso-morphological space, a non-neutral mutation will find one of these big iso-morphological spaces rather than a smaller one [Schuster et al. 1994, Huynen 1996, Reidys et al. 1997 Jörg et al. 2008, Aguirre et al 2011, Dingle et al 2015, Weiss and Ahnert 2020].

4. When considering the complexity of phenotypes on the GPMs several properties have been found.

   a. It has been shown that fewer genotypes exist for complex phenotypes and a mutational asymmetry, in which non-neutral mutations are more likely to reduce complexity rather than increase it (Salazar-Ciudad and Jernvall 2005, Fortuna et al. 2017, Dingle et al. 2018).
   b. Complex phenotypes tend to have neutral networks which are divided in several independent parts, i.e. they are not reachable by accumulating neutral mutations (Payne and Wagner 2013, Fortuna et al. 2017).
   c. Complex phenotypes exhibit lower evolvability, since their iso-morphological spaces are smaller, and a linear relationship between the size of the space and evolvability is explicitly assumed (Fortuna et al. 2017).

Table 1.**GPM models studied**

| **Based on natural systems** | | |
|---|---|---|
| **Genotype** | **Phenotype** | **Authors** |
| RNA sequence | RNA secondary structure | Shuster 1994, Aguirre 2011; Coperthwaite 2008 |
| Polar/Apolar aminoacids | Latice based structure | Lipman and Wilbur 1991; Bornberg-Bauer and Chan 1999 |
| Genotype circuits | Expresion genotype | Ciliberti 2007 |
| Metabolism | Nutrient use | Rodriguez and Wagner 2009 |
| Three gene networks | Stripe formation | Cotterel and Sharpe 2010 |
| Chain of characters and blocks (protein subunits) | Two dimensional polyomino (quaternary structure) | Greenbury 2014 |
| Parameter values in tooth development | Three-dimensional tooth morphology | Salazar-Ciudad and Jernvall 2010; Salazar-Ciudad and Marin-Riera 2013 |
| Genetic networks | Stripes formation in a one-dimensional array of cells | Salazar-Ciudad, Newman and Sole 2001 |
| Based on Non biological systems | | |
| 26 instruction alphabet | Combination of nine boolean operations | Fortuna et al 2017 |
| Program with four boolean instructions | Sequence of Boolean output | Hu et al 2012 |
| 2PDoL system | Sequence of letters | Lehre and Haddow 2005 |
| Fibonacci model: sequence of zeros and ones | Sequence of zeros and ones | Weiis and Ahnert 2017 |

These results underpin the importance of studying developmental dynamics, even if it's only simple gene networks [Cotterel and Sharpe 2010, Salazar-Ciudad et al. 2001] or more comprehensive developmental dynamics, such as a full tooth development [Salazar-Ciudad and Jernvall 2010, Salazar-Ciudad, Marin-Riera and Salazar-Ciudad 2013]. By studying individual genes, these results could not have been obtained, and their consequences for phenotypic evolution could not have been considered. Studying developmental dynamics and the general properties of their GPMs allows us to understand what kind of phenotypic variation is possible for a given genotype without having to make assumptions on how this variation should be, for example, should every mutation in the genotype lead to a phenotypic change? How much should the phenotype change given certain mutations? To understand phenotypic variation is especially powerful to study evolution, since variation is one of the main tenets of evolution.

By considering that phenotypic variation is limited, we can add an additional layer to evolutionary dynamics. This layer will be defined by the

phenotypic variation that is possible for a given developmental mechanism. The possible phenotypic variation will determine which phenotypes are realized and therefore, which phenotypes natural selection can act upon. Considering what phenotypic variation is possible, allows to combine the more common approach of "survival of the fittest" with the "arrival of the frequent" [Coperthwaite et al. 2008, Schaper and Louis 2014]. This implies combining natural selection ("survival of the fittest") with the phenotypic variation ("arrival of the frequent"). Recognizing what phenotypic variation is possible for different developmental mechanisms, could answer questions about the "tempo and mode of evolution".

### 1.4.1 GENOTYPE PHENOTYPE COMPLEXITY

How changes in the genotype affect the phenotype (the GPM) varies between different developmental mechanisms. A common way to describe GPM´s is by referring to its complexity. A simple or linear GPM results when small changes in the genotype produce small changes in the phenotype [Oster and Alberch 1982, Newman and Muller 2000, Salazar-Ciudad and Marin-Riera 2013]. On the other hand, the GPM is considered complex when small genotypic changes produce big phenotypic changes or vice versa, or when the relationship is not linear [Oster and Alberch 1982; Newman and Muller 2000, Salazar-Ciudad and Marin-Riera 2013].

Considering the complexity of the GPM when studying evolution is particularly interesting, since it has been shown to affect evolutionary dynamics. Most importantly, simple GPMs allow adaptation in small and gradual steps, allowing a faster and finer adaptation [Salazar-Ciudad and Jernvall 2005]. Complex GPMs on the contrary decrease the efficiency of natural selection since even small genetic differences between the parent and the offspring can result in big morphological differences [Salazar-Ciudad and Jernvall 2005, Wagner and Altenberg 1996; Lewontin 1974, Kauffman 1993]. This can result in genotypes in which none of its offspring is better adapted, effectively getting trapped in a local optimum. However, precisely because small genetic differences can lead to big morphological changes, it has also been shown that complex GPMs allow to explore wider areas of the phenotypic space, resulting in more novelties and the chance to occupy new niches [Salazar-Ciudad and Jernvall 2005]. Therefore, assuming that GPMs are always simple would hinder our understanding of evolution, especially considering that complex GPMs are very common [Alberch 1982, Wagner and Zhang 2011, Gjuvsland et al. 2013, Milocco and Salazar-Ciudad 2020].

In this section I have argued that in order to understand the relationship between genetic and phenotypic variation, we need to consider development. In a similar way, non-genetic variation, arising from environment or stochastic

fluctuations during development, are important to consider in conjunction with development. Since in these cases, development also establishes how these non-genetic variations affect the phenotype. In the following section I will introduce some concepts related to these non-genetic variations, specially focusing on developmental stability, which is the lack of phenotypic variation under stochastic fluctuations during development.

## 1.5 DEVELOPMENTAL INSTABILITY

An important component of phenotypic variation comes from environmental changes and stochastic fluctuations during development. As we have seen in the previous section, how genetic variation affects phenotypic variation depends on developmental dynamics. In the same way, how these other sources of variation affect the phenotype will also depend on development. However, even when considering these sources of variation, the Neo-Darwinian way of thinking is predominant. For instance, small genetic changes are expected to produce small phenotypic changes, when the phenotype does not change, we say the phenotype is mutationally robust or that there is canalization [Felix and Wagner 2008, Hallgrimsson et al. 2002]. Similarly, when environmental changes do not produce phenotypic changes, we say there is canalization [Hallgrimsson et al. 2002]. Low phenotypic variation under stochastic fluctuations during development is defined as developmental stability [Zakharov 1989, Hallgrimsson et al. 2002, Klingenberg 2019]. These concepts refer to a lack of phenotypic variation that in many cases was expected a priori.

In order to explain why this expected variation is not encountered, some have claimed that specific mechanisms exist, such as the Hsp90 chaperone or other canalizing gene products [Mayer and Bukau 1999, Young et al. 2001, Richter and Buchner 2001]. However, it is also possible that the lack of phenotypic variation is simply due to developmental dynamics [Klingenberg and Nijhout 1999, Salazar-Ciudad 2007, Green et al. 2017], which do not require any specific mechanism. Whether specific mechanisms are necessary or not is up to debate. Of course, both theories can coexist, as argued in [Milton et al. 2003] and [Salazar-Ciudad 2007]. In [Milton et al. 2003] they show that Hsp90 can reduce phenotypic variation arising from genetic variation, and therefore act as a canalization agent. However, phenotypic variation arising from random perturbations during development is not reduced by Hsp90. This latter phenotypic variation seems to depend on developmental dynamics. In other words, developmental mechanisms themselves can be associated with certain degrees of developmental instability, depending on how sensitive they are to stochastic fluctuations during development.

Stochastic fluctuations during development have different origins. At a biochemical or molecular level random thermal fluctuation can have important effects, especially when the number of molecules is low [Del-bruck 1940], but any process involving gene expression or other molecular dynamics will be affected by noise [Arias and Hayward 2006]. At a cellular level, stochastic effects can also be seen for example in cell migration, where cells move in a biased random walk due to the random protrusion of lamellipodia or filopodia in cells [Pézeron et al. 2008, Petrie et al. 2009]. Gene expression patterns in a tissue can also be affected by noise, which can originate from temporal fluctuations of signaling molecules, heterogeneity of receptors or from cells moving and dividing [Wartlick et al. 2009]. Cytoskeletal dynamics are also intrinsically stochastic [Fletcher and Mullins 2010]. As a consequence, cell shape can be affected, which can introduce noise in the configuration of cells, possibly resulting in macroscopic effects, such as variation at the level of tissues.

At the level of tissues, stochastic dynamics also appear, such as in buckling morphogenesis or wrinkling. Wrinkling is a mechanical instability that reduces the compression of a tissue by bending the tissue out of the plane [Sharon and Efrati 2010]. For example, in a rapidly growing epithelium, pressure will accumulate as more cells are forced to share a very close space, in order to release this compression cells will move up- or downwards relative to the plane of the epithelium, forming folds in the locations were stress has built up the most. In-plane compression can come from cell division or changes in cell shape, any irregularity in these can cause the folding to happen asymmetrically [Nelson 2016]. Examples of these can be seen in the cerebral cortex [Bayly et al. 2013] or intestinal tube [Savin et al. 2011, Thomason et al. 2012, Nerurkar et al. 2017]. In order to achieve folds in precise locations using these mechanisms, it has been suggested that spatial heterogeneities must exist, for example in stiffness, thickness or cell division [Nelson 2016].

## 1.6 COMPARING MORPHOLOGIES

In order to study developmental stability, methods are needed to compare different morphologies in a quantitative way. Some of the most popular methods for this are included under the morphometrics umbrella, which consist of several methods which provide a mathematical description of the morphology of organisms.

Traditional morphometrics, in the 1960´s and 1970´s, measured different morphological variables, such as length, width, height, ratios or angles. By collecting these data from different individuals, a multivariate analysis could be done that would uncover patterns of variation between and among samples. However, these methods have a big flaw, as they fail to capture the real shape

of morphologies. For example, if we try to differentiate an oval and a rectangle by just using height and width, it is possible that they will be identical. In order to be able to consider shape more accurately, the morphometric field developed the geometric morphometric methods, which can capture the overall geometry of the morphologies being studied.

In geometric morphometrics there are two popular approaches, the outline methods and the landmark methods. The outline methods focus on the bounding edge of morphologies. For this method points are collected along the outline of a morphology. These points are then fitted to a mathematical function, often a Fourier analysis, and then the coefficients of the curves of different shapes are compared [Sheets et al. 2006, Adams et al. 2004].

The landmark-based methods require a set of 2- or 3D coordinates of biologically identifiable features, i.e. landmarks. These landmarks cannot be directly studied, since the coordinates of the landmarks are highly affected by position, orientation and scale of the different individuals being studied. To avoid variation due to these factors, superimposition methods are applied, which result in the landmarks capturing only shape information. One of these methods is the Generalized Procrustes Analysis (GPA). This method superimposes landmarks by following several steps [Gower 1975, Rolf and Slice 1990]:

1. Centering: This is done by calculating the centroid (the center of the xyz coordinates, calculated as the mean of the x-, the y- and the z- coordinates for all the landmarks) of each landmark configuration and then making it the origin of a new coordinates system.
2. Re-sizing: The landmark configurations are resized so that they share a common centroid size, calculated as the square root of the sum of the Euclidean distances between each landmark and the centroid.
3. Rotation: the landmark configurations are rotated around the centroid in order to minimize the Euclidean distance between the homologous landmarks.

After this superimposition, shape differences can be studied by measuring the distance between the homologous landmarks. In essence, morphologies are being reduced to a set of landmarks. Therefore, it is extremely important to choose landmarks correctly, as they need to represent the most important aspects of a morphology. There are a couple of basic rules that help to identify possible landmarks. First, landmarks need to be easily identifiable, so they can be accurately found in different individuals, reducing variation in the shapes due to errors while recording the coordinates. Second, landmarks need to be present in all the morphologies that are being considered, in other words, every morphology needs to have the same set of homologous landmarks.

However, finding homologous landmarks is not always an easy task, especially when considering complex morphologies, in which exact homologies between landmarks might be difficult to establish.

So far, I have mentioned complexity several times in a rather loose way. As complexity is a central part of this dissertation, I will dedicate the next section to explain what complexity is, how it can be measured and how it has been studied in relation to evolution.

## 1.7 COMPLEXITY

One of the most striking changes in morphological evolution is how from simple unicellular organisms a huge diversity of complex multicellular organisms evolved. As we have seen, morphological evolution can only be understood "under the light of development", so to explore how complexity arises and evolves, we need to ask, at the level of development, what kind of mechanisms could allow for such an increase in complexity? And, is the GPM any different between phenotypes of different complexity? How diverse and disparate are the phenotypes produced by mutating phenotypes of different complexity? In order to approach these questions in a quantitative way it is first necessary to properly understand and define complexity.

### 1.7.1 WHAT IS COMPLEXITY

The complexity of an object or system can be defined, in its most general and intuitive way, by the size of its minimal descriptor [Papentin 1983, Hinegardner and Engleberg 1983]. Similarly, Kolmogorov [Kolmogorov 1965] defines complexity as the shortest algorithm capable of generating a system, therefore putting the focus on the process rather than on the object itself. These definitions are the basis for many of the current definitions of complexity, which aim to quantify complexity, although as we will see, there is no single definition that allows to measure complexity in a universal way.

Even though simple at first glance, to define complexity by its minimal descriptor has the unavoidable issue of the halting problem [Turing 1937]. Put simply, how are we sure we have found the shortest descriptor? If we have a program running until it finds the minimal descriptor, when will it be done running? Turing showed that there is no way to solve this issue reliably. Although it is not possible to be sure that the minimal descriptor of a system has been found, some approaches have been taken that assume that getting "close" enough to a very short descriptor in a reasonable amount of time is still useful [Boffetta et al. 2002]. Another difficulty is that to find the proper descriptor or algorithm of a system is not easy, not at a practical nor at a

theoretical level. Even when a definition is found, if the system is completely random it will be quantified as very complex. For example, the sequence of numbers 001100110011 could be summarized as 0011 x 3, but a random sequence like 010001110101 cannot be summarized, therefore the minimal description will have the same length as the actual sequence. Similarly, describing the location of bricks in a wall is easier than in a jumble of bricks.

A jumble of bricks requires a lot of information to be described accurately because every brick is in a different position relative to each other, so every brick requires a separate description. When bricks are organized in a wall, there is much less variation in the relative position of the bricks, so that by describing the position of one brick next to another, the position of the other bricks can be deduced. Similarly, if we were to measure the complexity of woodlice (Isopods) and lobsters (Nephropidae) just by considering their legs, we would consider lobsters more complex, as they have more types of legs, a higher "leg variation". The link between complexity and variation is strong, to the point that some consider variation and complexity to be interchangeable concepts [McShea 1991], and to be the opposite of order [Wicken 1979].

When studying complexity in organisms, how much of the phenotypic variation resulting from stochastic events should be considered? Stochastic events can affect development and introduce asymmetries in the morphology of organisms, which can increase the amount of variation, and therefore of complexity. Some argue [Lineweaver et al. 2010] that considering an increase in the diversity of parts of an individual as an increase in complexity is wrong, as it is simply an increase in entropy and therefore an approach towards thermodynamic equilibrium. Lineweaver argues that features that increase complexity should be considered only when they have been selected for, i.e. complexity is not necessarily being selected, but adaption happens to increase the complexity of the organisms. Others, like [McShea 2005] or [Stolzfus 2012], consider that an increase in the variation of parts in an individual implies an increase in the complexity of the organism, regardless of natural selection. In fact, they argue that natural selection often acts as a counterforce to an increase of complexity since phenotypic variation and asymmetries in some cases can be maladaptive [McShea 2005].

### 1.7.2 QUANTIFIYING COMPLEXITY

When trying to quantify complexity, there is only one aspect in which most methods can agree on. The resulting quantity should measure some kind of difficulty in achieving an object or system [Li 1991]. For example: how difficult is it to describe an object, constructing a system or reaching a goal.

However, in order to be able to study complexity, concrete methods of measuring complexity are needed. In biology, these methods generally focus on some specific level, such as genes, phenotypes (e.g. morphology) or ecosystems. In general, there is no consensus on which of these levels is more important. Some authors defend for example that an egg and an adult are equally complex, as they have the same DNA content [Hinegardner and Engleberg 1983]. Others argue that development is an organizational process that allows for morphological complexity to increase. As development proceeds, cells differentiate, and tissues form. Tissue morphology becomes geometrically more heterogeneous, as the epigenetic information unfolds during development [Apter and Wolpert 1965, Salazar-Ciudad et al. 2003]. As a result, a simple morphology like an egg, with a relatively homogeneous gene expression pattern[1] develops into a complex morphology with many genes expressed in different patterns and differentiated cells. Regardless, depending on the research question, there are different options.

1. Complexity at the genome level.

    a. Genome length [Sneath 1964]. The original assumption was that the genome contains all information necessary to construct an organism, that is, that genomes are the blueprint of organisms. This means that bigger genomes lead to more complex organisms, the logic being that more complex organisms need a bigger construction map (genome). The realization that this was not the case came to be known as the C-value enigma or paradox [Hahn and Wray 2002, Cavalier-Smith 1985]. It was then thought that by disregarding the "junk" DNA, the C-value paradox could be resolved [Cavalier-Smith 1985].

    b. Number of genes. Similarly to the genome length, it was assumed that if a genome contains more protein coding genes, the resulting organism should be more complex. The lack of correlation between number of genes and organismal complexity is in this case referred to as the G-value paradox [Hahn and Wray 2002]. This organismal complexity is often based on what as humans we expect should be more complex, which generally assumes that primitive taxa are simpler than more derived taxa. Therefore, bacteria are the simplest organisms while humans (of course) are among the most complex organisms. For example, [Comings 1972], although

[1] An initial asymmetric gene expression that defines the different axis of symmetry must exist if the organism is going to develop with different axis of symmetry. In other words, a system with perfect spherical symmetry, can only result in a spherical system, but never in an organism like a cow, which has different axes of symmetry [Turing 1952].

admitting a chauvinistic view towards humans, argues that humans, as one of the more complex species on earth, should have one of the biggest genomes. Comings then proceeds to describe how the "lowly liverwort" and the "slimy, dull salamander" have genomes, which are respectively 18 and 26 times bigger than the human genome. This "excess" in DNA, i.e. the surplus of DNA the organisms "should" have according to their expected complexity, is assumed to be junk DNA.

c. Regulatory sequences. There seems to be a correlation between the number of interactions between molecules (interactome complexity) and complexity, measured as the diversity of cell types. This can be seen at least between some broad groups. Prokaryotes have simpler interactomes, followed by unicellular eukaryotes and with the more complex interactome, the metazoan. However, Fernandez and Lynch [Fernandez, Lynch 2011] argue that this relation arises because of the size differences between these groups, prokaryotes are smaller than unicellular eukaryotes and metazoan bigger than these two groups. The size of organisms often relates with population size, bigger organisms usually have smaller population sizes. Smaller populations are less effective at eliminating mildly deleterious mutations, thus allowing for a faster accumulation of these mutations. This induces a secondary selection for more protein-protein interactions that stabilize some key functions [Fernandez, Lynch 2011].

In order to explain the G-paradox, several explanations have been proposed, and they all relate on how flexible the protein-coding genes are, for example:

- cis-Regulation. Genes are expressed during development in different places and at different times. This is possible due to the different ways in which genes can be regulated [Hahn and Wray 2002]. The more combinations of genes being expressed in different places and at different times, the higher the complexity of the phenotypes can be [Davidson 2001]. Therefore, even if only 5% of the human genome is transcribed to proteins, there is plenty of DNA left to include many cis-Regulatory sequences, which can potentially increase the number of combinations of genes being expressed [Hahn and Wray 2002]. Therefore, even if humans have less DNA than a "lowly liverwort", according to this explanation, humans are more complex because they have more genes being expressed in different combinations.

• Intergenic combinations and alternative splicing have been suggested to increase regulatory and functional protein diversity, which could increase complexity [Lander et al. 2001]. If all proteins could potentially interact, a slight increase in G-value, would mean a huge increase in the possible combinations of proteins. For example, the estimated G-value of the human genome is 31,000, which allows for about 480 million pairwise combinations; by adding 100 genes to the pool, this number goes up by three million. As in the previous point, here, humans are still more complex than the "lowly liverwort" because in humans, proteins can combine in many more ways. Additionally, this explanation assumes that once all the junk DNA is removed, even if humans have only slightly more protein coding DNA than the "lowly liverwort", it represents a huge amount of possible new functions.

By increasing the number of genes in these ways, we can justify why G-values and organismal complexity do not correlate positively. However, there is no reason for us to expect that G-values and organismal complexity should correlate in any way. As Kauffman argues [Kauffman 1993], due to the emergent properties of gene networks, there is no simple relationship between the complexity of the instructions (genes) and the complexity of the product (phenotype). Therefore, no matter how precise our estimates of genomic information become, we will not be able to directly correlate it with organismal complexity [Hahn and Wray 2002].

2. Cellular level: number of cell types. Morphological complexity can also be estimated by considering the number of cell types [Sneath 1964, Bonner 1988, Valentine et al. 1994]. This is an intuitive and at first glance easy way to measure complexity and it allows us to compare very diverse organisms. But as [Bonner 1988] points out, there are several difficulties in determining the precise number of cell types in an organism. First, the bigger the organism, the harder it will be to make precise measurements. The more organs made of different cell types are present, the more difficult it will be to precisely identify all cell types. Second, to agree on a way to compare different levels of cell differentiation is very complicated when comparing organisms of very distinct nature. Third, when measuring the complexity of unicellular organisms (which outside of life cycle phases only have one cell type), some authors suggest that the diversity of the microbiome should be taken into consideration [Smith 2010]. Finally, this method does not consider how cells are distributed in space, which can determine different functions or types of tissues [Valentine et al. 1994]. In any case, Bonner [Bonner 1988] showed that in some lineages the maximum number of cell types has increased during evolution. Although in the different lineages the cell number does not grow

indefinitely, it reaches a maximum early in the evolution of each lineage [Carrol 2001]. For example, in bacteria the maximum number of cell types found is three, while in protists it is four. Porifera and cnidaria have up to 12 cell types, and Bilateria, with the addition of the mesodermal germ layer, greatly increased their number of cell types. For example, Caenorhabditis elegans and Drosophila melanogaster have an estimated 50 cell types, while zebrafish and humans have about 120 cell types.

3. Morphological complexity. In this section I include some methods in which the shape of organisms or parts of them are used to measure how complex they are. Due to the technical difficulty of working with 3D shapes, such as organisms, little work exists in this field. However, the continuously improving technologies have helped to advance the study of 3D structures.

a. Orientation patch count [Evans et al. 2007]. This method was first used to measure the complexity of 3D tooth surfaces. It works by determining the orientation of each point in a surface (the orientation of the normal vector to the surface in that point) and then grouping all the contiguous points with the same orientation, each group forms a patch. The final complexity will be the number of patches.

b. By specific features: number of cusps, segments, etc. A popular approach for measuring morphological complexity is to count the number of certain features of interest. For example, for tooth complexity the number of cusps [Evans et al. 2007], the variation and number of leg-pair types in arthropods [Cisne 1974] or variation in homologous series like vertebra [McShea 1992]. These measures have the advantage of being easy to take. On the downside, they focus only on one aspect of the morphology, so they can miss other aspects of the morphology that could be important to consider when studying complexity. This can be especially problematic when choosing the feature of interest, which will generally have something that makes it attractive (like being diverse or intuitively complex), this will introduce an important bias in our generalizations of how complexity develops or how it evolves.

c. Outer shape complexity Based on Mutual Information. [Rigau 2005]. This method is based on measuring the mutual information between a morphology and its minimum circumscribing sphere (the smallest sphere that contains the shape being studied). This a robust method, in which similar

shapes will obtain similar values. With this method spheres and symmetrical objects will have low complexity values, however, it has the caveat that flatter morphologies will obtain high complexity values.

4. Functional complexity. Perhaps one of the most difficult to measure and therefore barely used in practice. Put simply, it defines complexity by the number of functions an organism has. This has the issue of defining and identifying functions, a rather difficult matter [McShea 2000].

### 1.7.3 COMPLEXITY TEMPORAL TRENDS

Traditionally, most scientists agreed that organismal complexity has increased during evolution. This trend was defended by many [Lamarck 1809, Darwin 1859, Rensch 1960, Saunders and Ho 1976, Saunders and Ho 1981, Wicken 1979, Bonner 1988]. However, some authors have noted that this trend might not exist at all [Williams 1966, Hinegardner and Engelberg 1983, Gould 1994]. Others consider that increases in complexity only happen in some groups, while decreases in complexity might also be common [McCoy 1977, Wake et al. 1986, Maynard Smith 1970, Simpson 1949]. Gathering the different explanations for a possible trend in complexity evolution, two main groups exist:

1. Passive trends. Trends due to a total increase in variance where a lower complexity boundary exists, also called “the uninteresting” explanation by Maynard Smith [Maynard Smith 1970]. The trend in increasing complexity can be explained by the fact that the first organisms started at the minimal complexity boundary. From this minimal complexity, organisms could only increase their complexity, increasing the mean and maximum complexity of Life. As more species appear and diversity increases, so does the maximum and average complexity. This is comparable to a diffusion-like process, in which an increase in variance and a lower complexity bound result in a directional increase in complexity [Stanley 1973, Fisher 1986, Gould 1988]. This way, if every time a new group evolves it has a fifty percent chance of increasing or decreasing their complexity, the average complexity of all the groups will initially increase, until at some point this increase would be negligible, although the maximum complexity would still rise. Gould [Gould 1988] underpins how this increase in maximum complexity is the base for many arguments about the continuous increase of complexity seen in different organisms. In fact, Gould argues that only these high complexity outliers are being

considered. Therefore, the whole discourse about how complexity increases is based solely on these outliers. But we still must recognize that most current species are at the lower spectrum of what we consider complex, i.e. unicellular organisms, so we can hardly argue that all organisms necessarily gravitate towards increasing their complexity.

2. Active trends. Trends due to the biased replacement of primitive forms by more derived ones, which are more complex.

a. Natural selection. Several authors [Rensch 1960, Bonner 1988] argued that more parts will allow a greater division of labor. Since according to these authors this greater division of labor will confer an evolutionary advantage, evolution naturally favors a continuous increase in complexity, measured by the number of components or features. Having more components is favored by natural selection because it allows an internal division of labor so that organisms can adapt each of these components individually, which leads to an increase in the fitness of the organism [Bonner 1988]. However multivariate selection studies, i.e. evolutionary studies in which more than one trait are considered, have shown that the optimization of multiple traits simultaneously can be very limited. Some authors claim that developmental dynamics preclude traits from varying independently, so that not all traits can be optimized at the same time [Oster and Alberch 1982]. Similarly, trade-offs between traits also limit the possible emergence of different trait combinations [Wake and Larson 1987, Shoval et al. 2012]. For example, in insects there is a trade-off between developmental time and reproduction. Fast developing embryos result in smaller adults that reproduce earlier, but due to their smaller size the number of offspring is reduced [Hoffmann 2014]. Moreover, it has been suggested that natural selection, even under the Neo-Darwinian paradigm, is unable to optimize all traits within an organism (Brun-Usan et al. 2014). The more traits an organism has, the more traits will be far from the optimum.

b. Accumulation of heritable variation drives a passive increase in complexity [Wicken 1979, Brooks and Wiley 1988, McShea 2005, Stoltzfus 1999, Lukeš et al. 2011]. The argument here is that from generation to generation neutral, or slightly deleterious mutations accumulate. These mutations result in an accumulative increase in the variation of the different parts of an organism, which will increase the complexity, measured as the number of different parts an organism has. For example

[Fleming and McShea 2013] show that D. melanogaster mutants in the laboratory, under no selection pressure, develop breakdowns in symmetry which increase their complexity. This breakdown in symmetry includes asymmetrical wings and irregularly long legs. Under this argument, natural selection acts more often by eliminating complexity, as irregular wings will often be an impediment for fliying and will probably decrease fitness. In certain cases, these asymmetries will be neutral or even increase fitness, as in the case of fiddler crabs (Uca pugnax) which have one claw bigger than the other; or the snail eating snake (Pareas iwasakii) which have asymmetric jaws.

c. Saunders and Ho [Saunders and Ho 1976] and Katz [Katz 1986] suggested that component additions are more likely than deletions because additions are less likely to disrupt normal function, resulting in an evolutionary increase in complexity, measured by the number of parts. This argument is used both for organisms close to the fitness peak and far from it. In organisms that are near a peak of fitness, every component has been optimized to interact with one another in a specific way, they are optimally organized. By removing a component of the system, the fitness will decrease, since otherwise, it would have been this reduced system the one found at the optimum. Although adding components will most likely also reduce fitness, it will potentially also allow for an increase in fitness, since novelties could find a new higher local fitness peak. A similar argument is presented by [Maynard Smith 1970]. He argues that while duplications or other events that increase the genetic content are common, large losses could easily lead to a loss of a function essential for the organism. In organisms far from a local fitness peak, since additions are expected to increase the set of tools organisms have, it is assumed that these new tools will increase the possibilities of finding a novelty. This line of argumentation has similar flaws as the first argument of this section (Natural selection). It fully accepts the adaptationist program, in which every trait is perfectly adapted by natural selection however, as previously explained, this is rarely the case [Gould and Lewontin 1979, Brun-Usan et al. 2014].

d. Several authors [Waddington 1969, Arthur 1994, Knoll and Bambach 2000, Heim et al. 2017, Doebli et al. 2019] argue that as diversity increases in the evolution of some ecosystems, niches become more complex and offer new ecological spaces that can be occupied. More specifically, [Knoll and Bambach 2000] discuss the existence of several

megatrajectories that explain some of the directional change we see in nature. Megatrajectories consist of evolutionary events that allow new niches to be occupied and these new niches will allow the next megatrajectory to occur. Megatrajectories follow a specific and necessary sequence, since for the latter megatrajectories to appear, the previous ones are necessary. The megatrajectories identified by Knoll and Bambach are: 1. From the origin of life to the last common ancestor. This megatrajectory is characterized by an increase in the efficiency of metabolic processes. 2. Prokaryote diversification. Metabolic diversity increases and primary ecosystem structures are formed. 3. Unicellular eukaryote diversification. Size increases as well as functional diversification. 4. Multicellularity. Open ended size scale, complex food chains, functional integration of cells via development. 5. Invasion of the land. 6. Intelligence. Each megatrajectory is characterized by establishing a new right complexity wall (a new maximum complexity) and a new left complexity wall (the minimal complexity) [2]. Inside the limits of these walls, complexity will increase passively, or in some cases it might be directed by some mechanism. This way, every time a new megatrajectory is established, it pushes the maximum complexity even higher. It is important to keep in mind that establishing new megatrajectories does not mean that the previous ones will disappear, therefore, similarly to the passive mechanism, the total complexity variance will increase.

Despite these arguments defending a trend of increasing complexity, there are also strong arguments defending that increases in complexity are rare when compared to decreases in complexity.

1. Fisher's geometric model. Fisher compares the functioning of a microscope to the fitness of an organism (Fisher´s argument refers to functional complexity). To illustrate the basic idea, if we were to randomly modify a microscope, it is more likely that the microscope will stop working rather than be improved. On the contrary, randomly modifying a simpler object has a higher chance of improving the object. If the microscope is represented as a point in a multidimensional space, where each dimension corresponds to a trait,

[2] For example, prokaryotes are between two complexity or size walls. On the minimal wall, prokaryotes cannot be smaller than 250 nm, since that is the smallest size that still allows to include ribosomes inside the cell. On the maximal wall, if cells become too large, the diffusion of metabolites will not be efficient. In order to increase size, an increase in complexity is needed, which in this example should include some new system to allow for a more efficient transport of metabolites [Heim et al. 2017].

the more independent dimensions of variation the phenotype has, the less likely it will be that a random change affects a trait combination in a way that improves the phenotype. Therefore, Fisher predicts that complexity has a price, the more complex organisms will evolve at a slower pace, since finding beneficial mutations will be increasingly more difficult. Additionally, Fisher also argued that the smaller the change, the more likely it would be to improve the fitness of the phenotype. This reinforced the very extended belief that evolution proceeds by small mutations with small phenotypic effects.

Orr [Orr 2007] further built on Fisher's geometric model. He argued that the price of complexity is even higher than Fisher had predicted, since small mutations have a harder time getting fixed in the population. The reason being that small mutations with small phenotypic effects will barely improve fitness and natural selection will barely select them over other phenotypes, especially in small populations [Crow and Kimura 1970, Kimura 1983, Lande 1976, Lacy 1987].

In summary, the more functions an organism has, the more difficult it is to find a mutation that increases fitness. Additionally, the bigger the phenotypic effect of a mutation, the less likely it will be that it increases fitness, but the smaller the effect of the mutation, the less likely it will be to get fixed in the population. These result in a complexity trap, in which increasing complexity becomes more and more unlikely [Orr 2007].

2. In vitro experiments in teeth have shown that increases in complexity (measured by the number of cusps) are rare. Most individual mutations in teeth cause the teeth to completely stop its development or result in a loss of cusps, with only a few mutations being able to increase the number of cusps [Bei 2009, Charles et al. 2009]. [Harjunmaa et al. 2012] have shown that by modifying single gene products only mild increases in cusps numbers are possible, for example adding 0.5 micrograms/ml of activin A increased the average cusp number by 2.2. By adjusting two gene products, activin A and cyclopamine, the average cusps number was increased by 4.5. However, the biggest increase in cusp number comes from a triple combination of activin A, cyclopamine and EDA, which boost the average cusp number by 7. This kind of highly complex mutants are never found in nature, probably because it is extremely unlikely that all these gene products are mutated in the necessary way in order to increase the number of cusps. These results seem to imply that in dental development, there is a bias against increasing complexity, while decreasing complexity seems to be rather easy.

3. In natural populations variation towards an increase in complexity is rare [Miles and Grigson 2003] and simplification can be quite common [Lahti et al. 2009]. When a trait becomes either neutral or nonfunctional, non-neutral mutations will accumulate and may result in the loss or reduction of the trait. For example, Astyanax mexicanus is a teleost that can be found in water surfaces and in caves. The cave form has completely lost the eyes, i.e. it has lost a function, therefore it is less complex [Lahti et al. 2009].

This argument seems to clash with McShea´s [Fleming and McShea 2013] hypothesis that the accumulation of mutations leads to an increase in phenotypic variation and thus to an increase of complexity. In fact, for Mc Shea, natural selection is acting as a brake against complexity, as this variation will often reduce fitness. The argument here defends that traits which are not under natural selection will lose their functionality, often resulting in the trait itself becoming completely reduced. The main difference between these arguments is that while Mc Shea focusses on morphology, Lahti mainly refers to function. An easy reconciliation point between these arguments is that once the function of a trait is lost, mutations can accumulate and possibly increase variation (and complexity), and only later, the trait might get completely reduced, which would be a reduction on complexity also under Mc Shea´s definition.

## 1.8 MODELING

In this final section, before explaining the methods used for this dissertation, I will briefly introduce mathematical modeling, since all the experiments are done with such an approach.

### 1.8.1 WHAT IS A MODEL

"Science can be understood as the interplay between reality and ideas" [Sharpe 2017]. On one hand, scientists observe reality and describe it, for which they use very diverse tools, such as telescopes to observe the cosmos or microscopes to observe cells and their content. But as information about reality accumulates, ideas are formed about what those observations mean, i.e. why is the apical side of the epithelium expressing that gene? And, how different observations relate to each other, i.e. why inhibiting gene X increases the expression area of gene Y? These ideas are translated into a hypothesis about the workings of a system which needs to be tested. In some cases, testing a hypothesis can be straightforward, but in many other cases, especially as the

hypotheses become more complex, they cannot be tested in a trivial way. To test complex hypotheses may require the experimentation of many factors, for example manipulating genes, biomechanical interactions or tissue geometry. The more factors we want to be part of our hypotheses, the more expensive and time consuming the experiments will be. Even more, when factor interactions (e.g. genes in a network) become highly complex, our minds will not be able to predict the outcomes of the hypothesis, for example a specific gene expression pattern. This poses a challenge when comparing the outcome of different hypotheses with the real outcome. In order to test if our hypotheses are feasible, we use computational models, a worthy tool in the kit of any scientist.

However, a computational model can never truly prove a hypothesis. In fact, it can only disprove them and direct the attention of the researcher to missing parts of the hypothesis that could explain why it fails or is incomplete [Voit 2019]. This can drive the improvement of hypothesis, as the feedback between the reality and the model can uncover how developmental mechanisms work. Therefore, modeling is not only useful to (dis)prove hypotheses, but also to guide their progress and inspire new experiments that deepen our understanding of different complex systems.

Another use for computational models, specifically regarding development, is to study how developmental mechanisms could have evolved and how that could have affected our current developmental mechanisms. For example, by using a model of tooth development [Salazar-Ciudad and Jernvall 2010] Salazar-Ciudad and Jernvall studied the effect on phenotypic variation of two distinct ways of how morphogenetic (changes in tissue shape) and inductive mechanisms (establishment of gene expression patterns) interact. By allowing morphogenetic and inductive mechanisms to act simultaneously, they interact with each other, which can result in big morphological changes when any of these two mechanisms is modified. If they act one after another, first inductive and then morphogenetic mechanisms, modifying any of these generally results in less drastic phenotypic changes. For example, if morphogenetic mechanisms and inductive mechanisms act simultaneously, increasing cell division affects not only the size of the cusps but also their relative positions. While in the case that they act at different times, only the height of the cusps are affected, whereas the cusps' relative positions in the teeth are maintained. These extreme cases of how morphogenetic and inductive mechanisms can interact reveals the importance of understanding developmental mechanisms. These different ways of interacting shed light on how development might evolve. Producing high phenotypic disparity in some cases, which could be advantageous to find novel phenotypes, or less disparate phenotypes which can be optimized more effectively. Similarly, how segmentation could have evolved was studied by using a computational model [Vroomans et al. 2016]. These models show that by knowing and

implementing some basic developmental rules, information about evolutionary dynamics can be uncovered, even if the models are built after specific organs or tissues.

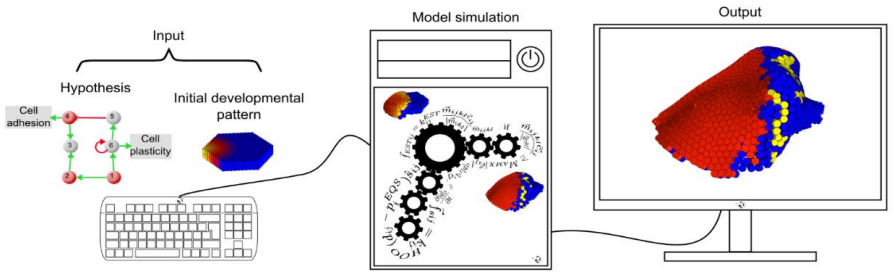



Fig 3. **Computational models receive an input, which is simulated to produce an output**. A crucial part of the input is the hypothesis, which in developmental biology can include, for example, a gene network and the regulation of some cell behaviors. This hypothesis can then be applied to an initial developmental pattern (a specific tissue geometry with some initial gene expression). The mathematical model will then apply the equations it is built on to determine, step by step, how the gene expression patterns and the morphology of the tissue changes over developmental time. As a result of the hypothesis affecting a specific initial pattern by the rules that the model uses, an output is reached.

### 1.8.2 BUILDING A MODEL

Models, at a basic level, work by applying a hypothesis to an input, which turns into an output. For example, given a developmental mechanism that describes how different gene products interact (the hypothesis) and an initial pattern of gene expression (the input), the model will apply the hypothesis to the input, which will result in a final gene expression pattern (the output), see Fig. 3.

When building a model, there are three parts we need to consider:

1. Parameters. They build the main frame of the hypothesis and are fixed, i.e. they do not change during the progression of the simulation. They generally specify the parts of the hypothesis and how they interact, for example the number of genes and their interactions, i.e., they specify the topology of the gene network and the interaction strength between the different gene products.

2. Variables. They define the current state of some changing value in the model, for example gene concentrations or the coordinates of cells. These are different from the parameters, as variables will change during the simulation, while parameters will not. E.g. while the topology of the gene network is not going to change during one simulation, the concentration of the different gene products will change as the simulation progresses. Variables are what models are trying to predict. For example, if a model is trying to predict the morphology of a tooth, it is basically modeling how some initial cell coordinate variables change into different cell coordinates, which should describe a tooth morphology.

3. Equations. A mathematical description of the relationship between the parameters and variables of the model. The equations determine how variables in the input change to result in the output. In general, equations do not change when the hypothesis changes (for example the topology of a gene network), but there are exceptions. For example, we might choose the Gierer-Meinhardt equations to model reaction-diffusion as an activator-inhibitor interaction, which uses the following set of equations:

$$\frac{\partial a}{\partial t} = \rho - \mu_a a + D_a \frac{\partial^2 a}{\partial x^2} + \rho_a$$

$$\frac{\partial h}{\partial t} = \rho a^2 - \mu_h h + D_h \frac{\partial^2 h}{\partial x^2} + \rho_h$$

Or we could use the Gray Scott equations, if we want to model reaction-diffusion as an activator-substrate system, which would use these equations:

$$\frac{\partial u}{\partial t} = r_u \nabla^2 u - uv^2 + f(1-u)$$

$$\frac{\partial v}{\partial t} = r_u \nabla^2 v - uv^2 - f(f+k)v$$

Both sets of equations determine how different gene products will interact, which is a fundamental part of any hypothesis.

To clarify and continue with the example of a reaction-diffusion system, the parameters determine which genes are the activator, the inhibitor or other genes in the gene network and the constants in the equations. The equations establish the rules which determine how the activator and the inhibitor

interact. And the variables represent the amount of activator and inhibitor present at every step of the simulation.

When building a model, the most important part to consider is the hypotheses in question. This generally requires knowledge about the system, not only to be able to consider all the necessary parameters, but also to know which ones can or should be left out of the hypotheses [Brodland 2015]. For example, when modeling the molecular interactions in a specific gene network, if all the known molecules and their interactions are used, it could result in a massive network, and the solution for our specific hypothesis will get lost in the midst of all the extra information. For instance, if a model of a gene network contains hundreds of genes, it will be more difficult to study its dynamics than if we can simplify the gene network to just a few genes. Therefore, if the hypothesis can be built with a reduced number of genes and interactions, the answer to our concrete question will be easier to elucidate. Although this simplification might look like a limitation, it forces the formulation of a specific hypothesis that can be tested and modified if required, thus a certain degree of simplification is desirable. The degree of the simplification will depend on the hypothesis at hand. If there is too much simplification, the behavior of the systems might not be properly captured, if there is too little simplification, it will be difficult to extract conclusions about the systems behavior.

### 1.8.3 MODELING EVOLUTION

Studying evolution is extremely difficult. One reason that makes it difficult is that in general, several generations of a population are needed, which in most cases means that only fast reproducing organisms can be studied in laboratory conditions. This introduces several biases in the dynamics we observe, for example in the size of the organisms, population size, complexity of the genome or phenotype, etc. All of which play an important role in evolutionary dynamics. If only fast reproducing and small organisms are studied, how much can we extrapolate to slow reproducing and big organisms? Also, there is the problem of what to select for, which can greatly affect the success, tempo and rate of phenotypic change. There might also be the problem that while artificially selecting for some feature there is some underlying selection for something else, for example, rare male advantage, in which rare males have a higher mating success (Partrigde and Halliday 1984, Barry and Kokko 2010) which could obscure some of the results. In nature it is even more complicated since the lack of control over reproduction or even of what features are being selected make it very difficult to obtain relevant results. Some studies exist that study evolution in a laboratory (Lenski et al.

1991) and even in nature (Grant and Grant 2006), but generally these experiments are still subject to the above-mentioned biases.

However, evolution of relatively complex systems can be computationally modelled. Multiple generations can be modelled quickly, they can be repeated and manipulated as necessary to study different dynamics, for example setting different optima, changing the mutation rate or selection strength. Computational models can help us understand evolutionary dynamics such as speciation, natural selection and coevolution.

### 1.8.4 EXPLOTING PHENOTYPIC VARIATION WITH MODELS

Modeling can also be used to study the statistical properties of different systems. This is done by building huge sets of randomly built systems, e.g. random networks, which then are simulated in a computational model so that some generic properties of that system can be extracted. This can be especially powerful when building similar systems but using some different rules of construction, allowing to compare if different properties appear, or which common properties are kept [Kauffman 1969, Salazar-Ciudad and Jernvall 2004]. This kind of approach is called an ensemble approach, used for example by Kauffman, when he studied the differences between continuous and discrete models. Ensemble approaches are a powerful tool to explore the general parameter space [Kauffman 1969], by allowing an overview of how particular ways of building developmental mechanisms affect the phenotypes that develop.

In addition to the ensemble approach, which can be used to explore the general parameter space, it can be useful to explore the local parameter space of different systems. These local explorations focus on the parameter space close to specific system configurations, e.g. a developmental mechanism. These kinds of explorations can tell us something about the variation that different developmental mechanisms can produce. For example, it has been found that developmental mechanisms which include reciprocal interactions between diffusible gene products are more prone to be affected by mutations than developmental mechanisms without these reciprocal interactions [Salazar-Ciudad et al. 2001]. By studying how different developmental mechanisms are affected by changes in their parameter values, e.g. how changes in the genotype affect the phenotype, allows us to learn something about how they could evolve. Under the Neo-Darwinian paradigm this approach would not make sense. It would not make sense because Neo-Darwinism explicitly assumes that natural selection is the only force that needs to be considered in order to understand evolution. Therefore, it implicitly assumes that variation is limitless, and that selection can optimize any phenotype. This would make the study of phenotypic variation possible by

different developmental mechanisms irrelevant. However, if we accept that different developmental mechanisms have different variational properties, i.e. the kind of phenotypes they can produce, we can consider which phenotypes would be more likely to be encountered by natural selection and therefore influence how evolution proceeds. Although to get the whole picture of how evolution proceeds both natural selection and variational properties should be considered, examining only the later has some benefits. For instance, when modeling evolution, individuals in a population all have relatively similar genotypes. This means that a lot of time is spent in modeling individuals which are very close in the parameter space or that are exactly the same. With the general and local explorations, very diverse developmental mechanisms can be explored in the same amount of time than a single evolutionary simulation.

# 2 METHODS

In this section I will describe the basic methods used in this work. For a detailed description and information about the statistical test used (Kolmogorov-Smirnov, permutation test, linear regression, etc.) see the corresponding study.

## 2.1 COMPUTATIONAL MODEL: EMBRYOMAKER

### 2.1.1 INTRODUCTION

The development model I used in this thesis was EmbryoMaker [Marin-Riera et al. 2016]. EmbryoMaker is a programming framework, designed to construct mathematical models of pattern formation and morphogenesis in animal development. For this project EmbryoMaker is particularly well suited, since it is not a model that is restricted to a single developmental process. Instead, EmbryoMaker specifies generic equations which describe how gene products and cells interact, biochemically and biomechanically. Importantly, EmbryoMaker considers the different biomechanical properties that exist between epithelial cells, extracellular matrix and mesenchymal cells, which are crucial to understand developmental processes. It also includes the basic animal cell behaviors, such as cell division and cell growth, cell adhesion, cell death, cell migration, polarization, ECM secretion, cell signaling and epithelial to mesenchymal transition (see Fig. 4).

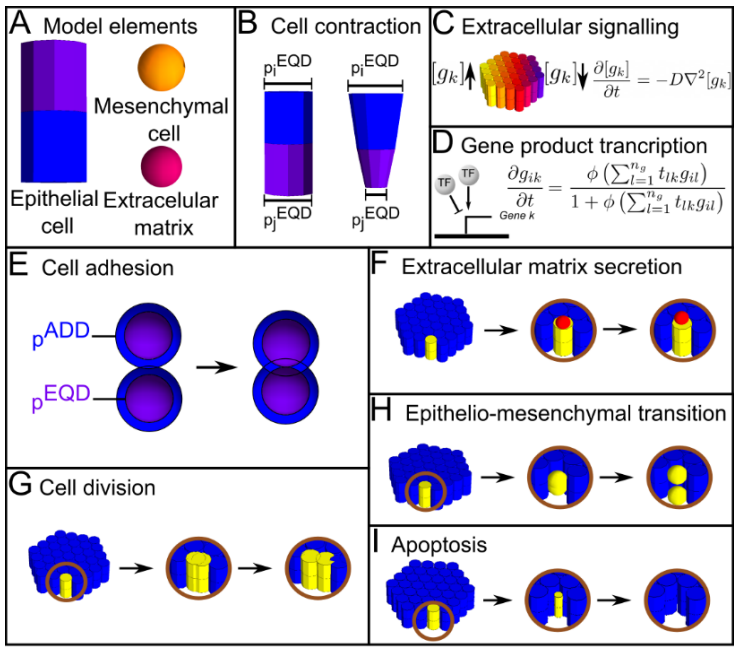



Fig 4. **EmbryoMaker**. **(A)** EmbryoMaker includes three types of elements or nodes. Epithelial cell cylinders are made of two nodes, the basal one in blue and the apical one in violet. Mesenchymal cells and extracellular matrix elements are made of single spherical nodes. **(B)** Cell contraction. Parts of a cell change their size by decreasing the equilibrium radius. As a result, epithelial cells may change from a cylinder shape to a cone shape. **(C)** Extracellular signaling. Diffusion is implemented as transfers of molecules between nodes using Fick's second law of diffusion, where $q_i$ is the concentration of gene product i in a node. **(D)** Gene product transcriptional regulation determines the rate of change in the expression of a gene in a cell over time. $g_{il}$ is the amount of transcriptional factor l in node i and each $t_{lk}$ term is the strength by which each specific transcriptional factor k activates or inhibits the transcription of gene l. **(E-J)** Cell behaviors implemented in EmbryoMaker. **(E)** Cell adhesion. Two cells that are in contact, i.e., their radius of adhesion overlap ($p^{ADD}$, blue sphere), will come closer to each other until their equilibrium distance is reached ($p^{EQD}$, purple sphere). **(F)** Extracellular matrix secretion. Any cell type can secrete extracellular matrix, given that there is expression of a molecule regulating this secretion. **(G)** Cell division. **(H)** Epithelial-mesenchymal transition. **(I)** Planar cell polarity. On the left, the cell marked in yellow produces an extracellular signal that diffuses to produce a gradient, as seen on the right. Polarization is in the direction (light blue lines) of the gradient (towards the steepest decreases in concentration). **(J)** Apoptosis. When a cell undergoes apoptosis, its size will decrease until it reaches a minimal value, at which point it is eliminated.

The network of interactions between gene products and the regulation of cell behaviors by the network is not fixed, any imaginable network topology can be built, and these networks can regulate any of the mentioned cell behaviors. Thus, while EmbryoMaker provides the generic equations, each system-specific model has to provide the values of the parameters in such equations and the number of such equations (since there are several equations per gene product, cell mechanical properties and behaviors).

EmbryoMaker requires two inputs (Fig. 3). First, it requires a developmental mechanism, which encompasses the network of gene products interactions and the regulation of cell behaviors by these gene products. The second input is a specification of the initial developmental pattern. A developmental pattern includes the spatial coordinates of the cells, i.e., the morphology of the starting tissue or organ, and the gene expression pattern in these. EmbryoMaker, by using the in-built equations, which describe how gene products and cells interact with one another in these two inputs, simulates the whole developmental process which transforms the initial developmental pattern into the final developmental pattern, which is the output that EmbryoMaker provides, generally a 3D morphology with some gene expression patterns. When studying how some organ develops, the developmental mechanism is the hypothesis that the model will test, which transforms a given input in an output.

As most models, EmbryoMaker includes variables and parameters. The parameters include the developmental mechanisms, i.e., the number of genes, which genes interact, how they interact (strength, activating or inhibiting), the cell behaviors that each gene regulates and other model specific values which are fixed, such as temperature, noise, etc. The variables include the concentration of each gene, which will presumably change from the initial values provided by the initial developmental pattern; the location and number of the cells which will also change as cells divide and move during morphogenesis; and the biomechanical properties of the cells, which can be regulated by gene products.

### 2.1.2 BASIC ELEMENTS IN EMBRYOMAKER

EmbryoMaker includes three types of elements: epithelial cells, mesenchymal cells and extracellular matrix. Each of these is made of one or more sub-elements called nodes and have their own biomechanical properties.

Epithelial cells are made of cylinders, and each cylinder includes two types of nodes, an apical node and a basal node. The basal node and the apical node of each cylinder are connected by an elastic link that keeps them together while allowing the nodes to modify the distance between each other if force is applied. Mesenchymal cells and extracellular matrix are made of spherical

elements, each of them composed by a single node. EmbryoMaker allows to build cells made of multiple elements (multiple cylinders per epithelial cells and multiple spherical elements for mesenchymal cells). This feature is very useful when the shape of the cells needs to be considered in detail, however, it does increase the running time of the simulations. Since this work requires a huge amount of simulations, cells are modelled using individual elements in order to decrease the time each simulation needs.

### 2.1.3 BIOMECHANICS

Nodes in EmbryoMaker move following an overdamped Langevin equation of motion, which means that nodes are not modelled as being in a vacuum, they rather move as in a viscous environment.

$$\frac{\partial \vec{r_i}}{\partial t} = \sum_{j=1}^{n_v} f_{A_{ij}} \hat{u}_{ij} \qquad (1)$$

Where $r_i$ is the position of node $i$ in three-dimensional space, $n_v$ is the number of nodes that are close enough to node $i$ to mechanically interact with it, $t$ is time, $f_{Aij}$ is the modulus of the force acting between node $i$ and $j$ and $u_{ij}$ is the unit vector connecting node $i$ and node $j$. The modulus and sign of the force is dependent on the distance between the two nodes:

$$\begin{cases} f_{A_{ij}} = \left(p_i^{REC} + p_j^{REC}\right)\left(d_{ij} - \left(p_i^{EQD} + p_j^{EQD}\right)\right) \;\; if \;\; d_{ij} < \left(p_i^{EQD} + p_j^{EQD}\right) \\ f_{A_{ij}} = k_{ij}^{ADH}\left(d_{ij} - \left(p_i^{EQD} + p_j^{EQD}\right)\right) \;\; if \;\; \left(p_i^{EQD} + p_j^{EQD} \le d_{ij} \le p_i^{ADD} + p_j^{ADD}\right) \\ f_{A_{ij}} = 0 \;\; if \;\; \left(p_i^{ADD} + p_j^{ADD}\right) > d_{ij} \end{cases} \qquad (2)$$

When the distance between nodes $i$ and $j$ ($d_{ij}$) is shorter than the sum of their radii at equilibrium (node property $p^{EQD}$), there is a repulsive force proportional to the sum of the node property $p^{REC}$ of each node (this coefficient determines their incompressibility). When this distance is longer than the equilibrium distance but shorter than the sum of the maximum radii of $i$ and $j$ (node property $p^{ADD}$), there is an attractive force between nodes i and j. This force is proportional to the amount of adhesion molecules expressed in the nodes. The direction of force vectors differs between mesenchymal-mesenchymal, epithelial-epithelial and the epithelial-mesenchymal node interactions, since vectors need to be normal to the contact interface between nodes and nodes have different shapes in epithelial cells and mesenchymal cells, see [Marin-Riera et al. 2016] for a detailed explanation.

Epithelial cells are subject to three additional forces. There is an elastic spring that connects the apical and basal nodes of the same cylinder, which opposes departures from the equilibrium distance between the nodes. The other two forces ensure that the epithelial cells are organized in sheets. A radial force ($f_{EST_{ij}}$) acts along the apical-basal axis of the cell and tends to restore displacements in that axis in respect to neighboring cells in the epithelium, whereas a rotational force ($f_{ERP_{ij}}$) acts tangentially to the surface of the epithelium and tends to orient the apical-basal axis of cells normal to the epithelial plane. See (Marin-Riera for details).

$$\frac{\partial \vec{r}_i}{\partial t} = \vec{f}_{s_{ik}} + \sum_{j=1}^{n_d} (f_{A_{ij}} \hat{u}_{ij} + \vec{f}_{EST_{ij}} + \vec{f}_{ERP_{ij}}) \qquad (3)$$

Nodes move as result of the combination of all these forces, but additionally there is some movement due to noise. At each time step, a proportion $M_{NOI}$ (a global model parameter) of the nodes are chosen at random and are tentatively moved in a random direction for a random distance between 0 and $p^{DMO}{}_i$, a mechanical property of each node. For each node the potential mechanical energy is calculated, by integrating the same force equation 1, in the new position. If the potential energy in the new position is smaller than in the old position the movement is accepted. If not, the movement is accepted with a probability proportional to the difference in potential energy between the new and old positions and inversely proportionally to a temperature parameter, model parameter $M_{TEM}$, plus a node property defining the node's propensity to movement ($p^{MOV}$ ). If the movement is not accepted the node is put back to its old position. This energy biased noise reflects the fact that noise can affect node positions, but it is unlikely to bring nodes into very energetically unfavorable positions (*e.g.* noise is very unlikely to bring a node from a cell inside another cell). This is a standard way to implement noise in many physical and biological systems, such as in the Pott's model [Graner and Glazier, 1992].

### 2.1.4 GENE EXPRESSION AND GENE NETWORKS

EmbryoMaker considers gene products but also other kinds of molecules that are not transcribed. In this work, for simplicity, we consider only gene products and only transcriptional regulation. Each gene product has a set of properties associated with it (genetic parameters). These include its intrinsic degradation rate ($\mu_i$) and how they affect transcription, node properties and

cell behaviors. The rate of change in the concentration of gene *k* in node *i* over time is:

$$\frac{\partial g_{ik}}{\partial t} = \frac{\Phi[\sum_{l=1}^{n_g} t_{lk} g_{il}]}{1 + \Phi[\sum_{l=1}^{n_g} t_{lk} g_{il}]} - \mu_k g_{ik} \qquad (4)$$

Where $\mu_k$ is the intrinsic rate of degradation of molecule *k*, $g_{il}$ is the amount of transcriptional factor *l* in node *i* and each $t_{lk}$ term is the strength by which each specific transcriptional factor *k* activates (positive $t_{lk}$) or inhibits (negative $t_{lk}$) the transcription of gene *l*. The sum is done through all the regulatory molecules and by definition only transcriptional factors have $t_{lk}$ terms different from zero. Φ is a function that is equal to 0 for values of x smaller than 0 and equals to x when x is greater than 0 ($\Phi(x) = 0 \ \ if \ x < 0$ and $\Phi(x) = x \ \ if \ \ x > 0$). This function is used to ensure that there is not such a thing as negative transcription (although $t_{lk}$ can be negative and thus repress transcription). Each $t_{lk}$ is a model parameter, the set of all the possible $t_{lk}$ is what we call the T matrix, a $n_g$ x $n_g$ matrix where $n_g$ is the number of gene products in a model. Matrix *T* defines a model's gene network.

Equation 4 represents the binding of several transcriptional factors to the promoter of gene *k*. This is a saturating process that, for simplicity, is represented by a Hill equation of order 1. This means that when there are few activator factors the rate of transcription increases with the amount of these factors. But when there are many of these factors the rate of transcription does not increase as much with the amount of activator factors since the binding sites are likely to be already occupied. Equation 4 also implies that the maximal rate of transcription of a gene is 1. The same [Salazar-Ciudad et al. 2000] or similar [Reinitz and Sharp 1995] equation has been used in previous models of gene networks in development.

### 2.1.5 CELL-CELL SIGNALING

In EmbryoMaker some gene products can diffuse between cells. EmbryoMaker includes ligand-receptor dynamics, but in this work, I consider the simplest case in which diffusible gene products can affect transcription directly, so each signal transduction pathway transmits a signal in a lineal way without amplification. Diffusion is implemented as transfers of molecules between nodes (including ECM nodes). This transport follows Fick's second law of diffusion:

$$\frac{\partial q}{\partial t} = -D\nabla^2 q \quad (5)$$

Where $q$ is concentration of a molecule, $D$ is the diffusion coefficient of that molecule and $\nabla^2 q$ is the second derivative of the concentration in 3D space. We calculate transfers of matter between pairs of nodes. Since we only make calculations in the nodes, diffusion is essentially discrete (although non-uniformly) and this equation is roughly approximated by:

$$d_{ik} = D_k \sum_{j=1}^{n_v} \left( \frac{g_{ik} - g_{jk}}{d_{ij}} \right) \quad (6)$$

Where $g_{ik}$ is the amount of molecule $k$ in node $i$, $t$ is time, $D_k$ is the diffusion coefficient of molecule $k$, $n_v$ is the number of nodes within the maximum radius of diffusion from node $i$ and $d_{ij}$ is the distance between node $i$ and $j$. Both this distance and $n_v$ depend on how nodes are arranged in space. The maximum radius of diffusion is two times the maximal $p^{ADD}$. This latter choice ensures an optimal accuracy even if there are changes in the sizes of the nodes in the embryo over time, see [Marin-Riera et al. 2016] for details. The change in concentration of diffusible molecule $k$ in node $i$ over time is then:

$$\frac{\partial g_{ik}}{\partial t} = \frac{\Phi\left[\sum_{l=1}^{n_g} t_{lk} g_{il}\right]}{1 + \Phi\left[\sum_{l=1}^{n_g} t_{lk} g_{il}\right]} - \mu_k g_{ik} + d_{ik} \quad (7)$$

### 2.1.6 CELL BEHAVIORS

EmbryoMaker includes several animal cell behaviors which can be regulated genetically. The cell behaviors included in EmbryoMaker are the ones considered as the most basic ones that can be found in animals. These cell behaviors are division and cell growth, cell adhesion, cell death, cell migration, polarization, ECM secretion, cell signaling and epithelial to mesenchymal transition. See Fig. 4 for a depiction of how they work in EmbryoMaker.

## 2.2 ENSEMBLES

Using the EmbryoMaker, several ensembles were built. An ensemble in this work is defined as a set of developmental mechanisms built using the same rules, which are unique for each ensemble. Although some rules do overlap between ensembles. By rules I mean for example, the number of genes per developmental mechanism or which cell behaviors will be considered when building the developmental mechanisms. All the analysis and results obtained in this work are based on these ensembles, they provide a window into a hypothetical realm in which I know every organism and their development. This allows me to study how a myriad of developmental mechanisms produce different phenotypes and look for commonalities and differences between the developmental mechanisms and between the ensembles. In this hypothetical realm, all developmental mechanisms are built randomly, which is not necessarily the case in real ones, which are the result of millions of years of evolution. But by studying random networks instead of current realized networks, allows to explore general and unbiased principles of developmental mechanisms.

Some of the rules between the ensembles are shared:

1. When building a random developmental mechanism, they always include exactly 10 genes. The only exception for this rule is the Autonomous biomechanics-only ensemble, see below.

2. The initial developmental pattern is always a flat epithelium with a single layer of mesenchyme on the basal side. However, the gene expression patterns will be different between some of the ensembles.

3. The topology of the gene network is built at random, with 20% chance of a gene regulating any of the other genes. A gene can activate or repress other genes with equal probability.

4. All genes need to be part of the network, i.e., genes cannot be detached from the network.

5. Genes can be extracellularly diffusible or intracellular with equal probability.

The basic properties of the ensembles can be found in table 2 and in Fig. 5. The different ensembles are:

1. Broad ensemble. The developmental mechanisms used in this ensemble are completely random, as described in the previous section. One gene is initially expressed in one cell in the center of the epithelium. This naive approach failed to find developmental mechanisms able to produce morphogenesis, or even non-trivial gene expression patterns in most of the 100,000 random developmental mechanisms explored.

2. Signaling only ensemble. In this ensemble, cells were not allowed to move, grow or divide, but otherwise used the same strategy to make developmental mechanisms described before, although with no cell behavior being regulated. With this ensemble we were able to identify 20,000 developmental mechanisms able to lead to changes in gene expression over space in a temporally stable fashion.

3. Signaling ensemble. This ensemble was constructed using the developmental mechanisms identified in the signaling only ensemble. However, random genes from these developmental mechanisms are forced to regulate some random cell behavior or node property. Additionally, the gene initially expressed, instead of being only in the central cell, is expressed in a gradient in the initial developmental pattern.

4. Planar cell polarity (PCP) ensemble. This ensemble is exactly as the signaling ensemble but includes nine cells at the margin of the epithelium that constitutively secrete an extracellular signal. This signal diffuses over the embryo producing a concentration gradient. Each cell's polarization vector points in the direction where the signal concentration decreases faster as shown in Fig. 5E. The polarization vector of each cell biases the direction of cell division and cell movement. This ensemble was built to explore the effect of PCP on morphological complexity and developmental instability.

The next ensembles were built to explore the morphological complexity and developmental instability that is possible without cell signaling.

5. Autonomous ensemble. In this ensemble there is no extracellular signaling and one gene is homogeneously expressed through all cells in the initial developmental pattern

whereas the rest are not expressed. Gene expression, thus, does not change in space but can change over time as a result of the model dynamics. Even if gene expression is homogeneous the biomechanical interactions between cells can lead to morphogenesis and symmetry breaks, which are induced by noise and boundary conditions (see results). This ensemble explores the morphogenesis that is possible from the uniform regulation of cell behaviors.

6. Autonomous biomechanics-only ensemble. This ensemble is like the autonomous ensemble but without a regulatory gene network. Thus, gene expression does not change in space or in time but, as in the previous ensemble, morphogenesis can occur. Developmental mechanisms include only three genes: one is expressed in the apical part of cells, one in the basal side and one in both. These genes activate the cell behaviors that were regulated by the original developmental mechanism in the signaling ensemble and with the same intensity. This ensemble explores the morphogenesis that is possible from the uniform regulation of cell behaviors with no changes in gene expression over time.

7. Gradient autonomous ensemble. This ensemble is just like the autonomous ensemble but only one gene is initially expressed, and it is expressed in a gradient over the epithelium. This ensemble explores the morphogenesis that is possible from the gradient regulation of cell behaviors.

Table 2. **Basic properties of the ensembles.**

| | **Properties** | | | | | |
|---|---|---|---|---|---|---|
| **Ensemble** | Gene network | Cell signaling | Initial condition gradient | Initial condition homogeneous | Polarization | Cell behaviors |
| Signaling only | ✓ | ✓ | ✓ | ✗ | ✗ | ✗ |
| Signaling | ✓ | ✓ | ✓ | ✗ | ✗ | ✓ |
| Signaling with polarization | ✓ | ✓ | ✓ | ✗ | ✓ | ✓ |
| Autonomous | ✓ | ✗ | ✗ | ✓ | ✗ | ✓ |
| Autonomous biomechanics-only | ✗ | ✗ | ✗ | ✓ | ✗ | ✓ |
| Gradient autonomous | ✓ | ✗ | ✓ | ✗ | ✗ | ✓ |

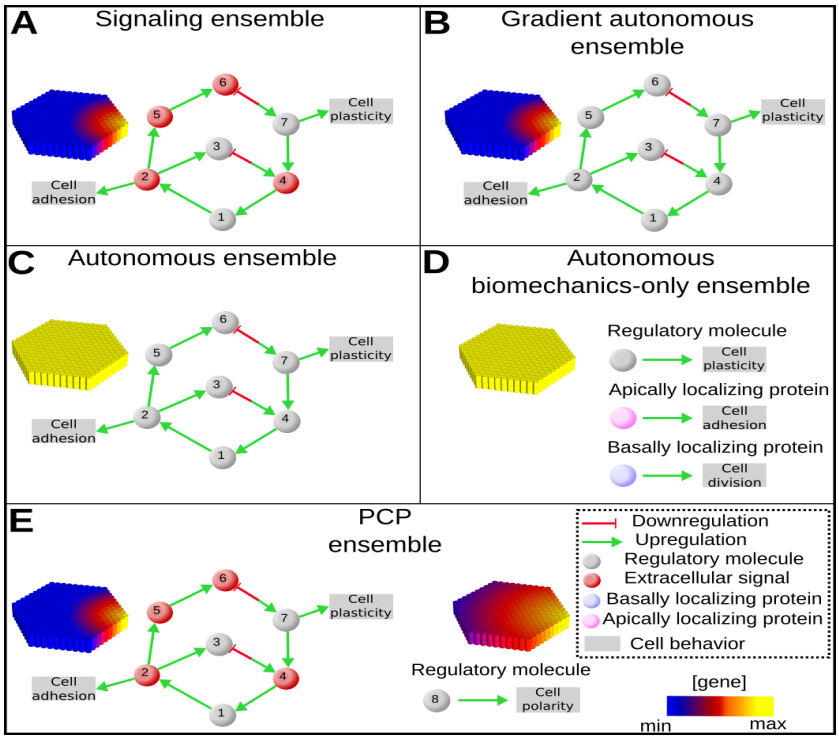



**Fig 5.** The initial developmental pattern of each ensemble is shown in the upper left side for each ensemble. In color we show the level of expression of the gene expressed in the initial developmental pattern (yellow is the maximal and blue is the minimal expression). Next to the initial conditions we depict idealized examples of developmental mechanisms for each ensemble. Notice that in A there are extracellular signals while in the autonomous ensembles (B, C and D) there are none. Also, notice that in C and D simulations start from a homogeneous gene expression pattern. In E there is a legend explaining each of the components of the network.

## 2.3 STUDY OF VARIATIONAL PROPERTIES

One of the most important conditions for evolution to occur is the existence of phenotypic variation. In the case of morphology, the way in which genotypic variation relates to phenotypic variation is through development or developmental mechanisms. By studying the variation that different developmental mechanisms can produce, we can learn what natural selection can select from and understand better some evolutionary dynamics. For

example, some developmental mechanisms might be more prone to produce huge phenotypic changes even under the slightest genotypic alterations, while other phenotypes will change proportionally to the changes in the genotype. All the possible phenotypes possible from altering a developmental mechanism (without changing the topology of the network) are included in the variational properties of that developmental mechanism [Salazar 2006].

In the following sections I will describe the methods used in this work to study the variational properties of different ensembles of developmental mechanisms. These methods were only applied to the signaling ensemble and only after pruning all the superfluous interactions of the developmental mechanisms. Superfluous interactions are those whose deletion does not affect the phenotype in any significant way, i.e., after being deleted, the resulting morphology remains the same (for details on this see study II).

### 2.3.1 ONE MUTANT NEIGHBORHOOD

With these methods the immediate genotypic surroundings of each developmental mechanism are studied, which allows us to study the phenotypic variation most likely to happen in each moment. A detailed description of these methods can be found in study II.

#### *2.3.1.1 Interaction strengh mutations*

In this screening I explore the morphological variation that arises from making single mutations in developmental mechanisms, i.e. by changing one parameter at a time. Each element or parameter in a developmental mechanism (gene interactions and cell behaviors) is mutated eight times, while keeping the other elements in the developmental mechanism the same, i.e., with the same values the developmental mechanism had before making any mutation. The developmental mechanism that has not been subjected to mutations we define as a parental. Each mutation affects a parameter proportionally to the value of that parameter in the parental. Each parameter is mutated eight times in this way in the following proportions: -80%, -60%, -40%, -20%, +20%, +40%, +60% and +80%.

#### *2.3.1.2 Topological mutations*

The one-mutant neighborhood of topological mutations explores the phenotypic variation of the neighbor developmental mechanisms. Although the idea is similar than with the IS-mutations, as the topology changes, the developmental mechanisms also changes (by definition). It has also been

hypothesized that mutations that change the topology of the network are in general less likely to occur or can have different effects on the variational properties [Salazar-Ciudad 2006, Coterel and Sharpe 2010, Payne et al. 2014]. Topological mutations were applied in two ways, by deleting and by adding a gene interaction.

*One-mutant deletion neighborhood*. As with the IS-mutations but eliminating one mutation at a time.

*One-mutant addition neighborhood*. As for the one-mutant deletion neighborhood but adding a random interaction per mutant, either between gene products or between a gene and a cell behavior or cell mechanical property. For each developmental mechanism we generated Nc mutant offspring, where Nc is the number of interactions in the parent developmental mechanism. This way we ensured that roughly the same number of deletions and additions where essayed for each developmental mechanism (i.e. the number of possible deletions per developmental mechanisms is the number of interactions it has).

### 2.3.2 ISO-MORPHOLOGICAL RANDOM WALKS

We use this approach to estimate the size of the region of the parameter space in which a specific morphology can be develop. Note that each developmental mechanism can have a different number of parameters, which results in the parameter space of different developmental mechanisms to differ in their dimensionality. Despite this we devise a way to estimate these sizes in a comparable way.

For one parameter at a time I performed an IS-mutation, which changed the parameter value by +/-200% of the parental original value, each of these mutations is one step in the walk. This mutation is accepted if it leads to no morphological change and rejected if it did. If the mutation is rejected, this mutation is reversed, and a new mutation is applied. If the mutation is accepted, the next mutation will be applied on the mutated developmental mechanism, i.e. mutations will accumulate if they do not change the morphology.

The size of the region leading to a morphology was then calculated as the proportion of mutations in a random walk that did not change the morphology. In other words, the larger the proportion of mutations that change a phenotype, the smaller is the region of the parameter space in which a morphology forms.

### 2.3.3 GENOTYPE-PHENOTYPE MAP (GPM)

To build GPM we use the IS one-mutant neighborhood screening. In this screening, each parameter was mutated eight times, proportionally to its value in the parent (from -80% to +80% in 20% steps). For each of these mutants, the morphological distance to the other mutants was measured. This way, we have a genotypic distance between the mutants (the percentage of mutation) and a phenotypic distance (the distance between the mutant morphologies). With these we built the GPM regression plots, by putting the genetic distance in the x-axis and the phenotypic distance in the y-axis. Notice that each parameter from each developmental mechanism will have its own GPM regression plot, so that each parental developmental mechanism will have one of these plots for each parameter.

With this method, when small genetic changes lead to big changes in the phenotype, the regression coefficient will be high, and we consider the GPM to be complex. Notice also that morphologies with higher developmental instability do not necessarily have higher regression coefficients, as we can see in Fig. 6B and 6C. Even though C has a higher developmental instability than B, as the morphological distances between the different mutants does not change with their genetic distance, they both have small regression coefficients. The regression coefficient is, thus, a measure of the GPM that is not affected by developmental instability. Having such a property is important because complex morphologies in the ensemble are more developmentally unstable than simple morphologies.

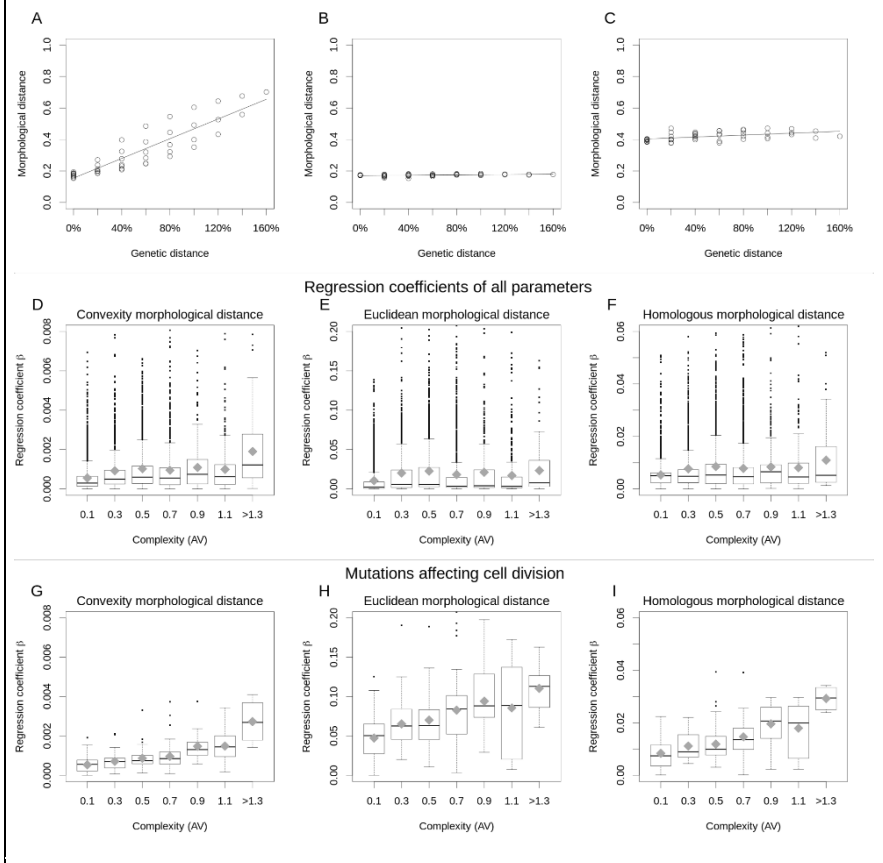



Fig. 6. **Genotype Phenotype Map. (A-C)** example of "GPM regression plots". The X-axis shows the "genetic distance" between each pair of mutant offspring of a given parent. Each offspring diverges from its parent in only one parameter and at different proportions of that parameter (from -80% to +80% in 20% intervals). The "genetic distance" is the change in a parameter value (in proportion to those in the parent) between each pair of mutant offspring. In the 0% position in the x-axis we have all the twins of the mutants and the parent (i.e. their developmental instability). In the 20% position we compare the mutants that are at a 20% genetic distance from each other, i.e. the mutants that are at a 40% distance to the parent versus the ones that are at a 20% distance to the parent, the ones that are at 40% versus those that are at 60%. Each point in the Y axis shows the average morphological distance between all the offspring with a parameter value and all the offspring with another parameter value at a specific genetic distance. The morphologies in **(C)** have a higher developmental instability than the morphologies on **(B)**, but since the morphological distances between the different phenotypes in each of these plots do not change, they both

have very small regression coefficients. Therefore, the regression coefficient is a measure of the GPM that is not affected by developmental instability. Having such a property is important because complex morphologies in the ensemble are more developmentally unstable than simple morphologies. **(D-F)** Plots of the GPM regressions coefficients, such as that in **(A-C)**, for each parent and parameter against the parental complexity. In other words, the slopes of the GPM plots are plotted against the complexity of the parent. In these plots there would be one point per parent and parameter, but they are binned into boxes of 0.2 AV complexity intervals. Boxes enclose 50% of the regressions per parent interval (i.e. for all the parameters of all the parents in an interval). The line in the box shows the median and the gray diamond the average for that interval. The whiskers extend 1.5 times the interquartile range of the box. **(G-I)** as in **(D-F)** but considering only the mutants that directly affect the cell phase cell property PPHA, and therefore affect cell division rates. We pinpoint the plots for this parameter because it is one of the parameters with the clearest effect on complexity. Spearman correlations. **(D)** rs=0.2635, pval<0.001, n=7009. **(E)** rs =0.1585, pval<0.001, n=7009. **(F)** rs =0.0781, pval<0.001, n=7009. (G) rs =0.5307, pval<0.001, n=691. **(H)** rs =0.4315, pval<0.001, n=691. (G) rs =0.5429, pval<0.001, n=691.

## 2.4 COMPLEXITY MEASURES

Our measures of complexity are related to predictability, i.e. how likely is it to predict the position of an epithelial cell knowing the position of its neighbor´s cells. For example, in a flat epithelium, the position of a cell can be easily predicted by its closest neighbors because it will have the same position in the z-axis. In a highly folded epithelium, this would be very difficult, unless the epithelium happens to fold regularly, for example following a sinusoidal wave. Thus, very complex morphologies are folded irregularly. We used two different measurements of complexity: angle distance variance and orientation patch count.

### 2.4.1 ANGLE-DISTANCE VARIATION (AV)

This measure is based on the variation of angles between epithelial cells. For details on how to calculate this complexity see Fig. 7. With this complexity measure, epitheliums with different morphological features will be considered complex, while a perfect sphere or flat epithelium will have zero complexity. For examples of how this measure captures morphological complexity see Fig. 7D.

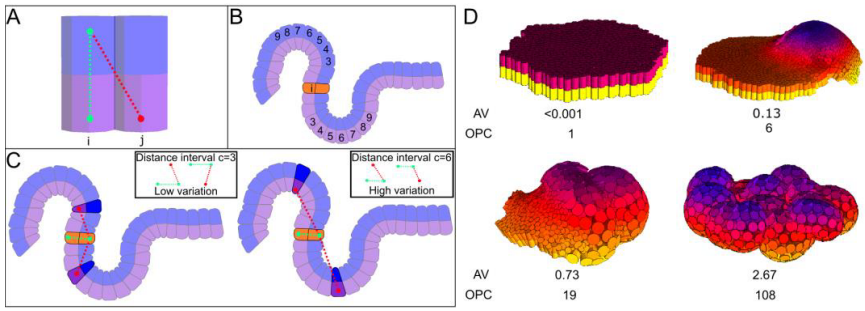



Fig. 7. **Angle variation (AV): method to measure the complexity of an epithelium.** It is based on the variation of angles between epithelial cells. **(A)** The angle between two cells $i$ and $j$ is calculated as the angle between two vectors, the vector between the apical and basal node of epithelial cell $i$ (green dotted lines) and the vector between the basal node of cell $j$ and the apical node of cell $i$ (red dotted line). **(B)** For each cell $i$ we calculate the angles to all other epithelial cells $j$ ( $j \in \{1, \dots, i-1, \dots, i+1, \dots, n\}$ where $n$ is the total number of epithelial cells). Each cell $j$ is grouped into a distance category based on its distance to cell $i$ (there are six of these distance categories). Each category includes the nodes at a given distance interval to $i$, defined as follows

$$D_c = \{c \times p^{\overline{ADD}}, (c+1) + p^{\overline{ADD}}\}; c \in \{3, \dots, 9\}$$

Where $D_c$ is the distance interval in which cell $j$ must fall in order to be included in the category $c$. $c$ defines the maximal and the minimal distance for each interval. $p^{\overline{ADD}}$ is the average distance of adhesion of all epithelial cells in the whole embryo. This is a measure of the average cell size. The minimal $c$ we use is 3. The largest value of $c$ is 9. This value allows us to consider the macro-structure of the embryo (i.e. large-scale morphological complexity). **(C)** We calculate the variance of each of the categories and add them together. All the steps are repeated for each cell in a morphology. The final angle variation complexity (AV) is:

$$AV = \frac{\sum_{i=1}^{n} \sum_{c=3}^{9} \quad V_{ic}}{7n}$$

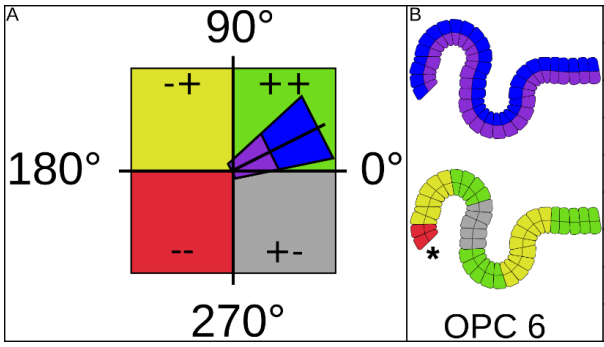
### 2.4.2 ORIENTATION PATCH COUNT (OPC)

Orientation patch count (OPC) is based on the number of differently oriented slope patches an epithelium has. This measure is a fully 3D version of

a measurement of tooth complexity that has been found to correlate with diet (Evans et al. 2007).

For this method, we first assigned each epithelial cell to one of eight categories. Each category corresponds to one octant (one of the eight divisions of a Euclidean 3D coordinate system defined by the signs of the coordinates, see Fig. 8A and right table). To determine in which octant the basal node is, we simply checked the sign of each of the dimensions of the vector from the apical to the basal node of each epithelial cell.

Each cell was then further classified as belonging to a specific patch. A patch is a set of cells belonging to the same orientation category (of the eight possible) and globally connected to each other. This means that one can go from any cell in a patch to any other cell in the patch through a sequence of contiguous cells belonging to the same orientation category (see Fig. 8B). By contiguous cells we mean cells that are in contact. To avoid that small irregularities in the epithelial cells increase complexity, we considered only patches with more than three cells. Finally, we simply counted the number of patches in a morphology, which gave us the OPC value.




| Octal | x | y | z |
|---|---|---|---|
| 1 | - | - | - |
| 2 | + | - | - |
| 3 | - | + | - |
| 4 | + | + | - |
| 5 | - | - | + |
| 6 | + | - | + |
| 7 | - | + | + |
| 8 | + | + | + |

Fig. 8. **Orientation patch count (OPC): method to measure the complexity of an epithelium.** This measure is based on the number of differently oriented patches an epithelium has. This measure is a full 3D version of a measure of tooth morphological complexity that has been found to correlate with diet [Evans et al. 2007]. In order to calculate the OPC we first group each epithelial cell in one of eight categories. Each category corresponds to one of the eight divisions of a Euclidean 3D coordinate system, defined by the signs of the coordinates (an octal). Each octal was defined as in in the table in **(B)**. In order to determine which octal an epithelial cell belongs to, we use the basal node (remember that epithelial cells are made of two nodes, an apical node and a basal node) as the center of coordinates, as in **(A)**. We then calculate the difference between the X-position of the basal node and the X-position of the apical node ($node^{x}_{basal}$-$node^{x}_{apical}$), considering only the sign of the difference (positive or negative). We repeat this for the Y and Z positions. Based on these differences we assign an octal orientation to each cell (as in the right table). Once the orientation of each cell is determined, cells are categorized into connected patches. A patch is a set of cells belonging to the same orientation category (of the 8 possible ones) and topologically connected to each other. This means that one can go from any cell in a patch to any other cell in the patch through a sequence of cells belonging to the same patch, as shown in **(B)**. Finally, we count the number of patches in a morphology, which will give us the OPC value. Only patches with more than 3 cells were considered. Notice that in **(B)** there are only 6 patches (OPC 6) because the patch next to the asterisk has only two cells.

## 2.5 MORPHOLOGICAL DISTANCE MEASURES

The distance between morphologies was calculated using three different methods, all of them, as with the complexity measures, consider only epithelial

nodes. As a reference of the values that the different measurements provide and for some example morphologies see Fig. 9.

### 2.5.1 EUCLIDEAN MINIMAL DISTANCE (EMD)

This measure allows to compare morphologies made of different numbers of cells and without having to arbitrarily pre-select landmarks or special morphological features [Salazar-Ciudad and Marin-Riera 2013]. This is very convenient for our study since embryos can be made of different numbers of cells. EMD is defined as the mean distance from one node in a morphology to the closest node in another morphology. More specifically, for each node in a morphology the distance to the closest node in the other morphology is calculated. Then the process is repeated for each node in the other morphology. All these distances are then averaged in respect to the sum of nodes in both morphologies. In other words, the distance between two morphologies is calculated as:

$$EMD = \frac{1}{n_1 + n_2}\left(\sum_{k=1}^{n_1} d_{k,min\,(k,2)} + \sum_{j=1}^{n_2} d_{j,min\,(j,1)}\right)$$

Where $n_1$ and $n_2$ is the number of nodes in morphology 1 and 2 respectively, $d_{k,min\,(k,2)}$ is the distance between node $k$ in morphology 1 and its closest node in morphology, $d_{j,min\,(j,1)}$ is the distance between node $j$ in morphology 2 and its closest node in morphology 1. Note that because one node in morphology 1 is the closest node to another node in morphology 2 does not imply that this latter node is the closest to the former, i.e. these minimal distance relationships are not symmetric.

EMD has the advantage that it can compare morphologies that differ in size and number of nodes. In addition, EMD can be calculated for any pair of morphologies without the need of establishing any correspondence or homology between points in the two morphologies being compared.

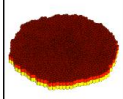
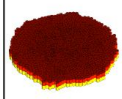
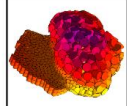
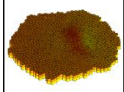
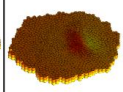
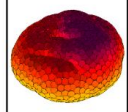
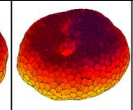
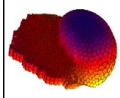
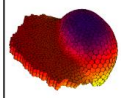
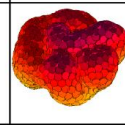
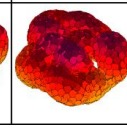
| Embryos | | Developmental instability | | | Embryos | | Developmental instability | | |
|---|---|---|---|---|---|---|---|---|---|
| Twin 1 | Twin 2 | EMD | HMD | CMD | Twin 1 | Twin 2 | EMD | HMD | CMD |
|  |  | 0.131 | 0.060 | 0.001 |  |  | 0.351 | 0.111 | 0.079 |
|  |  | 0.151 | 0.059 | 0.010 |  |  | 0.372 | 0.09 | 0.062 |
|  |  | 0.174 | 0.065 | 0.019 |  |  | 0.631 | 0.189 | 0.156 |

Fig. 9. **Examples of morphologies found in the ensemble and their developmental instability.** Developmental instability is measured as the distance between twins (i.e. morphologies arising from the same developmental mechanisms). We use three different methods to measure the distance between two morphologies: Euclidan Morphological distance (EMD), Homologous Morphological Distance (HMD) or Convexity Morphologicaly Distance (CMD).

### 2.5.2 HOMOLOGY BASED MEASURES

The next two methods are based on measuring the distance between homologous nodes. Since all initial developmental patterns have the same number of cells in the same positions, a clear homology between cells can be established. Each cell in the initial conditions has a numerical label, which is always the same for cells in the same initial position. Therefore, as cells move and divide, in order to find homologous cells between morphologies, we just need to find the cells that share the same label. Although cells move over simulation time, epithelial cells tend to keep the same cellular neighbors. In addition, there is a limited number of divisions in our simulations, i.e. we start with 542 epithelial nodes and finish simulations when there are 5000 nodes. This ensures that homologous cells will be in the same general area of the embryo.

#### *2.5.2.1 Convexity morphological distance (CMD)*

In this method we measure the local convexity of the epithelium around each epithelial node of a morphology and then compare it with that of each

homologous node in another morphology. The local convexity around a node is measured as follows. The set of initial epithelial nodes is S= {1, 2 ,3, …, 542}. For each node in S we calculate the unit vector between node $S_i$ and the other node in the same cell, this gives $v_1$ (note all epithelial cells are made of an apical and a basal node). We then calculate the unit vectors between node $S_i$ and each neighboring node that is of the same type (apical or basal). This gives us a set of vectors $V_i$. Next, we calculate the dot product between $v_1$ and each vector in $V_i$. The local convexity of node $i$ will be the average of these dot products. Irrespectively of the orientation of a morphology in 3D space the local convexity of a node is close to 0 if the neighboring nodes are in the same plane, 1 if the node is in an evagination of the epithelium and -1 if it is in an invagination (Fig. 10). The convexity of a node is:

$$l_{s_i} = \frac{1}{n_i} \sum_{k=1}^{n_i} (v_1 \cdot v_k)$$

Where $l_{s_i}$ is the local curvature of node $i$, $n$ is number of elements in $V_i$, $v_k$ is unit vector $k$ in set $V_i$. To obtain the distance between two morphologies, we calculate the mean absolute difference in convexity between the homologous cells of two morphologies.

$$d_{1,2} = \frac{1}{542} \sum_{i=1}^{n_i} |l_{1i} - l_{2i}|$$

Where $l_{1i}$ is the local convexity of morphology 1 at node $i$, $l_{2i}$ is the local convexity of morphology 2 at node $i$ and 542 is the number of initial epithelial nodes.

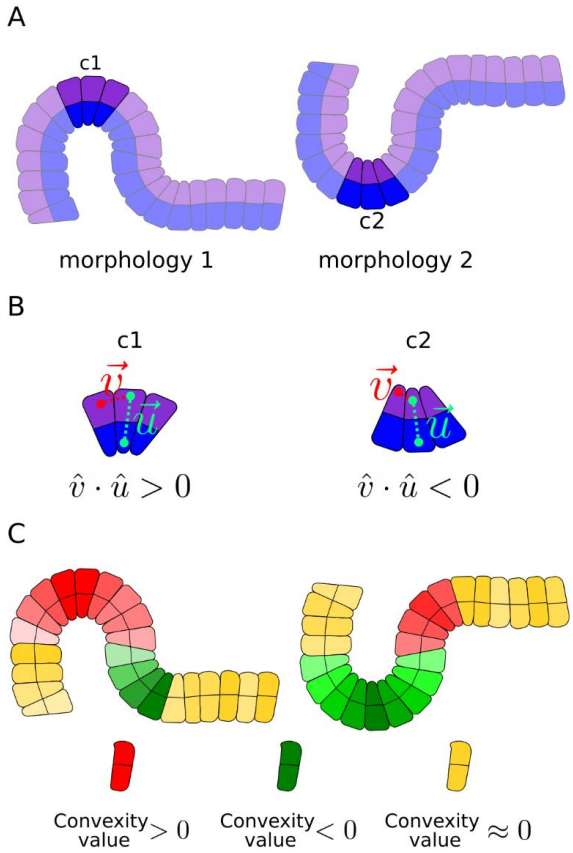



Fig 10. **Convexity morphological distance (CMD)**. Method to measure the distance between two morphologies based on their curvature differences. **(A)**. Two example morphologies, morphology 1 with an evagination and morphology 2 with an invagination. In purple we show the basal nodes of epithelial cells and in blue their apical nodes. **(B)**. Cut-outs of morphology 1 and 2. The green dotted line shows the vector $\vec{v}$, between the basal and the apical node of the same cell i. The red dotted line represents the vector $\vec{u}$, between the apical side of cell j and the apical node of a neighbor cell. By calculating the dot product between the unitary vectors $\vec{v}$ and $\vec{u}$ we obtain the convexity value between cells i and j. Notice that acute angles result in positive values, while obtuse angles result in negative values. The convexity value of a cell is the result of averaging all the convexity values of that cell with its neighbor cells (i.e. cells in physical contact). **(C)**. Morphologies 1 and 2 showing the convexity values of each of their cells. To calculate the distance between two morphologies, we add the convexity differences of the homologous cells (see section 7) between the two morphologies.

#### *2.5.2.2 Homologous Morphological Distance (HMD)*

For all the twins of a given combination of developmental parameters we calculate the mean morphology as the mean position of each homologous node. This helps to reduce the effect of developmental instability on morphology. In order to consider only the shape of the morphologies, these averaged morphologies undergo a Procrustes process, in which they are rotated, resized and centered in order to find the minimal Procrustes distance between them. The distance between two morphologies is this Procrustes distance, which is the square root of the sum of the square of the differences between the positions of each pair of homologous nodes.

# 3 DISCUSSION AND RESULTS

## 3.1 STUDY I

### 3.1.1 MOST DEVELOPMENTAL MECHANISMS DO NOT LEAD TO MORPHOGENESIS

The first ensemble that we built (the broad ensemble) explored 100,000 random developmental mechanisms, from which very few showed any morphological change compared to the flat initial condition. Also, at the gene expression level there was little change in relationship with the initial gene expression pattern, most genes were only expressed in the central cell or in its immediate vicinity.

In order to find developmental mechanisms able to change the gene expression pattern over space, we built the signaling only ensemble. This ensemble did not include morphogenesis, which allows the simulations to run faster and therefore we can explore a bigger number of developmental mechanisms. This search of developmental mechanisms able to produce non-trivial gene expression patterns, resulted in 20,000 developmental mechanisms which were used to build the other ensembles.

### 3.1.2 THE DEVELOPMENT OF COMPLEX MORPHOLOGIES DOES NOT REQUIRE CELL SIGNALING OR COMPLEX GENE NETWORKS

Complex morphologies can be found in autonomous ensembles, although they consist of mostly highly folded epithelia. In these highly folded epithelia, the folds never develop in the same location when the same developmental mechanisms are simulated several times, i.e. they are highly sensitive to noise. Therefore, these epithelia are highly complex but very developmentally unstable. Although mostly seen in the autonomous ensembles, these highly folded epithelia are also present in the ensembles with signaling. However, in the signaling ensemble, this kind of highly folded and noise sensitive epithelium is restricted to parts of the epithelium in which a gene is regulating some cell behavior which induces this folding, therefore, not the whole embryo is folded this way.

We found that in order to be complex, the decisive factor is that the regulation of cell behaviors changes over a large proportion of the embryo.

This is especially the case for two cell behaviors, cell contraction and cell division. Cell division can lead to buckling and wrinkling of the epithelium and then to some complexity, as in fact is also observed in the development of many animal organs [Bunn et al., 2011; Striedter et al. 2015]. Cell contraction has also been shown to lead to the formation of invaginations and tubes in animal development [Martin and Goldstein 2014].

To understand how from homogeneous initial developmental patterns, complex crumpled morphologies emerged, I followed the development of these kinds of morphologies in the autonomous ensemble. Initially, the epithelium homogeneously increases its curvature. This change in the curvature happens because cells are changing their geometry, as shown in the left panel of Fig. 11. As cells apically contract, an evagination will form (see center panel of Fig. 11). The size of this evagination is determined by the geometry of the cells, the more they contract, the smaller the evagination will be. As a consequence of evaginations being smaller, less cells will be able to be part of it. This way, a single evagination will form in a field of contracting cells which contains less cells that can be part of the evagination, as in Fig. 12A. However, if the field of contracting cells contains more cells that can be part of a single evagination, several randomly located evaginations will form, as in Fig. 12B and C. Evaginations will be randomly located because as cells contract and the epithelium bends, any small perturbation or noise will affect which cells will evaginate first. This will have an amplifying effect on the surrounding cells, whose orientation will change and favor an out of plane movement, which will form the evagination. Therefore, the location of these evaginations is highly sensitive to noise, resulting in evaginations being randomly distributed over the field of contracting cells. As a consequence, embryos which have large fields of gene expression, such as those in the autonomous ensembles, can result in complex morphologies.

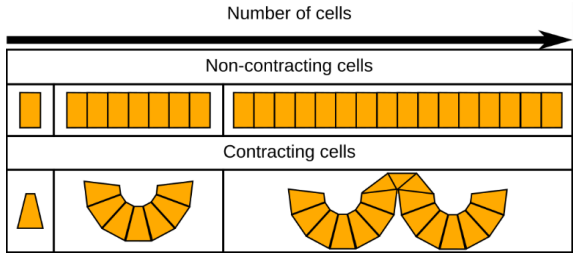



Fig 11. **Geometrical constraints on invagination**. The top panel shows idealized 2D sections of epithelia of different sizes. Each epithelial cell is represented by a rectangle. As shown in the lower panel, only a limited number of cells can fit into an invagination. As a result, when all cells in a large epithelium contract, several invaginations form. The larger the contraction and the larger the epithelium, the more invaginations will form..

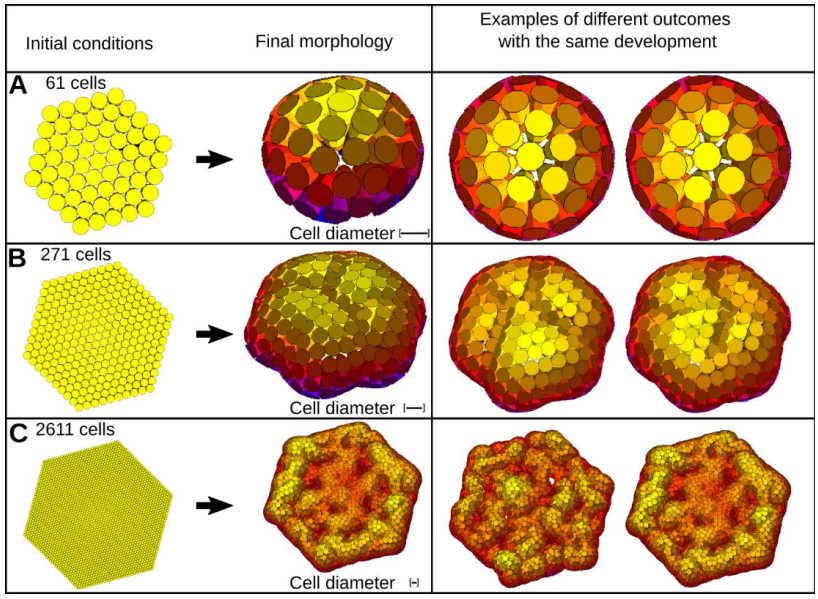



Fig 12. **The larger the number of cells contracting at the same time the larger the developmental instability**. **(A-C)** Three different simulations in which cell contraction occurs homogeneously over an epithelium are shown, each differing in the number of cells. The right-hand panels show two different runs from the same initial conditions. The different runs are identical except for noise. Cell color represents position along an arbitrary z-axis.

### 3.1.3 EXTRACELLULAR SIGNALING ENHANCES ROBUSTNESS THROUGH THE COMPARTMENTALIZATION OF THE EMBRYO INTO DIFFERENT REGIONS OF GENE EXPRESSION

We found that the developmental mechanisms producing complex morphologies tend to be more developmentally unstable than the developmental mechanisms producing simple morphologies. Interestingly, the ensemble that includes cell signaling produced, for the same complexity, morphologies that were on average more developmentally stable than the autonomous ensembles. This indicates that extracellular signaling is not necessary to develop complex morphologies, it is rather necessary for complex morphologies to be developmentally stable.

In order to understand how extracellular signaling enhances robustness against noise, we focused on developmental mechanisms which include cell contraction, as it is the cell behavior most often associated with complex morphologies in our simulations. Qualitatively, we found that if cells contract at the same time and with the same intensity over large regions of the embryo the resulting morphologies tend to be complex but unstable. If in contrast cell contraction occurs in different ways (at different moments or at different rates) over different regions of the embryo, development tends to lead to complex but also more stable morphologies.

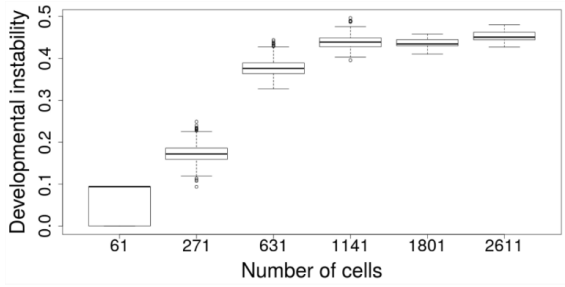



Fig 13. **The larger the number of cells contracting at the same time the larger the developmental instability**. To calculate developmental instability, we simulated 30 times each initial developmental pattern shown in Fig. 12 with noise (changing the random seed). We then measured the developmental instability (EMD) between all final morphologies. Developmental instability increases with the size of the epithelium, although EMD distances saturate with epithelium size. Boxes enclose 50% of the data points, the line in the box shows the median. Whiskers represent the nonoutlier points, which are within 1.5 the interquartile range of Q1 and Q2; circles represent; circles represent the outliers.

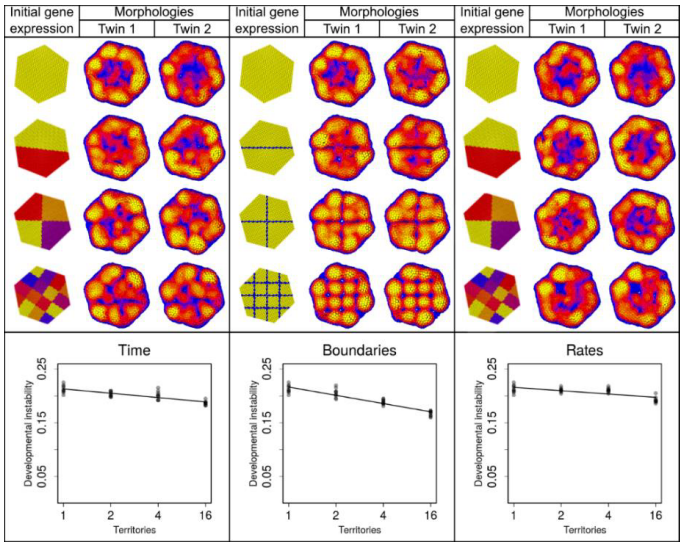



Fig 14**. Splitting the epithelium into regions of gene activity that regulate cell apical contraction can increase the developmental stability.** In each column we show the initial gene expression on the left (colors show the genes being expressed) and two of the final morphologies on the right, each of them we call a twin (color shows the cell position in the z-axis). The first column shows the results of splitting the embryo into regions that contract at different times (every 0.5 model time units), each of these regions will start contracting at different time points, but they all contract in the same way once they start contracting. The middle column shows the results of splitting the embryo into territories of contracting cells (yellow cells) with non-contracting boundaries (blue cells). All the contracting cells contract at the same time and in the same way. The right column shows the results of splitting the embryo into territories of cells that contract at different rates (although the variance was kept the same between the simulations of different territories). It is visually clear that splitting the embryos into territories increases the stability, especially when paying attention to the location of ridges (in yellow) and bumps (in blue). At the bottom of each column we show the results of quantifying the results. To calculate developmental instability, we simulate 5 times each initial condition with noise (changing the random seed). As before, developmental instability is measured by calculating the EMD distances between all the twins. Each point in the plot represents one of these distances. The intensity of the gray of the points indicates how many overlapping points there are. The line shows the linear regression. Time: Spearman correlation: $r_s$=-0.811, pval<0.001. Boundaries: Spearman correlation: $r_s$=-0.922, pval<0.001. Rate: Spearman correlation: $r_s$=-0.622, pval<0.001. The pval for the Spearman correlation was calculated with a permutation test.

To quantitatively support these qualitative observations, we ran several simulations in which epithelia of different sizes contracted all the apical side of their cells in the same way and at the same time (as in the cell behaviors-only ensemble). As can be seen in Figs 12 and 13, the larger the epithelium, the larger is its developmental instability. Splitting the epithelium into regions contracting in slightly different moments or at slightly different rates, however, decreased developmental instability (Fig. 14). The same occurred if the epithelium was split into equally contracting regions separated by narrow non-contracting boundaries (Fig. 14). In other words, compartmentalizing the embryo in different regions largely reduces developmental instability without precluding the development of complex morphologies.

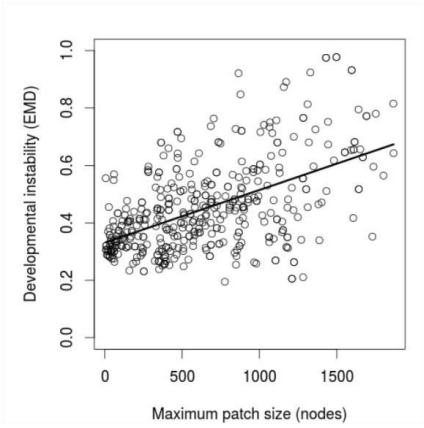



Fig. 15. **The number of cells contracting in the same way in an embryo correlates with developmental instability.** For all morphologies in the signaling ensemble, we calculated the number of epithelial cells that have a similar radius and are in contact with each other as forming a single patch. Then we calculated the size of the largest of such patches in each morphology in the signaling ensemble. Only morphologies with more than 1000 cells and an AV complexity larger than 0.3 were included. The black line shows the linear regression. Spearman correlation: rs=0.493, P<0.001, n=440.

If developmental instability is related to the size of the epithelial regions contracting in the same way, then developmental instability and the size of the compartments should correlate in the ensembles we simulated. Fig. 15 shows that this was indeed the case: the developmental stability of an embryo correlates with the size (in number of cells) of the largest compartments in which cells are contracting in the same way (i.e. changing their apical or basal side radius at the same rate). These regions are larger in the autonomous ensemble and cell behaviors-only ensemble because, since all cells behave in the same way. All the embryos in Fig. 15 (and most embryos in the ensembles)

have the same number of cells so the relationship we found was not due to larger embryos being less stable.

## 3.2 STUDY II

All the results of study II are based on developmental mechanisms from the "signaling ensemble", therefore in this study we simply refer to it as the "ensemble".

### 3.2.1 COMPLEX MORPHOLOGIES ARE RARE

We found developmental mechanisms producing complex morphologies in the ensemble, although with a frequency that decreased with complexity (Fig. 16). In addition, we found that a large proportion of the interactions in each developmental mechanism were not necessary for producing the observed morphology, i.e. the resulting morphology was unaltered if these interactions were deleted. This is perhaps not surprising given the fact that the networks were built at random. The number of interactions necessary for the development of a morphology increases with the complexity of such a morphology (Fig. 17).

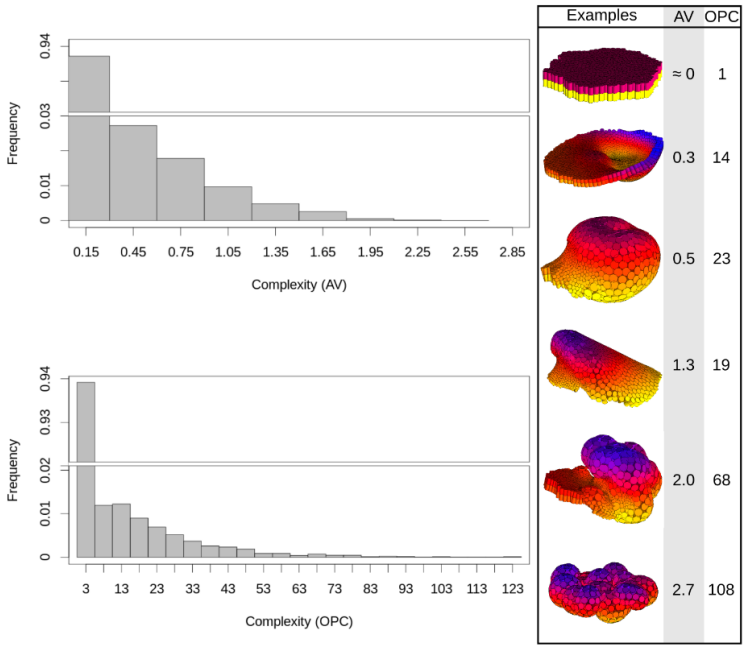



Fig 16. **Complex morphologies are increasingly less frequent.** The histograms show the distribution of complexity (for AV at the top and OPC at the bottom) in the morphologies found in the signaling ensemble. The right panels show example morphologies and their complexities.

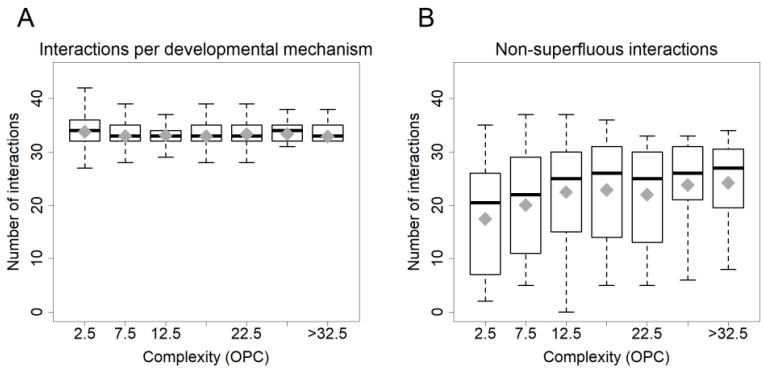



Fig 17. **The number of interactions that are non-superfluous in the development of a morphology increases with its complexity**. The developmental mechanisms in the ensemble are arranged in intervals along the X-

axis based on the complexity of the morphologies they produce (bin sizes: 5 OPC). **(A)** the Y-axis shows the number of interactions per developmental mechanisms. **(B)** the Y-axis is the number of non-superfluous interactions per developmental mechanisms. The boxes enclose 50% of the developmental mechanisms in each complexity interval. The black line in each box shows the median and the gray diamond shows the average of each interval. The whiskers extend 1.5 times the interquartile range of the box. Outliers not shown. Spearman correlations (A) $p=0.241$, $rs=-0.04$ (B) $p<0.001$, $rs=0.196$, $n=699$.

### 3.2.2 THE MORE COMPLEX A MORPHOLOGY IS, THE MORE FINELY TUNED ITS DEVELOPMENTAL PARAMETERS NEED TO BE

Each developmental mechanism in the ensemble was initially run with a specific combination of values in its parameters. We then introduced IS-mutations, i.e. changes in the values of the parameters without changing the topology of interactions in a developmental mechanism. The analysis of the morphologies resulting from these mutations showed that the simpler the morphology in the ensemble, the smaller the chances that an IS-mutation in its developmental mechanism would change that morphology (Fig. 18). Conversely, the more complex a morphology is, the higher the chances that IS-mutations will alter it. In other words, even when a given developmental mechanism can produce a complex morphology, this is only possible for a small range of values in its parameters. Thus, the most complex morphologies of a developmental mechanism tend to occupy small regions of its parameter space.

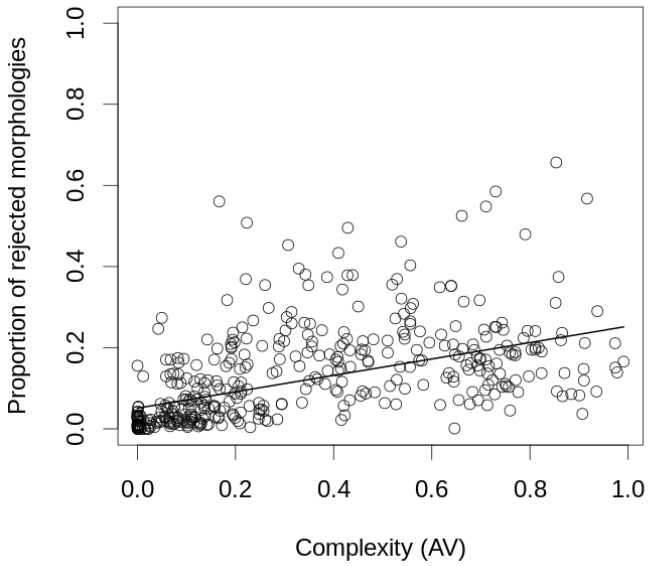



Fig. 18. **Developmental mechanisms producing complex morphologies occupy smaller regions of the parameter space than developmental mechanisms producing simple morphologies.** We performed an iso-morphological random walk for the developmental mechanisms in the parental set that are very stable developmentally (i.e. EMD distance between parental twins less than 0.3). In such a walk we mutated, one at a time and chosen randomly, gene-gene interactions or gene-cell behavior interactions in each developmental mechanism. If a mutation did not change in a significant way the phenotype (when compared to the original parental morphology in the walk) the mutation was kept, and a new mutation was applied. If the mutation did change the phenotype, this mutation was reversed, and a new mutation was applied. This way we calculated the proportion of mutations that changed the phenotype: the more mutations changed the parental phenotype, the smaller the region of the parameter space where a developmental mechanism can produce its parental phenotype. We performed 10 random walks per developmental mechanisms, each walk with 200 mutation steps. To minimize the effect of random developmental noise in our results each mutant was simulated 5 times. The morphological distance between the final developmental pattern of each of these 5 simulations and the parental was measured using CMD. The average of these distances was used to evaluate whether a mutation was accepted or not. In order to be considered different to the parent, the CMD had to be 0.01 higher than the developmental instability of the parental (measured in CMD). Spearman: p-val<0.0001, rs=0.65, n=422.

### 3.2.3 THE SIMPLER MORPHOLOGY, THE LARGER THE NUMBER OF DEVELOPMENTAL MECHANISMS THAT CAN PRODUCE IT

Our study also shows that there is a global degeneracy in the space of developmental mechanisms, i.e. very similar morphologies can be produced by different developmental mechanisms. However, the higher the complexity of the morphologies a mechanism can produce, the smaller the degeneracy (Fig. 19). Additionally, the morphological distance between complex morphologies produced by different developmental mechanisms tends to be larger than that between simple morphologies.

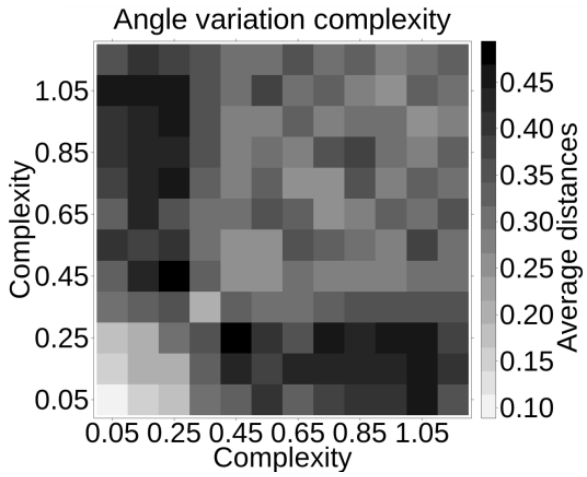



Fig 19. **Morphological degeneracy decreases with complexity.** We measured the pair-wise morphological distances between all the morphologies within a random subset of the signaling ensemble. Morphologies in such subsets were chosen to be evenly distributed across the complexity range. This range was subdivided into bins of 0.1 AV units. The 20 morphologies in each bin were compared with all other morphologies. The average of the distances between the morphologies of each pair of bins (20x20 distances) is plotted in the figure. For simplicity we only show the results for the morphological distance using HMD. The plot shows that simple morphologies tend to resemble each other while complex morphologies do not. Complex and simple morphologies also tend to be quite different from each other.

### 3.2.4 THE MORE COMPLEX THE MORPHOLOGY, THE LARGER THE MUTATIONAL ASYMMETRY

To study mutational asymmetry, we analyzed the complexity of the mutants in the IS one-mutant neighborhood. The results show a clear mutational asymmetry (Fig. 20A): the higher the complexity of the parent, the simpler the offspring will be. Most of the developmental mechanisms producing complex morphologies in the ensemble had some offspring with the minimal possible complexity (a flat epithelium). Thus, many complex morphologies are one mutation away from the simplest morphologies. The opposite is not true, the parents producing very simple morphologies were not one mutation away from very complex morphologies. This mutational asymmetry was even more evident when the mutational analysis was done with T-mutations (i.e. mutations changing which genes interact with which other genes, cell behaviors or cell mechanical properties, i.e. the topology of the gene network. Fig. 20B-C).

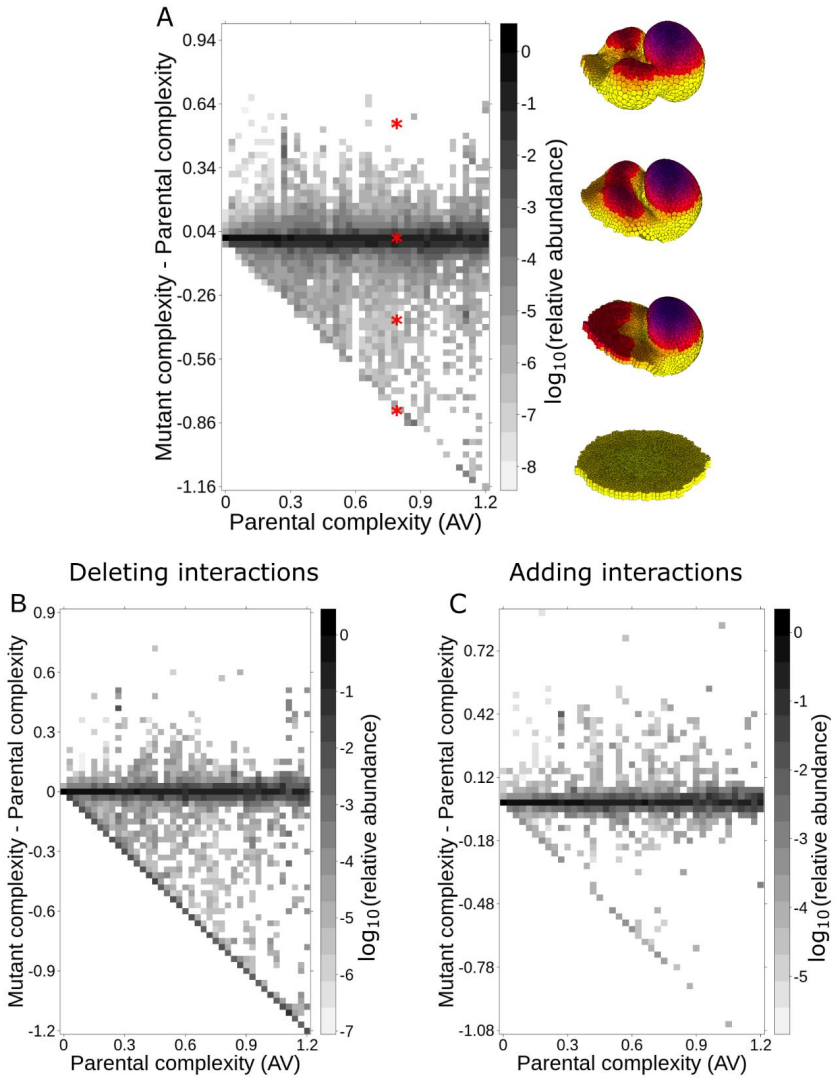



Fig. 20. **Mutations decreasing complexity are more frequent than mutations increasing it**. The plots show the distribution of the difference in complexity between each parent and its mutant offspring. Offspring is generated by mutating each parameter of the parent. **(A)** Each parameter was IS-mutated (i.e. without changing the topology of the gene network) the same number of times and each mutant offspring had only one mutation. The magnitude of these mutations was proportional to the value of that parameter in the parent and ranged from -80% to +80% in 20% intervals. In these plots I only show the results for AV complexity. Offspring is arranged along the y-axis according to their complexity minus their parental complexity and along the x-axis according to the complexity of their parents. The x-y plane is divided in square bins, the size of each bin is 0.03 AV units.

The darkness of each bin represents the logarithm of the relative abundance of offspring of a given parental complexity (x-axis). To calculate the relative abundance, for each column (x-axis), we divide the number of offspring falling in each bin by the total number of offspring in that column. Thus, the relative abundance of each column in the plot sums 1. The plot shows that most offspring have a complexity like that of their parents. It also shows that, for complex morphologies, there are more offspring that are simpler than their parents than offspring that are more complex than their parents. Notice that even the most complex parents can have very simple offspring but that simple parents rarely have very complex offspring. On the right we show some examples of offspring morphologies. The red asterisks mark the complexity of the examples. **(B)** and **(C)** are similar to **(A)** but instead of IS-mutations, topological mutations (T-mutations) are applied, which modify the topology of the gene network. In **(B)** these mutations delete interactions, in **(C)** the add interactions, in both cases, a similar trend as in **(A)** can be observed.

### 3.2.5 THE MORE COMPLEX MORPHOLOGY, THE MORE COMPLEX THE GPM

A direct correlation exists between the complexity of the GPM maps (calculated as described in Fig. 6) and the morphological complexity of the parental (Fig. 6). Which indicates that mutations on complex morphologies have larger phenotypic effects.

We also found that mutations in many parameters have no morphological effect (see Fig. 6 B), at least in the range of values studied. However, the interactions associated with those parameters cannot be deleted without the morphology changing dramatically. In other words, these interactions are required for the development of a morphology but do not contribute much to its variation. When we focus only on the GPM regression for the proliferation rate parameter, which tends to have a strong effect on complexity we see an even higher correlation (Fig. 6 G-I).

# 4 CONCLUSIONS

In this dissertation there are two overarching questions:

1. What can we say about the developmental mechanisms that allow complex morphologies to develop?
2. How does morphological complexity affect evolution?

For question one, it can be concluded that the main factor leading to complex morphologies is that cell behaviors are expressed over large areas of the embryo. This is especially the case for cell behaviors able to directly generate forces such as cell division and contraction [Bard 1990]. Other cell behaviors can have an important effect on morphogenesis, but mostly in conjunction with cell division and cell contraction. Another factor that was expected to contribute significantly to complexity in some way were gene networks. However, gene networks are not necessary to develop complex morphologies. Indeed, even developmental mechanisms completely lacking gene networks were able to produce highly complex morphologies. Alternatively, we found that gene networks in combination with cell signaling are necessary to develop developmentally stable morphologies. These results have some interesting implications on how development should be in order to achieve highly complex and stable morphologies, however we must consider several caveats before discussing these implications.

Admittedly, in the relatively simple ensembles used here, we do not find morphologies resembling a frog, a rose, or (probably) the reader. However, the aforementioned types of morphologies are unlikely to be present in the ensembles for a variety of reasons, primarily because the way we built the ensembles allows for a maximum of 10 genes in a given developmental mechanism. Although these developmental mechanisms can give rise to very diverse morphologies, the complexity that stable morphologies can have is limited, since the number of territories is limited by the complexity of the gene network and cell signaling interactions. On the other hand, although some of the morphologies in the ensemble are extremely complex, they are mostly composed of randomly folded epithelia, which if they resemble in any way the reader, it will be just by chance and it will most likely not happen again when the same developmental mechanism is simulated repeatedly.

Finally, a big limitation on the types of morphology and their complexity, which can be found in the different ensembles is related to how the embryos are modelled. For computational reasons, we modelled each cell as a single cylinder or sphere. This precluded us from simulating planar polarization in cases where the cells themselves needed to be polarized, as single cells cannot show any asymmetry in their biophysical properties or shape (only epithelial cells can show apical-basal polarity). Also, for computational reasons, the developmental simulations had time restrictions, in addition to a maximum number of cells that could be simulated, which also limits how complex the morphologies can become.

The next caveats relate mainly to ECM and mesenchyme. In EmbryoMaker, ECM is modelled as spherical nodes, which precludes an accurate simulation of the basal lamina. However, due to the importance of the basal lamina for epithelial morphogenesis, some of its defining properties are included in how we model the epithelium. One of the most important effects of the basal lamina on the physical properties of epithelial tissues is the stiffness it provides [Candiello et al. 2007]. In order to include this property, epithelial cells are modeled as cylinders, with a basal and an apical side, which have a tendency to avoid being bent. By regulating this tendency, epithelia with different stiffnesses are simulated in the ensemble. This is just an approximation and it is quite likely that a more accurate implementation of the basal lamina would increase the repertoire of morphologies obtained in our simulations.

Mesenchyme is also vital to understand how many tissues and organs develop. However, although we simulate the mesenchyme, we do not consider its morphology in our complexity analysis. This is because in most embryos in the ensembles, the morphology of the mesenchyme mimics that of the epithelium or it is very noisy. Mesenchyme is noisy because of the initial condition we use; mesenchyme is on one side of a flat epithelium. Under this open initial condition, mesenchyme is free to move without restrain, and will therefore become highly noisy. Thus, we fail to consider morphologies that arise from morphogenesis in mesenchymal tissues or even other non-epithelial tissues, such as blastomeres. For example, 3D condensates, rods and lumens within solid 3D tissues are not found in our ensemble. These caveats imply that some realistic morphologies are not going to be found in any of the ensembles. However, there is no reason to expect that these missing morphologies will have very different properties from the ones that were present.

The general results presented herein should be applicable at any scale of organismal complexity, which does not preclude that at different complexity and organization levels, additional requisites might be necessary. For instance, in order to develop a frog, a developmental mechanism which includes a gene network will be necessary. Otherwise, morphogenesis occurs mostly due to random epithelial folding, which will hardly ever result in morphologies resembling this kind of highly organized and complex morphologies, for example a frog. In other words, developmental mechanisms able to transform an embryo into a frog in a consistent way, will include cell signaling that will compartmentalize the embryo in such a way that cell behaviors are not expressed simultaneously over large areas of the embryo.

Whereas cell signaling does not directly affect the complexity of a morphology, the number of partitions or territories formed by cell signaling are an important indicator of the potential complexity a morphology can attain, at least when considering developmentally stable morphologies. This is because each territory can potentially change the morphology in a different way, which increases the variation of morphological features in the embryo and thus, increases morphological complexity. Therefore, for developmental mechanisms to be able to develop complex stable morphologies, they need to have the capacity of partitioning the embryo in different territories of gene expression. The capacity of developmental mechanisms to form different territories, depends on having cell signaling, whether the developmental mechanism is hierarchical or emergent [Salazar-Ciudad et al. 2000]. Additionally, in order to generate many territories which regulate different cell behaviors or the same cell behavior in different ways, it is necessary to have complex gene networks, i.e. networks with many genes and gene interactions. However, anyone familiar with emergent developmental mechanisms, which include for example those with reaction diffusion dynamics, could argue that even simple gene networks can generate many territories of gene expression. Although this is true, all the territories formed this way will be equal or will follow a repeated pattern. Additionally, once the equilibrium is reached, these territories will be uniformly distributed over space. Thus, these territories will regulate cell behaviors in the same way or in a repeatable and predictable way. If for example these territories are regulating cell contraction in the same way, it will result in many evaginations of the same size and form. Although this multi-evaginated tissue could be considered complex to some extent, its complexity is limited, since all the evagination will be equal. However, by including a hierarchic mechanism to this reaction diffusion system, a higher variation between the different evaginations could be achieved. For example, by adding a gene forming an anterior-posterior gradient, which regulates cell contraction, will introduce some variation in the size and shape of the

evaginations in that axis. By adding an additional gene forming a lateral gradient, complexity could be increased again. In summary, although reaction-diffusion systems can induce many different territories, in order to have highly complex and stable morphologies, it is necessary to have complex gene networks with many genes and interactions which include cell-cell signaling.

To sum up the answer to question one: What can we say about the developmental mechanisms that allow complex morphologies to develop? Two general characteristics about developmental mechanisms and the complexity of their phenotypes can be pointed out. On one hand, in order to produce complex morphologies, biomechanical interactions and cell behaviors can be sufficient, there is no need for complex gene networks. On the other hand, in order to make complex stable morphologies, gene networks and cell signaling which compartmentalize the embryo are necessary. These general results underpin how developmental mechanisms have a strong constraint on their architecture, due to the constant extracellular signaling required to progressively compartmentalize the embryo as it grows and deforms during morphogenesis.

Additionally, these results contribute to a hypothesis of how early metazoan might have evolved. According to a hypothesis by Newman, Müller and Comper [Newman and Comper 1990, Newman and Müller, 2000; Newman et al., 2006] early metazoans had relatively complex but unstable morphologies. These authors argue that the generic physical properties of animal cells allowed for a large repertoire of relatively complex morphologies. Newman and Comper [Newman and Comper 1990] define generic mechanisms as the physical processes that play a role in living and non-living systems. Generic mechanisms include processes like adhesion, surface tension, marangoni effect, viscosity, phase separation, convection or reaction-diffusion mechanisms. These generic physical properties have important morphogenetic effects when acting on living tissues. They can, without the need of any genetic regulation, give rise to very diverse morphological changes.

For example, interfacial tension arises at the interface of two immiscible liquids. Interfacial tension emerges when molecules in each liquid have a higher affinity to molecules of its same type. Therefore, molecules at the interface have fewer interaction partners or these interactions are weaker. Thus, the liquids will tend to form the smallest possible interface. As a consequence, in order to increase the surface of the interface, work must be applied. This gives rise to certain dynamics, some of which are very well

known, such as the behavior of water and oil when mixed. Water and oil will not mix under normal circumstances, they will end up forming two separate phases, in which droplets of oil will tend to fuse together. This is due to the interfacial tension arising from the fact that water is polar and highly attracted to itself, while oil is non-polar, and therefore not attracted to water molecules. In living tissues, the same dynamics can be observed. For example, in cell sorting cells reorganize themselves to form distinct phases made of cells of the same type. Depending on the adhesion molecules the different cells have, the behavior between the different phases (cells of the same type) can be very different. For instance, one of the phases could engulf the other, or they could promote the spreading of other phases. The importance of interfacial tension in morphogenesis has been clearly demonstrated in several experiments, for example the epiboly at the beginning of gastrulation [Armstrong and Child 1975, Steinberg 2007] or vertebrate limb formation [Gumbiner 2005].

These generic physical mechanisms are enough to produce a high diversity of morphologies, like rods, evaginations or cavities, which combined can form very complex morphologies. Newman, Comper and Müller argue that generic properties are the main responsible for morphogenesis, and that genetic programming are mainly responsible to conserve and support some morphogenetic tendencies [Newman and Comper 1990], in other words, to make morphologies more stable.

Strikingly, this hypothesis fits remarkably well with our results. First, in the autonomous ensembles, complex unstable morphologies arise by the activation of cell behaviors and physical interactions without extracellular signaling and without gene networks. Then, in the signaling ensemble, complex and stable morphologies arise due to cell signaling and gene networks regulating these generic mechanisms. Our results are consistent with their view that the early function of developmental gene networks and extracellular signaling may have been in stabilizing development rather than in building complex morphologies.

Admittedly, this hypothesis concerns mainly early metazoan evolution. In many current Eumetazoa, gene networks and extracellular signaling are pervasively important in the construction of morphology [Gilbert and Barresi, 2019]. In addition, current complex eumetazoan morphologies consist of more than folded epithelia and cell aggregates. These two facts suggest that beyond the earliest metazoan evolution the role of gene networks and extracellular signaling is not restricted to making complex morphologies stable, but also extends to further increasing possible morphological complexity. This could

be achieved by recombining existing developmental mechanisms and the morphological changes they regulate. For instance, the use of gene networks and extracellular signaling allows a finer partitioning of the embryo into territories. Each of these territories can then activate different developmental mechanisms. Therefore, in each of these partitions, the various developmental mechanisms can regulate how cell behaviors and generic physical properties induce the morphogenesis of some basic forms, such as rods, invaginations or cavities [Newman and Comper 1990; Newman and Müller, 2000]. The recombination of these basic forms through the different parts of the embryo, results in the construction of complex and modular anatomies, currently observed in many current Eumetazoa.

One important corollary of this hypothesis is that generic mechanisms give rise to certain morphological changes for free, i.e. without the need of any specific genetic program or need to evolve some coordination between macromolecules. Therefore, some of the morphologies that can be easily achieved by generic mechanisms are found repeatedly in all organisms. This way, during evolution, morphogenesis occurs due to generic mechanisms, while genetic mechanisms can direct or reinforce them in order to achieve stable morphologies. This means that the generic-genetic relationship builds morphologies based on a limited set of tissue transformations that the generic mechanisms can perform, which can be combined in many different ways to achieve a myriad of morphologies. This is in contrast with genetic programming hypotheses, such as neo-Darwinism, which assume that any pattern and morphology is eventually possible. This reinforces the importance of considering development, and not only genes or gene interactions, when studying morphological variation. We should not assume that any morphological change is possible and then be surprised when we find some "constraint" or "bias", this can produce problematic or inefficient research questions [Salazar-Ciudad 2006]. In fact, developmental mechanisms produce different types of morphological variation, depending for example on the generic mechanisms involved or on the topology of the developmental mechanism. Therefore, studying the morphological variation possible by different developmental mechanisms can help us to understand some evolutionary dynamics.

This is precisely the approach I took in the second study. In order to study how complexity itself could affect morphological evolution; I researched the phenotypic variation of developmental mechanisms leading to morphologies of different complexity. When searching for developmental mechanisms that give rise to morphologies of different complexity, one thing becomes apparent

very quickly. Complex morphologies are very rare in all the ensembles. Even if complex stable morphologies are limited by the size of the gene networks we use, why does the frequency of morphologies decrease as their complexity increases? There are two ways in which complex morphologies can form. Either by activating the same cell behaviors in all the cells of a morphology or by activating cell behaviors differently in different parts of a morphology. In the first case, specific cell behaviors must be regulated in order to achieve complex morphologies. Additionally, they need to be regulated inside certain parameter range in order to be able to induce morphogenesis leading to complex morphologies. In the next paragraphs I will concentrate in the second case, which can produce complex and stable morphologies. In the second case, the difference between the abundance of morphologies of different complexity can be understood when considering what is needed, as explained above, to make complex stable morphologies. Most importantly, gene networks and cell signaling that compartmentalize the embryo. As we will see, the answer to why complexity becomes increasingly rarer is related to the second question I proposed: How does genetic variation affect the phenotypic variation of developmental mechanisms leading to morphologies of different complexity?

There are several differences in how genetic variation can affect phenotypic variation of the developmental mechanisms that lead to morphologies of different complexity. First, for the same amount of genetic change, complex phenotypes undergo bigger morphological changes. Similarly, complex phenotypes are more affected by random fluctuations during development, therefore they are more developmentally unstable. Second, the range of parameter values that developmental mechanisms leading to complex morphologies can have is smaller than for simpler morphologies. In other words, developmental mechanisms leading to complex morphologies need to have their parameters more finely tuned in order to keep developing the same complex morphology. Accordingly, when genetic variation produces a change in the phenotype, the complexity of the resulting phenotype will be biased towards simplicity. However, this bias is not uniform for all phenotypes. The more complex a phenotype, the more of its mutant offspring will be simpler than itself.

These results are to be expected if studied under the umbrella of development. As explained above, gene networks are not necessary to make complex morphologies, however, they are necessary to make complex stable morphologies. This is the case because stable morphologies need to be compartmentalized. When the different compartments can induce morphogenesis in different ways, the resulting morphology will be complex

and stable. Therefore, when considering stable morphologies, the more complex the morphology, the more complex the gene network needs to be. This is because each territory forms due to gene interactions and cell signaling. As a result, the more gene interactions and genes a gene network has, the higher the potential of producing more territories. However, not all interactions will lead to the formation of a new territory. For an interaction to form a territory, the whole set of interactions must be inside a certain range of values (Fig. 21). Furthermore, the range of these values decreases as the number of territories increases, i.e. the parameters need to be more finely tuned. This can be easily understood with an example. In Fig. 21A, two gene products, B and C, are forming two territories. Both B and C are being activated by A in a dose-dependent manner, while B is inhibiting C. If we modify the number of enhancers for A that B or C have, or if we modify how strong B is inhibiting C, the most likely outcome is that B and C will still form two territories. It will take very big changes to make one of the territories to completely disappear. However, in Fig. 21B, a specific pattern of inhibitory interactions and of promoter affinities is necessary in order to form all the territories. In addition, the parameters of these interactions need to be within a specific range. Small deviations from this pattern or parameter values will most likely cause one or more of the territories to get lost, as in Fig. 21C. Thus, only a small fraction of the interactions that can be added to a developmental mechanism will lead to the formation of additional territories, and this fraction decreases with the number of territories. Since more territories means a higher potential to increase morphological complexity, it follows that the more complex a morphology is, the fewer the number of random developmental mechanisms that can produce it.

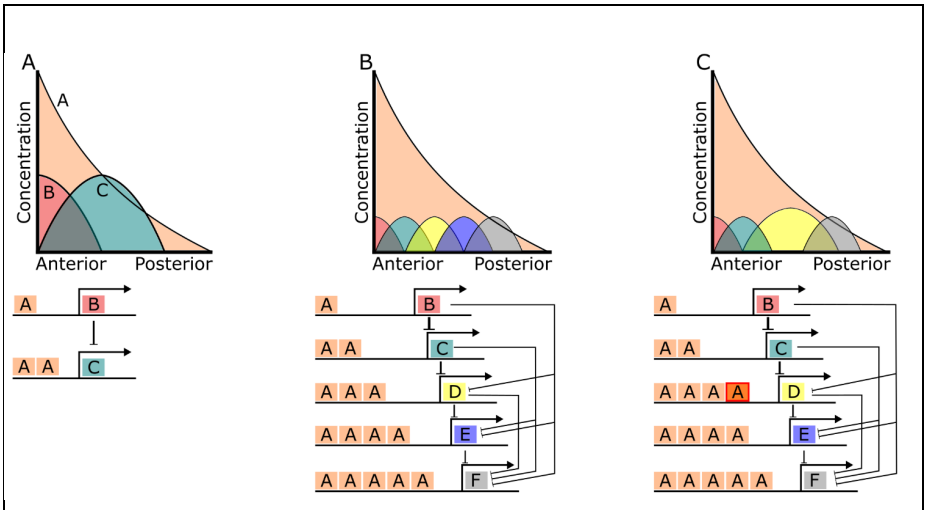


Fig 21. **Example developmental mechanisms**. **(A)** The plot shows the concentration of different gene products at different distances from the source of A. For simplicity we do not consider the signal transduction pathway of A, but we consider that this pathway leads to a dose-dependent increase in the concentration of a transcriptional factor that binds to a specific enhancer "A". The network below represents a schema of the network. The A orange-boxes represent enhancers for the transcriptional factor induced by A. B inhibits C's expression. **(B)** as **(A)** but for a more complex network leading to several distinct territories of expression. **(C)** as **(B)** but D has acquired a larger affinity for the transcriptional factor activated by A, which totally inhibits the expression of E. The more distinct territories of gene expression are to be formed in the same space, the more finetuned the different molecules need to be. For example, in **(C)**, an increase in the affinity of D to A, causes the total inhibition of E.

As a consequence of the decreasing range of values that genes can have to produce an increasing number of territories, complex morphologies occupy a smaller region in the parameter space of the developmental mechanisms that can produce them. Therefore, as complexity increases, it becomes more likely that mutant offspring will move to nearby regions of the parameter space where a different morphology will arise (see Fig. 22). As represented in Fig. 22, complex morphologies occupy smaller regions of the parameter space of a certain developmental mechanism, while simpler morphologies occupy a bigger region. This means that for the same amount of exploration in the parameter space, complex morphologies will be more likely to find a region of the parameter space in which a different morphology arises. Since simple morphologies occupy larger regions of the parameter space, chances are that these newly found regions correspond to simpler morphologies, explaining the mutational asymmetry. However, although developmental mechanisms can mutate to extremely simple morphologies in a single mutational step, they can also mutate to slightly less complex morphologies. This indicates that complex morphologies tend to cluster in the parameter space. Notice however that it is generally harder to find a slightly more complex morphology than a simpler one. These slightly less complex morphologies are found in large areas of the parameter space and are made possible by many more developmental mechanisms. Thus, the parameter space can be seen as having a structure in respect to morphological complexity (see Fig. 22 Complex morphologies form clusters of neutral networks, with the less complex morphologies bordering the more complex, while all of them are in contact with the neutral networks of the simplest morphologies.

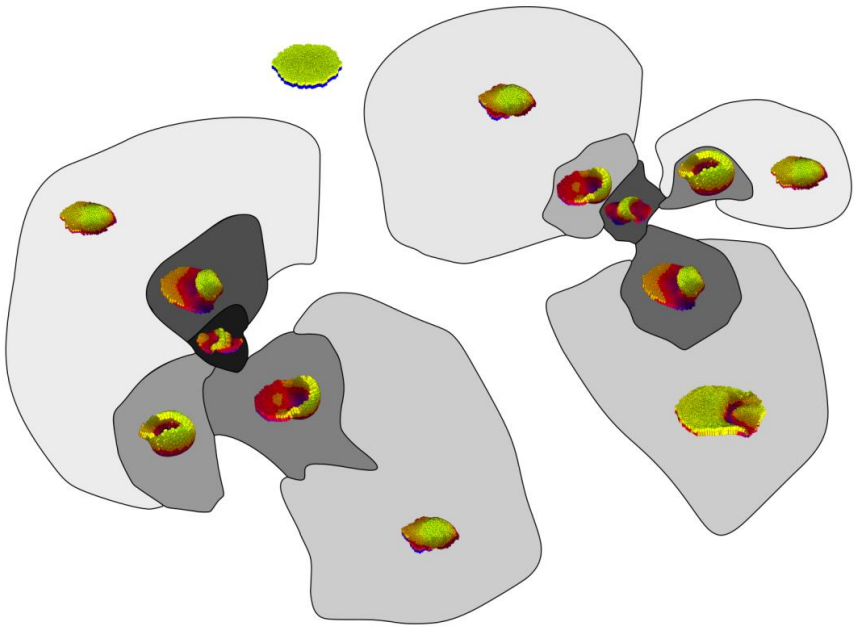


Fig 22. **Idealized schema of the developmental parameter space of a developmental mechanism.** Idealization of the developmental parameter space of developmental mechanisms. Each colored region represents a parameter region (i.e. neutral network) where a morphology of a given complexity would form. As in our results, the simpler morphologies occupy larger regions of the space and most such regions are in contact with the region producing the simplest morphology (in white). The regions with complex morphologies tend to neighbor regions that also produce complex morphologies.

If for most developmental mechanisms the regions of the parameter space that lead to complex morphologies cluster together, it can be expected that mutations in complex morphologies will result in a higher diversity of morphologies than mutations in simple morphologies. The reason being that complex morphologies occupy smaller regions in the parameter space than simpler morphologies, so that for a given amount of parameter space, there can be more types of morphologies. This explains why the GPM of complex morphologies is more complex, since mutations will easily reach new regions of the parameter space in which different morphologies are formed by the developmental mechanism.

These results have several evolutionary implications. In lineages in which complexity increases during evolution, it does so at a progressively slower rate. There are several reasons for this. On one hand, as complexity increases, the

mutations that further increase complexity become less frequent, therefore, it becomes difficult to change development in a way that the resulting morphology is more complex. Moreover, mutations that decrease complexity will become more frequent. On the other hand, as complexity increases, so does developmental instability and the complexity of the GPM, which makes it less likely that this complexity will be passed between generations and be selected.

Our results also imply that the evolution of complex and simple morphologies is qualitatively different. Complex morphologies evolve under a complex GPM and higher developmental instability. This reduces the efficiency of natural selection. From a classic neo-Darwinian paradigm this would imply that complex morphologies should evolve more slowly [Kauffman 1993, Wagner and Altenberg 1996]. However, complex morphologies produce a higher morphological diversity [Raff 1996] than simple morphologies for the same amount of genetic variation. This higher morphological diversity could allow for adaptation to a wider range of selective pressures on morphology [Salazar-Ciudad and Jernvall 2004].

The differences between complex and simple morphologies cannot be reduced to differences in evolutionary speed. There are differences in both the tempo and mode of evolution: simpler lineages can adapt faster but only within a smaller region of the morphospace; while complex lineages may evolve in wider regions of the morphospace. The observed evolutionary rates depend on the coarseness of the selective pressures on morphology, e.g., selection for precise small changes in a single trait versus selection for general features of overall morphology, the time scale considered and on how changes in morphology are measured.

It is relevant to stress that the evolutionary differences we encounter between complex and simple phenotypes are not due to natural selection. Our results are a mathematical necessity or constraint that becomes evident by considering how gene and cell interactions can be organized into networks to lead to pattern formation. In other words, the evolutionary differences between complex and simple morphologies are inherent to development, given the range of physical and logical properties of animal cells [Newman 2019]. These properties may themselves evolve but, at least over long-time scales, they should be considered inherent to development.

Finally, although the increase of morphological complexity during evolution becomes progressively slower as complexity increases, this does not imply that evolution will come to a halt. Since morphological complexity itself is most likely not being directly selected, it simply means that during evolution, there will be less morphological changes that increase complexity. Additionally, there are many phenotypic levels in which complexity can evolve

independently from each other, i.e. even if one phenotypic level complexity has come close to a maximum, other phenotypic levels might still increase their complexity. For example, metabolism, behavior, and culture have their own mechanisms of development with their own types of interactions [Jablonka and Lamb 2005], which determine their possible phenotypic variation. Although these other phenotypic levels have their own construction rules, they also use genetic, epigenetic and environmental information, in a process analogous to development. Therefore, we can expect that in these levels, if complexity increases for some reason, it will also slow down. However, innovations can eventually be found that facilitate an increase in complexity, at least until a new maximum is approached. For example, although gene networks might have been initially mainly responsible to stabilize and organize generic mechanisms, they eventually allowed to combine and regulate generic mechanisms in such a way that an increase in morphological complexity was possible. Thus, there might be some stepping-stones that open up new ways of increasing complexity, such as the evolution of multicellularity or certain adhesion molecules which enable tissues to behave as viscoelastic materials, opening a new repertoire of physical generic mechanisms and therefore new possible morphogenetic dynamics. After the evolution of one of these stepping-stones, we can expect an initial increase in complexity, at least at some phenotypic level. This is simply because as seen in Fig. 22, most of the parameter combinations form simple morphologies. Therefore, if this novelty evolves with some random initial parameter values (e.g. new adhesion molecules with random adhesion values), they will most likely fall in a space of low complexity. Starting from a low complexity, makes it more likely that complexity will increase over evolution, however, if complexity increases, it will do so at a progressively slower rate.

# REFERENCES


Adami C. What is complexity? BioEssays. 2002; 24, 1085-1094.

Adams DC, Rohlf FJ, Slice DE. Geometric morphometrics: ten years of progress following the 'revolution'. Italian Journal of Zoology. 2004; 71(1), 5-16.

Agassiz JLR. 1857. An essay on classification. London: Longman.

Aguilar-Rodríguez J, Peel L, Stella M, Wagner A, Payne JL. The architecture of an empirical genotype-phenotype map. Evolution. 2018; 72: 1242-1260.

Aguirre J, Buldú JM, Stich M, Manrubia SC. Topological structure of the space of phenotypes: the case of RNA neutral networks. PloS one. 2011; 6(10), e26324.

Alberch P. From genes to phenotype: dynamical systems and evolvability. Genetica. 1991;84: 5-11.

Alberch P. Ontogenesis and morphological diversification. Am. Zool. 1980; 20: 653-667

Alberch P. The logic of monsters: evidence for internal constraint in development and evolution. Geobios. 1989; 22, 21-57.

Alican NF, Thesleff H. Rethinking Plato's Forms. Arctos–Acta Philologica Fennica. 2013; 47, 11-47.

Apter MJ, Wolpert L. Cybernetics and development I. Information theory. Journal of theoretical Biology. 1965 8(2), 244-257.

Arias AM, Hayward P. Filtering transcriptional noise during development: concepts and mechanisms. Nature Reviews Genetics. 2006; 7(1), 34-44.

Armstrong PB and Child JS. Stages in the normal development of Fundulus heteroclitus. Biol. Bull. mar. biol. Lab. Woods Hole. 1965; 128, 143-168

Arthur, B. 1994. On the evolution of complexity. Complexity: Metaphors, Models, and Reality, Cowan, GA (ed), pp. 65–81, Addison-Wesley, Reading, MA.

Baer KE von, Dohrn A, Groeben C, Oppenheimer JM. 1993. Correspondence, Karl Ernst Von Baer, 1792-1876, Anton Dohrn 1840-1909. Philadelphia: American Philosophical Society.

Baer KE von. Über Entwickelungsgeschichte der Thiere [On the Development of Animals]. Königsberg: Borntraeger. 1828

Bard JBL. 1990. Morphogenesis: the cellular and molecular processes of developmental anatomy. Cambridge, UK: Cambridge University Press

Barry KL, Kokko H. Male mate choice: why sequential choice can make its evolution difficult. Animal Behaviour. 2010; 80(1), 163-169.

Bateson W. Evolutionary Faith and Modern Doubts. Nature. 1922; 109, 553–556.

Bayly PV, Okamoto RJ, Xu G, Shi Y, Taber LA. A cortical folding model incorporating stress-dependent growth explains gyral wavelengths and stress patterns in the developing brain. Phys Biol. 2013; 10(1):016005

Bei M. Molecular genetics of tooth development. Current opinion in genetics & development. 2009; 19(5), 504-510.

Boffetta G, Cencini M, Falcioni M, Vulpiani A. Predictability: a way to characterize complexity. Physics reports. 2002; 356(6), 367-474.

Bolker JA, Raff RA. Developmental genetics and tradi- tional homology. Bioessays. 1996; 18:489–494.

Bonner JT. 1988. The evolution of complexity by means of natural selection. Princeton University Press.

Brodland GW. 2015. How computational models can help unlock biological systems. In Seminars in cell & developmental biology (Vol. 47, pp. 62-73). Academic Press.

Brooks DR, Wiley EO, Brooks DR. (1988). Evolution as entropy. Chicago: University of Chicago Press.

Brun-Usan M, Marin-Riera M, Salazar-Ciudad I. On the effect of phenotypic dimensionality on adaptation and optimality. Journal of evolutionary biology. 2014; 27(12), 2614-2628.

Bunn JM, Boyer DM, Lipman Y, St Clair EM, Jernvall J, Daubechies I. Comparing Dirichlet normal surface energy of tooth crowns, a new technique of molar shape quantification for dietary inference, with previous methods in isolation and in combination. Am J Phys Anthropol. 2011; 145(2):247–261

Burns J. The synthetic problem and the genotype-phenotype relation in cellular metabolism. In: Waddington CH, editor. Towards a theoretical biology. 3. Drafts. An I.U.B.S. symposium. Chicago, USA: Aldine Publishing Company; 1970. pp. 47–51.

Candiello J, Balasubramani M, Schreiber EM, Cole GJ, Mayer U, Halfter W, Lin H. Biomechanical properties of native basement membranes. FEBS journal. 2007; 274(11): 2897-2908

Carroll SB. Chance and necessity: the evolution of morphological complexity and diversity. Nature. 2001; 409(6823), 1102-1109.

Cavalier-Smith T. 1985. The evolution of Genome Size. New York: John Wiley & Sons.

Charles C, Lazzari V, Tafforeau P, Schimmang T, Tekin M, Klein O, Viriot L. Modulation of Fgf3 dosage in mouse and men mirrors evolution of mammalian dentition. Proceedings of the National Academy of Sciences. 2009; 106(52), 22364-22368.

Charlesworth B, Lande R, Slatkin M. A Neo-Darwinian commentary on macroevolution. Evolution. 1982; 36: 474–498

Cisne JL. Evolution of the world fauna of aquatic free-living arthropods. Evolution. 1974; 337-366.

Comings DE. (1972). The structure and function of chromatin. In Advances in human genetics (pp. 237-431). Boston: Springer

Corning PA. Beyond the modern synthesis: A framework for a more inclusive biological synthesis. Progress in Biophysics and Molecular Biology. 2020; 153,5-12

Cotterell J, and Sharpe J. An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. Molecular systems biology. 2010;6(1), 425

Cotterell J, Sharpe J. An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. Molecular systems biology. 2010; 6(1), 425.

Cottingham MR, Stoothoff R, Kenny A. 1988. The Philosophical Writings of Descartes. Cambridge, UK Cambridge University Pres.

Cowperthwaite MC, Economo EP, Harcombe WR, Miller EL, Meyers LA. The ascent of the abundant: how mutational networks constrain evolution. PLoS Comput Biol. 2008; 4(7), e1000110.

Crick FHC. 1966. Of Molecules and Men. Seattle: University of Washington Press

Crow JF, Kimura M. 1970. An introduction to population genetics theory. An introduction to population genetics theory.

Darwin C. 1859. On the Origin of Species, London: John Murray.

Davidson, E. H. (2001). Genomic regulatory systems: in development and evolution. Elsevier.

Delbrück M. Statistical fluctuations in autocatalytic reactions. J. Chem. Phys. 1940; 8, 120–124.

Dingle K, Camargo CQ, Louis AA. Input-output maps are strongly biased towards simple outputs. Nature Communications. 2018;9(1).

Dingle K, Schaper S, Louis AA. The structure of the genotype–phenotype map strongly constrains the evolution of non-coding RNA. Interface focus. 2015; 5(6), 20150053.

Dobzhansky T. 1937. Genetics and the origin of species. Columbia: Columbia University Press.

Evans AR, Wilson GP, Fortelius M, Jernvall J. High-level similarity of dentitions in carnivorans and rodents. Nature. 2007; 445(7123), 78-81.

Evans AR, Wilson GP, Fortelius M, Jernvall J. High-level similarity of dentitions in carnivorans and rodents. Nature. 2007;445(7123):78–81

Evans ND, Minelli C, Gentleman E, LaPointe V, Patankar SN, Kallivretaki M., Chen X, Roberts CJ, Stevens MM. Substrate stiffness affects early differentiation events in embryonic stem cells. Eur cell mater. 2009; 18(1), e13.

Félix, M. A., & Wagner, A. (2008). Robustness and evolution: concepts, insights and challenges from a developmental model system. Heredity, 100(2), 132-140.

Fernández A, Lynch, M. Non-adaptive origins of interactome complexity. Nature. 2011; 474(7352), 502-505.

Fisher DC. 1986. Progress in Organismal Design, in Raup DM and Jablonski D (eds.), Patterns and Processes in the History of Life, Springer-Verlag, Berlin, pp. 99-117.

Fisher RA. 1930. The genetical theory of natural selection. Oxford: Claredon Press.

Fleming L, McShea DW. Drosophila mutants suggest a strong drive toward complexity in evolution. Evolution & Development. 2013; 15(1), 53-62.

Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. Nature. 2010; 463(7280), 485-492.

Fortuna MA, Zaman L, Ofria C, and Wagner A. The genotype-phenotype map of an evolving digital organism. PLoS computational biology. 2017;13(2), e1005414.

Garstang, W. The theory of recapitulation: A critical restatement of the Biogenetic Law. Proc. Linn. Soc. Lond. Zool. 1922; 35: 81–101

Gilbert SF and Barresi MJF. Developmental biology. 12th ed. New York: Sinauer Associates; 2019.

Gilbert SF, Opitz JM, Raff RA. Resynthesizing evolutionary and developmental biology. Developmental biology. 1996; 173, 357-372.

Gilbert SF, Sarkar S. Embracing Complexity: Organicism for the 21st Century. Developmental Dynamics. 2000; 219:1–9

Gilbert SF. Expanding the temporal dimensions of developmental biology: The role of environmental agents in establishing adult-onset phenotypes. Biological Theory. 2011; 6, 65-72.

Gilbert SF. The morphogenesis of evolutionary developmental biology. International Journal of Developmental Biology. 2003; 47, 467.

Gillispie CC. 1960.The edge of objectivity; an essay in the history of scientific ideas. Princeton University Press.

Gjuvsland AB, Wang Y, Plahte E, Omholt SW. Monotonicity is a key feature of genotype-phenotype maps. Frontiers in genetics. 2013; 4, 216.

Goldschmidt RB. 1940. The Material Basis of Evolution. Yale University Press.

Goldschmidt RB. Chromosomes and genes. Cold Spring Harb Symp Quant Biol. 1951; 16:1-11

Gould SJ, Lewontin RC. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. Proceedings of the royal society of London. Series B. Biological Sciences. 1979; 205(1161), 581-598.

Gould SJ. 1988. On Replacing the Idea of Progress with an Operational Notion of Directionality, in Nitecki NH (ed.), Evolutionary Progress, University of Chicago Press, Chicago, pp. 319-338.

Gould SJ. 2002. The structure of evolutionary theory. Cambridge, MA: Harvard University Press.

Gould SJ. The evolution of life on the earth. Scientific American. 1994 271(4), 84-91.

Gower JC. Generalized procrustes analysis. Psychometrika. 1975; 40(1), 33-51.

Graner F, Glazier JA. Simulation of biological cell sorting using a two-dimensional extended Potts model. Phys. Rev. Lett. 1992; 69(13):2013–2016.

Grant PR, Grant BR. Evolution of character displacement in Darwin's finches. Science. 2006; 313(5784), 224-226.

Green RM, Fish JL, Young NM, Smith FJ, Roberts B, Dolan K, Choi I, Leach CL, Gordon P, Cheverud JM, Roseman CC, Williams TJ, Marcucio RS, Hallgrímsson B. Developmental nonlinearity drives phenotypic robustness. Nature communications. 2017; 8(1), 1-12.

Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. Nature reviews Molecular cell biology. 2005; 6(8), 622-634.

Haeckel E. 1866. Generelle morphologie der organismen. Berlin: G. Reimer 1866.

Hahn MW, Wray GA. The g-value paradox. Evolution and Development. 2002; 4(2), 73-75.

Hallgrímsson B, Willmore K, Hall BK. Canalization, developmental stability, and morphological integration in primate limbs. Am J Phys Anthropol. 2002; 35:131-158.

Harjunmaa E, Kallonen A, Voutilainen M, Hämäläinen K, Mikkola ML, Jernvall J. On the difficulty of increasing dental complexity. Nature. 2012; 483:324–7.

Heim NA, Payne JL, Finnegan S, Knope ML, Kowalewski M, Lyons SK, ..., Wang SC. Hierarchical complexity and the size limits of life. Proceedings of the Royal Society B: Biological Sciences. 2017; 284(1857), 20171039.

Hertwig O. Urmund und Spina bifida. Archiv fur mikroskopische Anatomie. 1892; 39,353-503

Hinegardner R, Engelberg J. Biological complexity. Journal of Theoretical Biology. 1983; 104(1), 7-20.

Hoffmann AA. 2014. Evolutionary limits and constraints. Pp. 247–252 in J. B. Losos, ed. The Princeton guide to evolution. Princeton Univ. Press.

Horder T. 2010. History of developmental biology. In Encyclopedia of Life sciences. Chichester: Willey and Sons.

Huxley J, De Beer G. 1963. The elements of experimental embryology. New York: Hafner Pub. Co.

Huynen MA. Exploring phenotype space through neutral evolution. Journal of molecular evolution. 1996; 43(3), 165-169.

Ispolatov I, Alekseeva E, Doebeli M. Competition-driven evolution of organismal complexity. PLoS computational biology. 2019; 15(10), e1007388.

Jablonka E, Lamb MJ. Evolution in four dimensions: genetic, epigenetic, behavioral, and symbolic variation in the history of life. MIT Press Cambridge; 2005

Jablonka E, Lamb MJ. The changing concept of epigenetics. Annals of the New York Academy of Sciences. 2002; 981, 82-96.

Jaeger J, Surkova S, Blagov M, Janssens H, Kosman D, Kozlov KN, Platt OM, Myasnikova E, Vanario-Alonso CE, Samsonova M, Sharp DH, Reinitz J. Dynamic control of positional information in the early Drosophila embryo. Nature. 2004; 430, 368-371.

Jörg T, Martin OC, Wagner A. Neutral network sizes of biological RNA molecules can be computed and are not atypically small. BMC bioinformatics. 2008; 9(1), 1-12.

Katz MJ. 1986. Templets and the explanation of complex patterns. Cambridge: Cambridge Univ. Press.

Kauffman SA. Origins of Order. Oxford University Press; 1993

Kauffman, S. A. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. Journal of theoretical biology, 22(3), 437-467.

Kimura, M. 1983. The neutral theory of molecular evolution. Cambridge Univ. Press, Cambridge, U.K.

Klingenberg CP, Nijhout HF. Genetics of fluctuating asymmetry: a developmental model of developmental instability. Evolution. 1999; 53(2), 358-375.

Klingenberg CP. Phenotypic plasticity, developmental instability, and robustness: The concepts and how they are connected. Frontiers in Ecology and Evolution. 2019; 7, 56.

Knoll, AH, Bambach RK (2000). Directionality in the history of life: diffusion from the left wall or repeated scaling of the right? Deep Time: Paleobiology's Perspective, Erwin, D H and Wing, S L (eds), Vol. 26, 1–14

Kolmogorov AN. Three approaches to the quantitative definition of information'. Problems of information transmission. 1965; 1(1), 1-7.

Lacy RC. Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. Conservation biology. 1987; 1(2), 143-158.

Lahti DC, Johnson NA, Ajie BC, Otto SP, Hendry AP, Blumstein DT, Coss RG, Donohue K, Foster SA. Relaxed selection in the wild. Trends in ecology & evolution. 2009;24: 487-496

Lamarck J. Phylosophie zoologique. 1809.

Lande, R. Natural selection and random genetic drift in phenotypic evolution. Evolution. 1976; 314-334.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., ... & Funke, R. (2001). Initial sequencing and analysis of the human genome.

Laubichler M, Maienschein J. 2007. From embryology to evo-devo: a history of developmental evolution. Massachusetts: MIT Press Cambridge.

Lenski, RE, Rose MR, Simpson SC, Tadler SC. Long-term experimental evolution in Escherichia coli. I. Adaptation and divergence during 2,000 generations. The American Naturalist. 1991; 138(6), 1315-1341.

Lewontin RC. 1974. The genetic basis of evolutionary change. London: Columbia University press.

Li W. On the relationship between complexity and entropy for markov chains and regular languages. Complex Systems. 1991; 5(4):381–399,

Lillie FR. 1898¬¬¬¬. Adaptation in cleavage. In Biological Lectures from the Marine Biological Laboratory, Woods Hole, Massachusetts. Ginn, Boston, pp. 43–67.

Lineweaver CH, Davies PC, Ruse M. What is complexity? Is it increasing? In: Complexity and the Arrow of Time. Cambridge University Press; 2010. pp. 3–16

Lorenz K, In: The Butterfly effect, book: The Chaos Avant-garde: Memories of the Early Days of Chaos Theory eds. Ralph Abraham, Yoshisuke Ueda. 2001.

Lukeš J, Archibald JM, Keeling PJ, Doolittle WF, Gray MW. How a neutral evolutionary ratchet can build cellular complexity. IUBMB life. 2011; 63(7), 528-537.

Manuel EF. 1975. The religion of Isaac Newton. London, Oxford University Press.

Marin-Riera M, Brun-Usan M, Zimm R, Välikangas T, Salazar-Ciudad I. Computational modeling of development by epithelia, mesenchyme and their interactions: a unified model. Bioinformatics. 2016;15: 219-25

Martin AC, Goldstein B. Apical constriction: themes and variations on a cellular mechanism driving morphogenesis. Development. 2014; 141(10):1987–98

Mayer MP, Bukau B. Molecular chaperones: the busy life of Hsp90. Current Biology. 1999; 9(9). 1999; R322-R325.

Maynard Smith J. Studium Generale. 1970; 23, 266.

Mayr E. 1963. Animal species and evolution. Cambridge, MA: Harvard University Press.

Mayr E. 1981. The Growth of Biological Thought. Cambridge: Harvard

Mazzocchi F. Complexity in biology: exceeding the limits of reductionism and determinism using complexity theory. EMBO reports. 2008; 9, 10-14.

McCoy JW. Complexity in organic evolution. Journal of theoretical biology. 1977; 68(3), 457-458.

McShea DW. A metric for the study of evolutionary trends in the complexity of serial structures. Biological Journal of the Linnean Society. 1992; 45(1), 39-55.

McShea DW. Complexity and evolution: what everybody knows. Biology and Philosophy. 1991; 6(3), 303-324.

McShea DW. Functional complexity in organisms: parts as proxies. Biology and Philosophy. 2000; 15(5), 641-668.

McShea DW. The evolution of complexity without natural selection, a possible large-scale trend of the fourth kind. Paleobiology. 2005; 31, 146-156.

Miles AEW, Grigson C. (Eds.). 2003. Colyer's Variations and Diseases of the Teeth of Animals. Cambridge University Press.

Milocco L, Salazar-Ciudad I. Is evolution predictable? Quantitative genetics under complex genotype-phenotype maps. Evolution. 2020; 74(2), 230-244.

Milton CC, Huynh B, Batterham P, Rutherford SL, Hoffmann AA. Quantitative trait symmetry independent of Hsp90 buffering: distinct modes of genetic canalization and developmental stability. Proceedings of the National Academy of Sciences. 2003; 100(23), 13396-13401.

Morgan TH. 1932. The scientific basis of evolution. New York: WW Norton & Co.

Müller Fritz. 1864.Für Darwin.Leipzig: Wilhelm Engelmann.

Müller GB, Wagner GP. Novelty in evolution: Restruc- turing the concept. Annu Rev Ecol Syst. 1991; 22:229–256.

Nelson CM. On buckling morphogenesis. Journal of biomechanical engineering. 2016; 138(2).

Nerurkar NL, Mahadevan L, Tabin CJ. BMP signaling controls buckling forces to modulate looping morphogenesis of the gut. Proc. Natl. Acad. Sci. 2017; 114(9):2277–2282.

Newman SA, Comper WD. 'Generic' physical mechanisms of morphogenesis and pattern formation. Development. 1990; 110(1), 1-18.

Newman SA, Forgacs G, Muller GB. Before programs: the physical origination of multicellular forms. International Journal of Developmental Biology. 2003; 50(2-3), 289-299.

Newman SA, Müller GB. Epigenetic mechanisms of character origination. Journal of Experimental Zoology. 2000; 288, 304-317.

Newman SA. Inherent forms and the evolution of evolution. J Exp Zool B Mol Dev Evol. 2019; 332: 331-338

Nijhout HF. Metaphors and the roles of genes in development. Bioessays. 1990; 12:441–446.

Odell G, Oster G, Burnside B, Alberch P. A mechanical model for epithelial morphogenesis. Journal of mathematical biology. 1980; 9, 291-295.

Orr HA. Adaptation and the cost of complexity. Evolution. 2000; 54: 13–20.

Oster G, Alberch P. Evolution and bifurcation of developmental programs. Evolution. 1982; 444-459.

Oyama S. 1985. The ontogeny of information. Cambridge: Cambridge University Press.

Papentin F. Binary sequences. I. complexity. Information Sciences. 1983; 31(1), 1-14.

Partridge L, Halliday T. 1984. Mating patterns and mate choice In: Krebs JR, Davies NB (eds) Behavioural ecology. An evolutionary approach.

Payne JL, Moore JH, Wagner A. Robustness, evolvability, and the logic of genetic regulation. Artificial life. 2014; 20(1), 111-126.

Payne JL, Wagner A. Constraint and Contingency in Multifunctional Gene Regulatory Circuits. PLOS Computational Biology. 2013; 9(6): e1003071.

Petrie RJ, Doyle AD, Yamada KM. Random versus directionally persistent cell migration. Nature reviews Molecular cell biology. 2009; 10(8), 538-549.

Pézeron G, Mourrain P, Courty S, Ghislain J, Becker TS, Rosa FM, David NB. Live analysis of endodermal layer formation identifies random walk as a novel gastrulation movement. Current Biology. 2008; 18(4), 276-281.

Pigliucci M. Genotype–phenotype mapping and the end of the 'genes as blueprint' metaphor. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010; 365, 557-566.

Raff RA, Kauffman NTC. 1983. Embryos, Genes, and Evolution. New York: Macmillan.

Raff RA. The Shape of Life. University of Chicago Press; 1996

Reidys C, Stadler PF, Schuster P. Generic properties of combinatory maps: neutral networks of RNA secondary structures. Bulletin of mathematical biology. 1997; 59(2), 339-397.

Reinitz J, Sharp DH. Mechanism of eve stripe formation. Mech. Dev. 1995; 49(1–2):133–58.

Rensch B. The laws of evolution. 1960. University of Chicago Press.

Rensh B. 1954: Neuere Probleme der Abstammungslehre: die transspezifische Evolution. Stuttgard: Ferdinand Enke.

Richter K, Buchner J. Hsp90: chaperoning signal transduction. Journal of cellular physiology. 2001; 188(3), 281-290.

Riedl R. 1975: Die Ordnung des Lebendigen. Systembedingungen der Evolution. Berlin: Paul Parey.

Rigau J, Feixas M, Sbert M. Shape complexity based on mutual information. In International Conference on Shape Modeling and Applications 2005 (pp. 355-360). IEEE.

Rohlf FJ, Slice D. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Biology. 1990; 39(1), 40-59.

Ruse M. Charles Darwin and artificial selection. Journal of the History of Ideas. 1975; 36, 339-350.

Salazar-Ciudad I, Garcia-Fernández J, Solé RV. Gene networks capable of pattern formation: from induction to reaction-diffusion. Journal of Theoretical Biology. 2000;205: 587-603.

Salazar-Ciudad I, Jernvall J, Newman SA. Mechanisms of pattern formation in development and evolution. Development. 2003; 130: 2027-37

Salazar-Ciudad I, Jernvall J. A computational model of teeth and the developmental origins of morphological variation. Nature. 2010; 464, 583–586.

Salazar-Ciudad I, Jernvall J. Graduality and innovation in the evolution of complex phenotypes: insights from development. J Exp Zool B Mol Dev Evol. 2005;304: 619-31

Salazar-Ciudad I, Jernvall J. How different types of pattern formation mechanisms affect the evolution of form and development. Evolution and Development. 2004;6: 6-16.

Salazar-Ciudad I, Marin-Riera M. Adaptive dynamics under development-based genotype-phenotype maps. Nature. 2013;497: 361-364.

Salazar-Ciudad I, Newman SA, Solé RV. Phenotypic and dynamical transitions in model genetic networks I. Emergence of patterns and genotype-phenotype relationships. Evolution and Development. 2001;3(2):84-94

Salazar-Ciudad I. On the origins of morphological disparity and its diverse developmental bases. BioEssays. 2006; 28(11), 1112-1122.

Salazar-Ciudad I. Developmental constraints vs. variational properties: How pattern formation can help to understand evolution and development. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution. 2006; 306(2), 107-125.

Salazar-Ciudad I. On the origins of morphological variation, canalization, robustness, and evolvability. Integrative and Comparative Biology. 2007; 47(3), 390-400.

Saunders PT, Ho MW. On the increase in complexity in evolution II. The relativity of complexity and the principle of minimum increase. Journal of Theoretical Biology. 1981; 90(4), 515-530.

Saunders PT, Ho MW. On the increase in complexity in evolution. Journal of Theoretical Biology. 1976; 63(2), 375-384.

Savin T, Kurpios NA, Shyer AE, Florescu P, Liang H, Mahadevan L, Tabin CJ. On the growth and form of the gut. Nature. 2011; 476(7358), 57

Schaper S, Louis AA. The arrival of the frequent: how bias in genotype-phenotype maps can steer populations to local optima. PloS one. 2014; 9(2), e86635.

Schuster P, Fontana W, Stadler PF, Hofacker IL. From sequences to shapes and back: a case study in RNA secondary structures. Proceedings of the Royal Society of London. Series B: Biological Sciences. 1994; 255(1344), 279-284.

Sharon E, Efrati E. The mechanics of non-Euclidean plates. Soft Matter. 2010; 6(22), 5693-5704.

Sharpe J. Computer modeling in developmental biology: growing today, essential tomorrow. Development. 2017; 144(23), 4214-4225.

Sheets HD, Covino KM, Panasiewicz JM, Morris SR. Comparison of geometric morphometric outline methods in the discrimination of age-related differences in feather shape. Frontiers in zoology. 2006; 3(1), 15.

Shoval O, Sheftel H, Shinar G, Hart Y, Ramote O, Mayo A, ... & Alon U. Evolutionary trade-offs, Pareto optimality, and the geometry of phenotype space. Science. 2012; 336(6085), 1157-1160.

Simpson GG. The Meaning of Evolution. 1949. New Haven: Yale University Press.

Smith E. Emergent order in processes: the interplay of complexity, robustness, correlation, and hierarchy in the biosphere In: Complexity and the Arrow of Time. Cambridge University Press; 2010. pp. 191–223

Sneath PHA. 1964. Comparative biochemical genetics in bacterial taxonomy. Pp. 565–583 in C. A. Leone, ed. Taxonomic biochemistry and serology. Ronald, New York.

Stanley KO. Compositional pattern producing networks: a novel abstraction of development. Genet. Program. Evolvable. 2007; 8, 131–162

Stanley SM. An Explanation for Cope's Rule. Evolution. 1973; 27, 1-26.

Steinberg MS. Differential adhesion in morphogenesis: a modern view. Current opinion in genetics & development. 2007; 17(4), 281-286.

Stoltzfus A. On the possibility of constructive neutral evolution. Journal of Molecular Evolution. 1999;49: 169-181.

Stoltzfus, A. Constructive neutral evolution: exploring evolutionary theory's curious disconnect. Biology direct. 2012; 7(1), 35.

Striedter GF, Srinivasan S, Monuki ES. Cortical Folding: When, Where, How, and Why? Annu. Rev. Neurosci. 2015; 38(1):291–307

Svoboda P, Di Cara A. Hairpin RNA: a secondary structure of primary importance. Cellular and Molecular Life Sciences CMLS. 2006; 63, 901-908.

Takeuchi N, Hogeweg P. Multilevel Selection in Models of Prebiotic Evolution II: A Direct Comparison of Compartmentalization and Spatial Self-Organization. PLOS Computational Biology. 2009; 5(10): e1000542.

Thomason RT, Bader DM, Winters NI. Comprehensive timeline of mesodermal development in the quail small intestine. Dev. Dyn. 2012; 241(11):1678–94.

Turing AM. On computable numbers, with an application to the Entscheidungsproblem. Proceedings of the London mathematical society. 1937; 2(1), 230-265.

Turing, A. (2013). The chemical basis of morphogenesis (1952). B. Jack Copeland, 519.

Uller T, Moczek AP, Watson RA, Brakefield PM, Laland KN. 2018. Developmental bias and evolution: A regulatory network perspective. Genetics, 209(4), 949–966.

Valentine JW, Collins AG, Meyer CP. Morphological complexity increase in metazoans. Paleobiology. 1994;20(2):131–42

Voit, EO. Perspective: Dimensions of the scientific method. PLoS Computational Biology. 2019; 15(9), e1007279.

von Bertalanffy L, Pirozynski WJ. Ontogenetic and evolutionary allometry. Evolution. 1952; 387-392.

Vroomans RM, Hogeweg P, and ten Tusscher KH. In silico evo-devo: reconstructing stages in the evolution of animal segmentation. EvoDevo. 2016;7(1), 14

Waddington CH. 1968. The basic ideas of biology. In: Towards a Theoretical Biology, Vol. 1: Prolegomena. C.H. Waddington, Ed.: 1–32, Edinburgh: Edinburgh University Press.

Waddington CH. Canalization of development and the inheritance of acquired characters. Nature. 1942; 150(3811), 563-565.

Waddington CH. Genetic assimilation of an acquired character. Evolution. 1953: 118-126.

Wagner GP, Altenberg L. Perspective: complex adaptations and the evolution of evolvability. Evolution. 1996; 50: 967-976

Wagner GP, Zhang J. The pleiotropic structure of the genotype–phenotype map: the evolvability of complex organisms. Nature Reviews Genetics. 2011; 12(3), 204-213.

Wake DB, Connor EF, de Ricqles AJ, Dzik J, Fisher DC, Gould SJ, LaBarbera M, Meeter DA, Mosbrugger V, Reif WE, Rieger RM, Seilacher A, Wagner GP. 1986, 'Directions in the History of Life', in Raup DM and Jablonski D (eds.), Patterns and Processes in the History of Life, Springer-Verlag, Berlin, pp. 47-67.

Wake DB, Larson A. Multidimensional analysis of an evolving lineage. Science. 1987; 238(4823), 42-48.

Wartlick O, Kicheva A, González-Gaitán M. Morphogen gradient formation. Cold Spring Harbor perspectives in biology. 2009; 1(3), a001255.

Webster and Goodwin. 1996. Form and transformation. Cambridge University Press

Weiß M, Ahnert SE. Using small samples to estimate neutral component size and robustness in the genotype–phenotype map of RNA secondary structure. Journal of the Royal Society Interface. 2020; 17(166), 20190784.

Wicken JS. The generation of complexity in evolution: A thermodynamic and information-theoretical discussion. Journal of Theoretical Biology. 1979; 77(3), 349-365.

Williams GC. 1966. Adaptation and Natural Selection. Princeton: Princeton University Press, pp. 42-55.

Williams, CA. Neo-Darwinism is just fine. Journal of Experimental Biology. 2015; 218, 2658-2659.

Wilson EB. 1896. The Cell in Development and Inheritance. New York: Macmillan, 1928

Young JC, Moarefi I and Hartl FU. Hsp90: a specialized but essential protein-folding tool. The Journal of cell biology. 2001; 154(2), 267-274.

Zakharov VM. Future prospects for population phenogenetics. Soviet Scientific Reviews F Physiology and General Biology. 1989; 4: 1-79