Estimating complexity and adaptation in the embryo: a statistical developmental biology approach

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ACADEMIC DISSERTATION

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Contents

List of publications

This thesis is based on the following original publications which are referred to by their roman numerals in the text:

I **Salvador-Martínez, I.**, Salazar-Ciudad, I., 2015. How complexity increases in development: An analysis of the spatial-temporal dynamics of 1218 genes in Drosophila melanogaster. *Dev. Biol.* **405**, 328-339. doi:10.1016/j.ydbio.2015.07.003

II **Salvador-Martínez, I.**, Salazar-Ciudad, I. How complexity increases in development: An Analysis of the Spatial-Temporal Dynamics in *Ciona intestinalis*. Manuscript.

III **Salvador-Martínez, I.***, Coronado-Zamora, M.*, Castellano, D.*, Barbadilla, A., and Salazar-Ciudad, I. Mapping Natural Selection within *Drosophila melanogaster* embryo's anatomy. Manuscript.

IV Coronado-Zamora, M.*, **Salvador-Martínez, I.***, Castellano, D., Barbadilla, A., and Salazar-Ciudad, I. When in development: Estimating natural selection from the DGRP Estimating natural selection through *Drosophila* development from population genomics. Manuscript.

Author contributions

In study I, I. S-C. and I. S-M. conceived the study, I. S-M. analyzed the data and performed the statistical analysis, I. S-C. and I. S-M. wrote the manuscript.
In study II, I. S-C and I. S-M. conceived and designed the study. I. S-M. analyzed the data and performed the statistical analysis, I. S-C. and I. S-M. wrote the manuscript.
In study III, ISM analyzed gene age, gene expression and the relationship between codon usage gene expression and adaptation. MCZ calculated the selection estimators. DC help MCZ. ISC, AB , ISM and DC designed the study. ISC conceived the study. ISC wrote the article with the help of all co-authors.
In study IV, ISM analyzed the gene expression data through the life cycle and performed the clustering. ISM and MCZ performed the statistical analysis. MCZ calculated the selection estimators and the genomic determinants. DC help MCZ. ISC, AB, ISM and DC designed the study. ISC conceived the study. ISC wrote the article with the help of all co-authors.

*These authors contributed equally to this work.

Abbreviations

α	Proportion of adaptive nucleotide substitutions
ω	dN/dS ratio
ω_α	Proportion of adaptive non-synonymous substitutions
Dn	Non-synonymous (inter-specific) divergence per site
Ds	Synonymous (inter-specific) divergence per site
N_e	Effective population size
Pn	Synonymous (intra-specific) polymorphism per site
Ps	Synonymous (intra-specific) polymorphism per site
s	Selection coefficient
2D	two-dimensional
3D	three-dimensional
A/P	anterior/posterior
ANOVA	Analysis of Variance
BDGP	Berkeley Drosophila Genome Project
bp	base pairs (nucleotides)
CNS	Central Nervous System
D/V	dorsal/ventral
DFE	Distribution of Fitness Effects
DGRP	The *Drosophila melanogaster* Genetic Reference Panel
DNA	Deoxyribonucleic acid
DNE	Dirichlet Normal Energy
Fop	Frequency of optimal codons (a measure of codon bias)
GF	Growth Factor
GIS	Geographic Information Systems
GRN	Gene Regulatory Network
HG	Hourglass model
IQR	Inter Quartile Range

Contents

KW Kruskal-Wallis test

miRNAs Micro RNAs

MKT MacDonald-Kreitman test

PCA Principal Component Analysis

PNS Peripheral Nervous System

RNA Ribonucleic acid

RTK receptor tyrosine kinase

SEM Standard error of the mean

SFS Site Frequency Spectrum

SIGs Signaling molecules

siRNAs Small interference RNAs

SNP Single nucleotide polymorphism

ST Synexpression territories

TAI Transcriptome Age Index

Abstract

Embryonic development has amazed scientists for centuries. Many reasons have been suggested for the perceivable increase in complexity in development, during which a single cell into a larva or an adult. At the level of gene expression, it its assumed that genes change from being expressed in large spatial domains of the embryo in early development to spatially restricted domains (e.g., tissues, cells) in late development. For many developmental genes, the spatio-temporal expression dynamics have been thoroughly described. It is not clear however, if the global dynamics are similar, or if there are differences between types of genes or between species.

Adaptive reasons have been also said to be the cause for the increase in complexity. Adaptations could be estimated with molecular evolution methods based on the analysis of genes expressed in different developmental stages or regions in the embryo. These methods estimate adaptive changes at the DNA sequence level assuming that a positive selected site would show less variance than other sites evolving neutrally. Different developmental stages might show distinct levels of positive or stabilizing selection, that could be related to inter-specific divergence patterns proposed by the von Baer's laws or the hourglass model. The former states that the development of two species of a phylogenetic group would be very similar in early stages and increasingly divergent in subsequent stages. In contrast, the latter states that development is less divergent (more conserved) at mid development.

In here, I analysed gene expression information to estimate both complexity and adaptation in the embryo using a statistical approach. To measure complexity, I developed quantitative measures of spatial complexity and used them in publicly available gene expression data (thousands of in situ hybridization experiments) in *Drosophila melanogaster* and *Ciona intestinalis* from the BDGP/FlyExpress and ANISEED databases respectively. To estimate adaptation, I combined diverse *D. melanogaster* gene expression data (modENCODE, in situ images from the BDGP/FlyExpress and gene expression data based on a controlled vocabulary of the embryo anatomy) with population genomic data (from the DGRP project). Using the DFE-alpha method (which uses coding-region polymorphism and divergence to estimate the proportion of adaptive changes) , I charted a spatial map on adaptation of the fruit fly embryo's anatomy. Finally, I analysed the pattern of positive selection on genomic coding regions of genes expressed through the entire life cycle of *D. melanogaster* and how it correlated with specific genomic determinants (e.g., gene structure, codon bias).

Briefly, I found that *Drosophila* and *Ciona* complexity increases non-linearly with the major change in complexity being before and after gastrulation, respectively. In both species, transcription factors and signalling molecules showed an earlier compartmentalization, consistent with their proposed leading role in pattern formation. In *Drosophila*, gonads and head showed high adaptation during embryogenesis, although pupa and adult male stages exhibit the highest levels of adaptive change, and mid and late embryonic stages show high conservation, showing an HG pattern. Furthermore, I propose that the Hourglass model can be predicted by specific genetic and genomic features.

1 Review of the literature

During the last decades the scientific community has witnessed the flourishing of modern developmental biology (although developmental biology can not be considered a young scientific discipline, as its roots come from centuries ago from embryology and anatomy). Since the 1980's crucial discoveries (Gilbert, 1998) have improved our understanding of the developmental process in many model organisms.

Most of the modern developmental biology studies use an "individualistic" approach (Davidson, 2009), e.g., focusing only on the description of some gene's effect on the development of a specific structure or the role of a gene in a specific signalling pathway. This individualistic approach has increased substantially the knowledge in the developmental biology field and has accumulated a great amount of gene expression information in many years of collective efforts of the developmental biology community. The emergence of methods like DNA microarrays extended the determination of the expression of a single gene to a genomic level, allowing new systemic approaches to study gene expression during development. An example of the results obtained by these approaches is the identification of groups of temporal co-expressing genes during development (e.g., Arbeitman et al., 2002; Hooper et al., 2007).

The majority of the systemic approaches on gene expression during development have focused on the temporal analysis of expression, without considering the spatial distribution of the expressing genes in the embryo (there are however some noteworthy studies that have analysed the spatial patterns of gene expression during development, e.g., Gurunathan et al., 2004; Tomancak et al., 2007; Frise et al., 2010; Crombach et al., 2012; Konikoff et al., 2012).

The analysis of the spatial patterns of gene expression is now facilitated by recent high-throughput in situ hybridization approaches (Tomancak et al., 2002; Pollet et al., 2003; Imai et al., 2004; Christiansen et al., 2006; Lécuyer et al., 2007; Tassy et al., 2010), which have not only further increased the amount of spatio-temporal gene expression data during development of some model organisms, but also allow straightforward comparisons between gene expression patterns using computational methods. Therefore, the availability of gene expression at a genomic level allows to shift the focus of developmental biology from the study of single genes to a systemic approach in which the global statistical properties of development can be investigated.

The individualistic approach is also common in studies that aim to detect natural selection. Most studies that directly search for adaptation at the phenotypic level analyse only a single trait or a small number of traits (Hoekstra et al., 2001; Hereford et al., 2004). However, there is no study that has estimated natural selection over the entire body of an organism. As any adaptive change in the phenotype is expected to be partially caused by genetic mutation, an alternative to detect natural selection is the analysis of DNA sequences of genes expressing differentially in different parts of an organism's body. This could be extended to different stages in the life cycle of an organism if there is enough spatio-temporal gene expression information.

In the next subsections, I will make an introduction of the study of complexity and adaptation during embryonic development, emphasizing the methods and concepts that have been previously (or could be potentially) used to analyse both. Before I do this, it might be useful to define what is development. So firstly, I will address this apparently simple question.

What is development?

> *" It is not enough to see that horse pulling a cart past the window as the good working horse it is today; the picture must also include the minute fertilised egg, the embryo in its mother's womb, and the broken-down old nag it will eventually become."*
>
> C. H. Waddington 1957

It seems that there is no unique or straightforward answer to this question. Sometimes, the study of development is implicitly considered to be the same as the the study of embryology (Horder, 2010). This could be problematic when considering organisms with complex life cycles. For example, holometabolous insects, in addition to embryonic development, undergo a complete metamorphosis (from pupa to adult). This post-embryonic development shows clear similarities to its embryonic counterpart, specially in the imaginal disc pattern formation.

Currently, the most common definition of development refers to the set of processes through which an egg is transformed into an adult (Horder, 2010; Minelli, 2011). Already in 1880, Ernst Haeckel defined development in similar terms: "individual development, or the ontogenesis of every single organism, from the egg to the complete form is nothing but a growth attended by a series of diverging and progressive changes" (Haeckel, 1880).

Some authors criticize this egg-to-adult view to be an "adultocentric" view of development, and suggest instead to consider within the boundaries of development the whole life cycle of an organism (Gilbert, 2011; Minelli, 2011). Julian S. Huxley and Gavin R. de Beer said that development "is not merely an affair of early stages; it continues, though usually at a diminishing rate, throughout life" (Huxley and De Beer, 1963).

There have been recent attempts to construct a broader concept of development (Griesemer, 2014; Moczek, 2014; Pradeu, 2014) For example, Armin P. Moczek defines development as "the sum of all processes and interacting components that are required to allow organismal form and function, on all levels of biological organization, to come into being" (Moczek, 2014). The main challenge on adopting a new concept of development which is more inclusive, is to maintain its intuitiveness and applicability in scientific research.

Throughout this dissertation I will use the "common view" of development (Minelli, 2014), that considers the egg and the adult as the start and end of individual development respectively. However, and mainly for practical reasons, the major part of the analyses presented here (sudies I-III) are restricted to embryonic development.

1.1 Complexity

> *"The embryo in the course of development generally rises in organisation (...) I am aware that it is hardly possible to define clearly what is meant by the organisation being higher or lower. But no one probably will dispute that the butterfly is higher than the caterpillar."*
>
> Charles Darwin 1859

In this section, I will talk about the increase in complexity during embryonic development. A common intuitive notion of complexity relates to a system composed of

many elements with multiple interactions between these elements. However, some could consider something to be complex while other consider it to be simple. Is important to mention that there is actually no consensus in the definition of complexity, or how to measure it. It is indeed hard to find a definition of complexity that could be applied to the many different phenomena. Also, it could be that a specific method to estimate complexity only account for the complexity at a given system level. It would be more appropriate to use therefore several measures of complexity instead of only one. Consequently, in this work I will use three different measures that relate to different intuitive aspects of complexity during embryonic development. But first, I will review some of the current definitions (and measures) of complexity that have been applied to organisms. Then, I will explore the notion of complexity increase during development, the relation between complexity in evolution and development, and discuss the possibility of a trend in terms of complexity increase through evolution.

1.1.1 Different definitions of complexity

Complexity in informational terms

The use of informational terms (e.g., transcription, translation and code) in biology are widespread, specially in molecular biology (Smith, 2000; Yockey, 2005) More than just the use of informational terms in biology, information theory concepts like Shannon's entropy and mutual information have been used as a proxy to measure complexity. In the following paragraphs, I will briefly describe briefly these concepts and provide some examples of their use to address biological complexity.

Shannon's entropy Shannon's entropy is a measure of uncertainty. Given a set of n possible events whose probabilities of occurrence are $p_1, p_2..., p_n$, Shannon's entropy (H) can be defined as:

$$H = -\sum_{i=1}^{n} p_i \log p_i$$

Therefore, for a given n, the maximum H is equal to $\log n$ when all the events have the same probability (i.e., $\frac{1}{n}$) (Shannon, 1948). The logarithmic base of 2 corresponds to binary digits units, or *bits*.

As an example, the entropy (H) for a nucleotide position in the DNA sequence. As in principle, each DNA site can take four possible values (A, T, G or C), its maximal entropy can be calculated as:

$$H_{max} = -\sum_{i=A,T,G,C} p(i) \log_2 p(i) = \log_2 4 = 2bits$$

Cristoph Adami have used Shannon's entropy to define a "physical complexity" measure, that refers to the "amount of information that is stored in that sequence about a particular environment" (Adami, 2002). More specifically, Adami's complexity measure compares the maximum entropy of a specific DNA sequence with the "actual" entropy based on the actual probabilities $p_j(i)$ for each position j in the sequence. Given a pool of N sequences, $p_j(i)$ is estimated by counting the number $n_j(i)$ of occurrences of nucleotide i at position j, so that $p_j(i) = n_j(i)/N$ (for all positions $j = 1, ..., L$ of

the sequence with length L) (Adami et al., 2000). The information content of a DNA sequence is then $I = H_{max} - H$ where:

$$H = -\sum_{j=1}^{L} \sum_{i=A,T,G,C} p_j(i) \log_2 p_j(i)$$

Adami assumes that if a sequence has not been under selective pressures each position in the sequence would have any of the four nucleotides with the same probabilities, so the actual entropy would be equal to the maximal, and consequently the information would be zero (Adami, 2002). He also considers that the "physical complexity" would serve as a good predictor of functional complexity (Adami, 2004). His information measure is related to the degree of conservation of a given sequence, which in the case of protein sequence has indeed been used to identify its functionality (Casari et al., 1995; Kellis et al., 2003; Hannenhalli and Russell, 2000).

Mutual information A concept related to Shannon's entropy that has been used in biological sciences is the concept of mutual information. Mutual information is a measure of the information in one variable about another. It is measured using the "conditional entropy" concept (the entropy of a variable Y given that X is known) also introduced by Shannon (1948). The mutual information $I(X;Y)$ of variables X and Y can be expressed as:

$$I(X;Y) = H(X) - H(X|Y)$$

where $H(X)$ is the Shannon's entropy of X and $H(X|Y)$ is the conditional entropy of X given Y. As Shannon's entropy, the conditional entropy is a measure of uncertainty. In this case, $H(X|Y)$ measures how uncertain we are of Y on the average when we know X (Shannon, 1948).

Therefore, mutual information measures how much information of one variable is contained in the other. It can also be though as a similarity measure (Yockey, 2005), as if X is identical to Y, the information of knowing X determines the value of Y. Some decades ago, there were great expectations on the use of informational measures to predict some features of an organism based on its DNA or protein sequences. For example, it was thought that the DNA sequence of a coding gene would determine not only the sequence of a protein, but also its 3D folded structure (Anfinsen, 1973). However, it is nowadays clear that other factors like post-translational modifications and the physico-chemical environment of the protein affect its structure (Kang and Kini, 2009) and that the DNA sequence is not sufficient to predict it.

This lack of correspondence between DNA and proteins have restricted the application of the mutual information as a similarity measure that compares only DNA (Lichtenstein et al., 2015) or protein sequences (Gloor et al., 2005) separately.

Although it has been proved useful to analyse different aspects of these molecular sequences, the informational approach has not been successfully applied to higher organisation levels (i.e., cells, tissues, organism) (Longo et al., 2012).

Algorithmic complexity Another definition of complexity that has been very popular is the algorithmic complexity. The algorithmic complexity (also called Kolmogorov complexity) of a data string would be the shortest algorithm necessary to describe such

string. As the description of a string can be thought as a program to produce the data (Kolmogorov, 1963; Wolfram, 2002), the algorithmic complexity can also be defined as the shortest program that can produce the data.

Algorithmic complexity is related to randomness: if a program is as long as the data itself, then the data is considered to be algorithmically random (Wolfram, 2002). This measure of complexity that seems specially suited to analyse data strings is not easily applicable to other systems, like measuring phenotypic complexity. Even in the case of strings, it has been said that it is impossible to distinguish if most long sequences are random or not (Wolfram, 2002).

Also, it has been proposed that it is impossible to calculate the program-size complexity of anything, as it is impossible to prove that certain program is the shortest to produce some object (it would be only possible to prove upper bounds in its complexity if a program that produce the desired output is found; Chaitin, 1999). Some other authors have also said that algorithmic complexity, in which randomness is equated to high complexity, does not correspond to an intuitive notion of biological complexity (Adami, 2002), as living organisms are expected to show organization or order, far from randomness.

Spatial information There are also information-based measures applied to spatial data. For example, Michael Batty (1974) used Shannon's definition of information and applied it to "spatial systems". He was specially interested in applying his measure to systems such as a cities (Batty is a geographer). A brief description of Batty's method follows, for a full description see (Batty, 1974; Batty et al., 2014). For a certain location i, with a population P_i, the probability p_i is defined as the proportion of the population in i: $p_i = P_i/P$. To extend this to a probability density, the p_i is divided by the space available for the population, which is Δx_i. So the spatial entropy formula becomes:

$$S = -\sum_i p_i \log \frac{p_i}{\Delta x_i}$$

With this measure, the spatial entropy (and therefore spatial complexity) will reach its maximum when the probability is uniform $p_i = n$, and the distribution of land X in the different locations is also uniform $\Delta x_i = \sum_i \Delta x_i / n$. One application of this measure is to calculate if the entropy of the distribution density in a city has increased over time (Batty, 2010).

Another measure of spatial complexity based on information theory that has been proposed is the "spatial joint information" (Salazar-Ciudad et al., 2001) which indicates the relative entropy of a 1D arrangement, between cells with different "states" (based on the expression level of some gene). Salazar-Ciudad et al. (2001), used this measure to estimate the complexity of gene expression patterns in 1D cell arrangements produced with model genetic networks.

Computational metaphors

Many authors have used computational analogies to define development (Apter and Wolpert, 1965; Monod, 1963; Mayr, 1997; Davidson, 2001). Eric H. Davidson used the gene regulatory network (GRN) concept and a computational metaphor to explain development (and evolution). A GRN consists of DNA cis-regulatory elements, i.e.,

the regions in the vicinity of each gene which contain the specific sequence motifs at which those regulatory proteins which affect its expression bind; plus the set of genes which encode these specific regulatory proteins (i.e., transcription factors) (Davidson, 2001). For Davidson, development is then the outcome of spatial and temporal series of differential gene expression, that is controlled by a "regulatory program" (the GRN) built into the DNA.

A computational program, that is part of a computer system, contains a set of instructions that perform a specific task. The computer program needs a hardware, the set of physical objects that compose the computational system and where the computational program can be stored and execute. If the cell is considered as a computer system with the GRN as the computer program, then the hardware would be all the components of the cell including the genomic and cell structure and all the molecules present in the cell. However, in contrast to a computer system, the separation between the program and the hardware is not clear in a cell. The "genetic program" is affected by the components present in the cell ("hardware"), which in turn changes depending on the program (Oyama, 2000; Jaeger and Sharpe, 2014). For example, it is acknowledged that a cell might elicit different responses after the binding of an extracellular growth factor to a receptor at its membrane, depending on the presence or relative abundance of key signal transducer molecules (Dailey et al., 2005). In other words, it can be said that the set of gene products within a cell define its "state" (Forgacs and Newman, 2005), and depending on the current state of a cell (which is in turn the product of the previous cell state plus extracellular signals), it will respond differently to a specific extracellular signal. This is the case of the very early stages of development, as the zygote transcription is regulated by the gene products that are maternally deposited. Also, it is important to mention that cells not only change their state during development, they also change their spatial distribution. A change in the spatial distribution (at a specific time during development) might have an affect in the outcome of development process (Salazar-Ciudad, 2010), for example, if some cells migrate or invaginate while producing a growth factor ligand, the cells that will receive the signal will be different. Thus, it is clear that not all the information necessary for the development is contained in the genome or GRNs.

Using again the computational metaphor, Davidson considered that these programs of gene expression, which are "installed and executed" as the embryo develops, could serve as a metric of complexity (Davidson, 2001). For illustrating his point Davidson describes an imaginary example of a GRN that increases its complexity in evolution: first, there is a set of downstream genes activated by a small network of TFs (each of them with only one *cis*-regulatory element), which in turn is controlled by a single upstream TF; then, TFs of the network gain cis-regulatory elements (so the circuitry is more intricate) and newly recruited intermediate regulatory TFs activate a different set of down-stream genes. Otherwise, the initial set of downstream genes is still controlled by the single upstream TF (Davidson, 2001).

Thus, in Davidson example, a small hierarchical network changes so that an additional layer is gained (intermediate TF) and the topology of the network changes: instead of one outcome (the initial set of downstream genes), now two outcomes are possible (with the additional set of downstream genes activated by the new intermediate TF).

McShea's view of complexity

Daniel W. McShea has provided some useful definitions of biological complexity. According to one of his definitions, "complexity of an organism is the amount of differentiation among its parts or, where variation is discontinuous, the number of part types" (McShea, 1996, 2015). This definition can be used at different hierarchical levels of biological organization, e.g., tissues, cells, genes. Indeed, a measure of morphological complexity that has been favoured by some authors is the number of cell types that compose an organism (Valentine et al., 1994; Bell and Mooers, 1997; Bonner, 2004). This definition of complexity is not exempt of complications, as there is no clear criteria of how to define a cell type or how to determine, during development, when a new cell type has formed.

Importantly, with this definition (complexity as the number of parts), the complexity at different levels are not necessarily correlated. This lack of correspondence at different levels becomes evident when comparing the number of genes with the number of cell types. Before the release of the first eukaryotic genome sequences, it was expected that the number of genes would correlate with an intuitive perception of organismal complexity, ranking complexity as yeast $<$ nematodes $<$ flies $<$ humans (Hahn and Wray, 2002) (this intuitive notion of complexity correlates with the number of cell types in metazoans; Valentine et al., 1994). However, this expectation was proved to be wrong and this lack of correlation between "intuitive complexity" and genes number was called the "G-value paradox" (Hahn and Wray, 2002). Before that, the lack of correspondence between genome size and organism complexity (using again an intuitive notion of complexity), or "C-value paradox", was also noted. The lack of correspondence between the number of genes with an intuitive notion of complexity is now partly explained by some authors by the amount of post-transcriptional regulation (Sempere et al., 2006). This paradox can also be partially explained by the currently acknowledged notion that during development, genes do not act individually, but they act within gene networks. Therefore, the phenotypic complexity is affected not only by the number of genes involved in its development, but also by the topology of the gene networks.

This relates to McShea's distinction between "object complexity" that refers to the number of parts of a system and "process complexity" that refers to the interaction among parts in a system (McShea, 1996). This could be illustrated with the number genes (object complexity) and the number of gene-gene interactions (process complexity). Gene-gene interactions would refer to the regulation of a gene expression by the binding of another gene product (transcription factor) to its promoter region. Using this definition, two different organisms would have the same object complexity if they have the same number of genes, but one would have a higher process complexity it has more gene-gene interactions than the other.

1.1.2 Complexity Increase in Development and Evolution

The increase in complexity in an organism during embryogenesis is probably one of the most intuitive processes of animal development.

It is commonly seen even as one of its defining characteristics. Eric H. Davidson described the progressive increase in complexity as the "essence" of development (Davidson, 2001). Despite of the widely accepted view of complexity increase in development, there is no consensus of how to define it, much less on how to quantify it (Oyama, 2000).

Using the number of cell types, the increase of complexity during development is self-

evident: in vertebrates, the embryo begins with one cell type (the zygote) and concludes with more than 200 cell types (Alberts et al., 1994).

On the relationship between the increase of complexity in Evolution and Development

The connection between the increase in complexity during development and evolutionary time has been largely discussed. Haeckel was one of the first who made explicit hypotheses about the connection between the development and evolutionary patterns in his "Biogenetic Law" (see Box 1). These laws imply that the increase in complexity we see during development is a reflection of a similar increase in complexity that has occurred through evolution. Early views of evolution saw the increase in complexity as inexorable, with all the species descending from simpler ancestral forms (Lamarck, 1809; Haeckel, 1874), and with the human species as the latest and more perfect product of the evolution of animals (Haeckel, 1874).

Recent views recognize that complexity can increase or decrease in a lineage. Using the number of cell-types as complexity measure, there are clear examples of taxa that have decreased their complexity over time, specially in parasites (Canning and Okamura, 2003; Arthur, 2010) (although morphological simplification can not be considered universal in parasitic taxa Poulin, 2011). On the other hand, there are many lineages that have remained unicellular (i.e., their complexity would have remained constant), while some lineages (e.g., vertebrates) have increased their complexity. Hence, it seems that there is no unique trend to increase the complexity over time, in other words, the complexity of a specific lineage might decrease, increase or stay the same (see Figure 1.1).

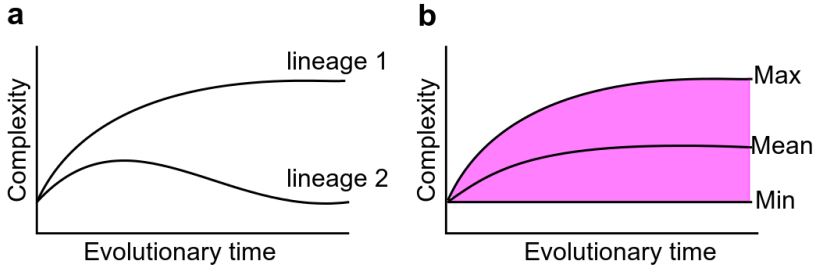

Figure 1.1: Two lineages with different complexity change through their evolutionary trajectories. b) Representation of the minimum, mean and maximum complexity of many lineages over evolutionary time in which the minimum stay constant while the mean and maximum increase. Redrawn from (Arthur, 2010).

If we consider uni-cellularity as the minimum complexity, it can be said that minimum complexity has remained more or less constant in evolution, as unicellular organisms like bacteria, have been present since 3.5 billion years. However, if we consider instead maximum complexity a trend for increasing such complexity would be apparent, as complexity would have increased with the appearance of simple multicellular organisms (only few cell types) and would have further increased until the appearance of organisms composed of hundreds of cell types. For some authors this apparent trend of increasing complexity is the product of natural selection (Bonner, 1988; Carroll et al., 2001).

In contrast, other authors consider that this apparent trend does not necessarily imply that it has been selected for (McShea, 2015), and that this trend might appear

even in scenarios without natural selection. For example, if we consider an evolutionary scenario in which the complexity of the organisms follow a random walk scenario without any selection regime but with the condition of a lower boundary (i.e., is not possible to have less than once cell), and starting from unicellular organisms, we will see an increase of the maximum complexity, with an initial increase in the mean complexity (in a random walk the expected distance of a point after n time steps is $\sqrt[2]{n}$, but when considering many points the mean distance at any time point is 0) (Gould, 1996).

Compartmentalization in development

The notion of an increase in complexity during embryogenesis is tightly related to the concept of embryo compartmentalization. In here, I will refer to compartmentalization as the subdivision of the embryo in different parts (or compartments) during development. How the different parts of the embryo can be defined based on the cell-phenotype or gene expression profile.

It is usually considered that the earliest compartments that are formed in the embryo define the main body axis, i.e., the anterior/posterior (A/P) and dorsal/ventral (D/V) axes. Later on, smaller compartments of the embryo would be formed, e.g, limbs, eyes or internal organs. In this manner as development proceeds, it is expected that spatial compartments would be progressively specified at an increasing finer resolution (Davidson, 2001). Furthermore, the increasing compartmentalization of the embryo during development can be conceptualized as the progressive spatial restriction of gene expression to subsequently smaller regions in the embryo. Sean Carroll defines this process(Carroll et al., 2001) as:

i. In early development, genes have a broad expression in the embryo and define the main axes of the body.
ii. Later, genes define smaller compartments like organs and appendages (field-specific selector genes).
iii. Finally, genes become expressed in specific cell types like muscle and neural cells (cell-type specific selector genes).

It is important to note that this would imply that, in general, the area of expression of a gene in the embryo would decrease during development (relative to the area of the whole embryo).

A well known definition of developmental compartment was proposed by García-Bellido et al. (1973). They defined compartments as differentiated populations of cells (at the gene expression level) that do not intermix between them and that these are formed from initially homogeneous contiguous cells. The definition used in here is related to the one of Garcia Bellído et al., but in contrast to it, does not rely on the identification of a boundary formation between different cell populations that would prevent cell mixing between them.

Complexity at the molecular level

For some authors, the increase in complexity in an organism during development (reflected by the increase of number of cell types), should be associated with an underlying complexity at the molecular level (Davidson, 2001; Arthur, 2010), following the reasoning that:

i. In development, complexity increases with time as new cell types form.

ii. Different cell-types are characterized by the differential expression of genes.
iii. Therefore, a complex organism (composed of many cell-types) has to contain a complex gene expression regulatory machinery to produce the different combinations of expressed genes in each cell-type (Davidson, 2001).

Gene expression regulation It is widely acknowledged that the spatio-temporal regulation of gene expression in development is crucial for the progressive compartmentalization of the embryo. More than fifty years ago, Jacques Monod and François Jacob (Jacob and Monod, 1961) published in a seminal work a model of the genetic regulatory mechanism in bacteria. The most important conclusion of this paper was the existence of "regulator" genes that control the production rate of proteins from "structural" genes, and that mutations in "regulator" genes affect the regulatory mechanism but not the structure of the regulated protein. In the same paper they suggested that these regulator genes may affect the synthesis of several different proteins (Jacob and Monod, 1961).

Nowadays the process of gene activation is known in great detail. The "regulator genes" Jacob and Monod studied are transcription factors, proteins that bind to DNA to promote or repress the transcription of a gene.

This transcriptional regulation represents however only one level of gene expression regulation. There are many other mechanisms that regulate the production of gene products. These include 3' untranslated regions (UTR) (Grzybowska et al., 2001), small interference RNAs (siRNAs) (Filipowicz et al., 2005), translational (Kozak, 1992; Kapp and Lorsch, 2004) and post-translational (Mann and Jensen, 2003) regulation of gene expression.

At least two regulatory levels have been explicitly suggested to have a causal role in the increase in complexity in different lineages: transcriptional regulatory level (as mentioned above) (Davidson, 2001) and miRNAs. The role of miRNAs (non-coding RNA molecules that negatively regulate gene expression) was proposed after the observation that miRNAs are found only in protostomes and deuterostomes and not in sponges or cnidarians, and that they are specifically expressed in certain cell-types, tissues or organs (Sempere et al., 2006). It could be expected however that the complexity of an organism could be reflected at any level of gene expression regulation, whether transcriptional, post-transcriptional, translational or post-translational.

1.1.3 Shape complexity

Until this point, I have focused on the concept of compartmentalization as one aspect that reflects the increase in complexity during development. Another aspect that is intuitively related to the increasing embryonic complexity is the shape (or form) of the embryo. Focusing on the shape of the embryo, embryonic development can be thought as a process that starts with a simple spherical or oblate fertilized egg and that ends with complex shapes and forms (in the adult or larva) (Forgacs and Newman, 2005).

In addition to the external shape of the embryo, the shape complexity of its internal structures (e.g., organs) is expected to increase during development (Sharpe, 2003).

However, it is not always possible to appreciate the morphological change of inner structures at simple view. The first attempts to describe the shape of internal organs during the development in vertebrates was in the 19th century, and it required section cutting and wax reconstruction (Hopwood, 2007). The use of staining techniques have facilitated the visualization of the inner morphology of the embryo. Techniques such

as the horseradish peroxidase staining facilitated not only the visualization of the inner morphology, they were also crucial to create the first fate maps, while being able to trace the cell-lineage of different organs.

Since now is known that many genes are expressed in a tissue/cell type specific manner, another useful technique to visualize inner structures is the use of labelling techniques that highlight the distribution of such tissue-specific gene products (e.g., whole-mount in situ mRNA hybridization or immuno-histochemistry techniques). If the embryo external and internal morphology are expected to increase their spatial complexity, and some gene expression patterns (visualized with a labelling technique) is expected to correspond to specific regions (e.g., internal organs) or the whole embryo (in case the expression is ubiquitous), consequently, the shape of gene expression patterns could be used to describe the morphological complexity of the embryo. It is important to mention that even when some gene expression patterns might reflect (and could partially explain) the organ distribution and form, usually there is no simple one-to-one correspondence between genes and organs. Indeed, many developmental genes are expressed in many organs at different developmental stages.

The study of morphometrics refers to the quantitative analysis of morphological shape. In the last decades, morphometric tools have been used widely as a tool to quantify, characterize and compare biological shapes (James Rohlf and Marcus, 1993). In the next paragraphs I will briefly explain the most important morphometric methods. For an extensive review, see (Bookstein, 1997; Dryden and Mardia, 1998; Zelditch et al., 2008; Slice, 2005).

Morphometrics

In morphometrics, shape refers to the geometric properties of an object that are invariant to location, scale and orientation (Slice, 2005). Many of the modern morphometric analyses are based on the use of "landmarks", which refer to precisely located points that establish a clear one-to-one correspondence between the samples under study (Klingenberg, 2010). To extract only the shape information from the landmarks, the variation in size, position and orientation are usually removed with a technique called "Procrustes superimposition" (Dryden and Mardia, 1998). Although there are many different morphometric methods, they can be divided in four main categories: "traditional morphometrics", "geometric morphometrics", outline analysis and surface analysis (Slice, 2005).

The **"traditional methods"** refer to the application of multivariate statistics, like Principal Component Analyses (PCA), to the direct measurement of lengths, widths or ratios of specific structures. Some typical applications of these methods are the classification of species or sexes (Jolicoeur and Mosimann, 1960) using lengths, widths or angles between landmarks (Dryden and Mardia, 1998).

Geometric morphometrics analyses use instead geometric coordinates of morphological landmarks (Mitteroecker and Gunz, 2009; Zelditch et al., 2012). As with the traditional methods, PCA can be used for analysing the shape variation in the dataset (Klingenberg, 2010). A variant of landmark analyses is the use of "semi-landmarks". Semi-landmarks are equally spaced points around an outline, usually between "real landmarks". Semi-landmarks are therefore used when only a few landmarks are recognisable. For example, in the analysis of the shape of hands, "real landmarks" can be placed in the tip of the fingers, and the semi-landmarks would be placed along the hand outline. After recording the semi-landmarks, procrustes superimposition and multivariate analyses can

be used as with ordinary landmarks (Dryden and Mardia, 1998).

Outline analyses are specially relevant when it is not possible to identify comparable landmarks between samples. One type of outline analyses is the Elliptic Fourier description (Kuhl and Giardina, 1982), which uses an orthogonal decomposition of a curve into a sum of harmonically related ellipses (Ferson et al., 1985). The extracted harmonics can then be analysed with PCA. A classical example of the Fourier description is the analysis of mussel shells (which can be represented as a closed outline) done by Ferson et al. (1985). Another outline method is the Eigenshape analysis, which uses outline coordinates to calculate angles between points to provide a map around the outline. More specifically, shape is represented as the shape function $\phi^*(l)$, the normalized net angular change in direction ϕ at each step around the perimeter (l) (the normalization can be based on the deviation from a circle, or from the sample mean) (Lohmann, 1983). Then, the major directions of observed and measured shape variation is analysed by means of eigenanalysis. Eigenshape analysis (a type of Principal Component Analysis) derives a set of empirical orthogonal shape functions by an eigenfunction or PCA of a matrix of correlation between shapes (Lohmann, 1983).

Surface analyses are used when comparing 3D objects (usually represented as Cartesian coordinates x,y and z of points on the object's surface) with limited landmark information. For example, a vertebrate skull has many identifiable anatomical landmarks in the face but only a few can be defined unambiguously on the smooth braincase (Mitteroecker and Gunz, 2009). In order to deal with this, Gunz et al., 2005 extended the use of 2D outline semi-landmarks to 3D surfaces. 3D semi-landmark methods are based on allowing the points to "slide" along the surface until some measure of shape difference (e.g., bending energy of a thin-plate spline) among the configurations is minimized (Mitteroecker and Gunz, 2009).

Topographic anaytical methods

Another approach to 3D surfaces is the use of topographic analytical methods. These methods, which apply concepts from Geographic Information Systems (GIS), have been used to quantify teeth surfaces as if they were landscapes (Jernvall and Selänne, 1999; Winchester et al., 2014).

New topographic analytical methods that do not rely on GIS have been recently developed. This is the case of the Dirichlet normal energy (DNE), a method for quantifying surface bending using concepts from differential geometry (Bunn et al., 2011). This method quantifies the deviation of a surface mesh from being planar (Bunn et al., 2011). A brief explanation of the DNE follows, for a complete description see (Bunn et al., 2011; Winchester, 2016). For each polygon in the surface mesh, DNE calculates its energy value $e(p)$. The energy value quantifies change in the normal map around a polygonal face. The DNE value of the whole surface is the sum of all the energy values $e(p)$ of the polygonal mesh surface. DNE values increase with both convexities and concavities on a surface. This measure has been used in the shape quantification of mammals tooth crowns, for dietary inference (Bunn et al., 2011).

1.2 Adaptation

In this section, I will start with the definition of the concepts of adaptation (although there are more than one definition of adaptation; Endler, 1986), and natural selection. Then I will introduce some of the methods that are used to estimate adaptation, with a focus on molecular methods.

On the concept of adaptation

Usually adaptation refers to two different things, to an adaptive trait or to the process to become adapted (Endler, 1986). George Gaylord Simpson (1953) defined adaptation in the following way:

"*an* adaptation is a characteristic of an organism advantageous to it or to the conspecific group in which it lives, while adaptation or the process of adaptation is the acquisition within a population of such individual adaptation" (italics by the author)

An adaptation (i.e., an adaptive trait) is usually related to a specific function of the organism. For example, the beak variations (in size, width and depth) in the Darwin's Galapagos finches, a classic example of adaptive traits, are related to the alimentary function of the finches, so that each species is specialized in a specific diet. The notion of adaptation existed before Darwin, but since Darwin it is closely related to the concept of natural selection. Under the current evolutionary framework, an adaptation, arising due to intrinsic natural variation, will be fixed in a population by natural selection due to the advantage it confers to their bearer organisms.

Natural selection

Charles Darwin, in its 1859's *Origin*, defined Natural selection as follows:

> *" Owing to this struggle (for life), variations, however slight and from whatever cause proceeding, if they be in any degree profitable to the individuals of a species (...) will tend to the preservation of such individuals, and will generally be inherited by the offspring. The offspring, also, will thus have a better chance of surviving, for, of the many individuals of any species which are periodically born, but a small number can survive. I have called this principle, by which each slight variation, if useful, is preserved, by the term Natural Selection"* (Darwin, 1859).

More recently, and following the Darwininan concept of natural selection, Jhon A. Endler (1986), defined it as a process in which, given that a population has:

a. **variation** among individuals in some attribute or trait;
b. **fitness differences** (consistent relationship between that trait and mating ability, fertilizing ability, fertility, fecundity, and, or, survivorship);
c. **inheritance** (consistent relationship, for that trait, between parents and their offspring, which is at least partially independent of common environmental effects).

Then:

i. the trait frequency distribution will differ among age classes or life-history stages, beyond that expected from ontogeny;
ii. if the population is not at equilibrium, then the trait distribution of all offspring in the population will be predictably different from that of all parents, beyond that expected from conditions a and c alone.

Conditions a, b, and c are necessary and sufficient for the process of natural selection to occur, and these lead to deductions i and ii (Endler, 1986).

Condition a relates to phenotypic changes across generations. Importantly, phenotypic changes, whether new characters or modifications of existing characters in the adult/larva, are produced from changes in development. For example, the difference in the beak size and shape between Galapagos Darwin's finches has been shown to be regulated by the differential expression of the genes CaM and BMP4 during development (Abzhanov et al., 2006).

Therefore, even when natural selection acts in the adult/larva phenotype, the changes that lead to an adaptation should be traceable during the development of such trait.

Methods to detect natural selection

There are many different methods designed to detect natural selection in natural populations. Jhon A. Endler classified ten different methods with diverse ability to detect natural selection (Endler, 1986). Some of these methods test directly the conditions (b and c) required by natural selection, while others test the predicted outcome of natural selection in a population.

There are many studies that have aimed to detect natural selection directly in the phenotype. Usually, these studies are based on the estimation of selection gradients of a quantitative trait (a measurable phenotype that usually depends on the cumulative actions of many genes and the environment). A selection gradient of a trait refers to the relation of the trait values and fitness. Is calculated as the slope of a linear regression of fitness on the trait value (Barton et al., 2007). Most of these studies are based on the analysis a single trait or a small number of traits of an organism (Hoekstra et al., 2001; Hereford et al., 2004) which are usually selected already under the suspicion to be adaptive. However, there is practically no study that has attempted to estimate natural selection over the entire organism.

Among the methods that test the predicted outcome of natural selection we find the molecular methods. The molecular methods are based on the assumption that changes leading to an adaptation are (at least partially) caused by DNA mutations and that the effects of natural selection could be traceable looking at the DNA sequence. There is an entire field within evolutionary biology, namely molecular evolution, dedicated to explain the evolutionary sequence changes in molecules as DNA, RNA and proteins.

In the next sections, due to its relevance in this work I will only focus on the molecular methods to detect natural selection.

1.2.1 Molecular evolution

The theoretical basis of the molecular evolution field includes concepts from evolutionary biology and population genetics. At the DNA level, any transmissible change in the sequence is considered a mutation. The most simple change is a point mutation, also called single nucleotide polymorphism (SNP), which is a change in a single nucleotide in the DNA sequence of a locus in an individual.

Variation at a particular DNA site within the individuals of a species or population is referred as polymorphism, while divergence refers to variation at a specific DNA site in individuals from different species. SNPs occur in non-coding and coding DNA sequences. A SNP that occurs in a coding sequence is classified in two categories, depending on its

effect on the protein sequence: i) synonymous mutation and ii) non-synonymous mutation. A synonymous mutation does not affect the amino-acid sequence of the protein (although it can affect its function (Kimchi-Sarfaty et al., 2007) or the gene transcriptional efficiency (Xia, 1996). A non-synonymous mutation affects the amino-acid sequence of the protein whether by changing a single amino-acid (missense mutation) or by producing a stop codon (non-sense mutation) which results in a truncated version of the protein. As the non-synonymous mutations can affect dramatically the structure and function of the protein, it is expected that most non-synonymous mutations have a negative fitness effect. However, it is also expected that a fraction of non-synonymous mutations would have a positive fitness effect that, depending on the strength of the fitness effect and several population genetics parameters, could lead to the fixation of that mutation in the population (i.e., adaptive substitutions).

An important branch of the molecular evolution field is dedicated to the identification of adaptive substitutions in a species, which has lead to the development of many statistical tests, which are based on the neutral theory of evolution, proposed by Kimura (Kimura, 1968).

Neutral theory of evolution

In 1968, Mooto Kimura calculated the average rate of nucleotide substitutions in the evolutionary history of mammals. The result of his calculations was that, on average, one nucleotide has been substituted every 2 years. For him, this very high rate of substitution was only explainable if most mutations were almost neutral in natural selection (Kimura, 1968). which was in contradiction with the prevailing view at the time that practically no mutations were neutral.

In 1969, Kimura proposed that the majority of amino acid substitutions that occurred in proteins are the result of random fixation of selectively neutral or nearly neutral mutations (Kimura, 1969). In the same year, King and Jukes (King and Jukes, 1969) independently proposed practically the same hypothesis. Two important assumptions of the neutral theory of molecular evolution were:

i. Deleterious and adaptive mutations are rapidly purged and fixed in a population respectively.
ii. Polymorphism is a transitory phase between random fixation or extinction due to genetic drift.

Importantly, the neutral theory provided a set of testable predictions, providing a null-hypothesis for adaptive molecular evolution.

From neutral to nearly neutral theories

In the subsequent decades after the proposal of the neutral theory of molecular evolution, much more protein sequence data became available, which made evident the great variation in the evolution rate of proteins. To account for this, Kimura and Ohta stated that "functionally less important molecules or parts of a molecule evolve faster than more important ones" (Kimura and Ohta, 1974). Then, Ohta propose that slightly deleterious mutations might be common in amino acid substitutions (Ohta, 1973). Later, it was proposed that half of the protein substitutions would be advantageous and the other half deleterious (Gillespie, 1994). Therefore, the neutral model was replaced by a

nearly neutral model with only deleterious substitutions, which in turn was replaced by one with a mixture of positive and negative effects (Ohta and Gillespie, 1996).

At the end of the 1970's comparative analyses of protein sequence data began to be replaced for analyses on DNA sequence data, which revealed that synonymous substitutions within coding regions are more frequent than non synonymous (those that change an amino acid) substitutions. From the early 1990s, the expectations of the nearly neutral theory at the DNA sequence level are that substitutions in non coding DNA and synonymous substitutions in coding regions are neutral and amino acid substitutions can be deleterious, neutral or advantageous (Ohta and Gillespie, 1996). Statistical methods were then devised to test such expectations.

1.2.2 Estimating adaptation at the molecular level

One of the most popular tests to estimate adaptation at the molecular level has been the McDonald-Kreitman test (MKT), which is used to detect adaptive substitutions comparing the relative numbers of synonymous and non-synonymous differences within a species with those numbers between closely related species.

McDonald-Kreitman test

John H. McDonald and Martin Kreitman developed this test in 1991 when analysing the divergence in the Alcohol dehydrogenase (Adh) locus in three *Drosophila* species (McDonald and Kreitman, 1991). The main assumption of the MKT is that the substitutions in a protein are neutral if the inter-specific ratio of non-synonymous (Dn) to synonymous (Ds) changes is equal to the intra-specific ratio of non-synonymous (Pn) to synonymous (Ps) changes (i.e. $Dn/Ds = Pn/Ps$). Any departure from this equality would imply the action of positive or negative selection. Importantly, MKT assumes for simplicity that non-synonymous mutations are either strongly deleterious, neutral or strongly advantageous (McDonald and Kreitman, 1991).

In other words, this method assumes that a protein in a given phylogeny has a specific substitution rate for synonymous and another for non-synonymous substitutions. Therefore, when comparing two proteins from two different species that have evolved under neutral conditions, the *total* number of each type of substitutions would depend on the time since the separation between species. If the proteins are from individuals from the same species, it would depend on the time elapsed since the separation of the within-species branches of the phylogeny. However, the ratio of non-synonymous to synonymous substitutions is expected to be the same in both inter-specific and intra-specific cases. In the case of non-synonymous mutations under positive selection (synonymous mutations are expected to be always neutral), the equality of these ratios would disappear. As mutations under positive selection are expected to spread through a population rapidly (and are not expected to be very common) they are not expected to be present as polymorphic (i.e., intra-specific) variation, but only as divergent (i.e., inter-specific) one.

Therefore, in the presence of mutations under positive selection, the ratio of non-synonymous to synonymous variation within species should be lower than the ratio of non-synonymous to synonymous variation between species (i.e. $Dn/Ds > Pn/Ps$; see Fig. 1.2). On the contrary, if the observed ratio of non-synonymous to synonymous

variation between species is lower than the ratio of non-synonymous to synonymous variation within species (i.e., $Dn/Ds < Pn/Ps$) then negative selection is at work.

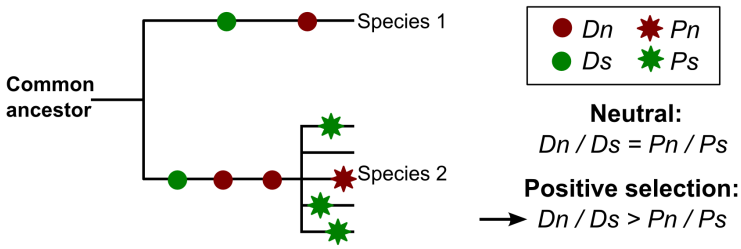

Figure 1.2: McDonald-Kreitman Test (MKT). MKT compares the ratio of non-synonymous (Dn; red circle) to synonymous (Ds; green circle) divergence (Dn/Ds) to the ratio of non-synonymous (Pn; red star) to synonymous (Ps; green star) polymorphic changes (Pn/Ps). Positive selection is detected when $Dn/Ds > Pn/Ps$, as in the example shown in the left.

Although the MKT has been proved robust to many sources of error (e.g., variation to mutation rate across the genome), it can be affected by the presence of slightly deleterious mutations or demography (Messer and Petrov, 2013; Eyre-Walker et al., 2006). The effect of slightly deleterious mutations is related to the effective population size (N_e). In a population with a low N_e, slightly deleterious mutations would have more probabilities of fixation by random genetic drift contributing more to polymorphism than to divergence, underestimating the proportion of adaptive changes (Messer and Petrov, 2013).

Recently, sophisticated methods based on the MKT have been developed to correct for underestimation of adaptive evolution in the presence of slightly deleterious mutations.

Distribution of Fitness Effects

Even when for simplicity the mutation effects are usually classified in strongly advantageous, neutral, and strongly deleterious, there is actually a continuum of selective effects, from strongly deleterious, to highly adaptive mutations, with weakly deleterious, neutral and slightly adaptive mutations in between (Eyre-Walker and Keightley, 2007).

The relative frequencies of all these types of mutations is called the Distribution of Fitness Effects (DFE). In order to know the DFE, a few experimental approaches exist. The most direct method is whether to induce (Sanjuán et al., 2004) or to collect (Mukai, 1964) spontaneous mutations and assay their effects (fitness) in the laboratory. As it can be expected, these experiments require many generations to gather sufficient data, so these approaches have been used mainly in micro-organisms (Eyre-Walker and Keightley, 2007). A caveat of these experimental approaches is that, in order to identify the effect of a mutation, its effect has to be detectable in a fitness assay. In fitness assays however, only effects with relatively large effects are usually detected. Therefore, these methods give valuable information for mutations with relatively large effects.

An alternative approach is to infer the DFE by analysing patterns of DNA sequence differences at intra and inter-specific level (polymorphism and divergence respectively). The methods using this approach rely mainly on two assumptions:

i. the probability that a mutation spreads to a certain frequency in a population (or to fixation) depends on the strength of selection (positive or negative) acting on it. Severely deleterious mutations have lower probability to reach a high frequency in a population.

ii. the efficiency of selection depends on the effective population size. With a high effective population size, selection is more efficient and a smaller proportion of mutation will behave as effectively neutral.

The "absolute strength" of selection on a mutation is then measured as $N_e s$, the product of the effective population size (N_e) by the selection coefficient (s) of the mutation. Mutations with $N_e s$ much less than 1 are effectively neutral, while $N_e s$ greater than 100 have no chance to appear as polymorphism (Eyre-Walker and Keightley, 2007).

DFE-alpha method

Eyre-Walker and collaborators proposed a method to estimate both the DFE and the proportion of adaptive nucleotide substitutions (α) using polymorphism and divergence data (Eyre-Walker and Keightley, 2009). More specifically, they use the polymorphism site frequency spectrum (SFS) to estimate the DFE and then use this estimated DFE to estimate the proportion of substitutions under positive selection between species. To estimate the DFE from the SFS, they developed a maximum likelihood method using the expected allele frequency distribution based on a variation of the Fisher-Wright transition matrix (for more details, see Keightley and Eyre-Walker, 2007; Eyre-Walker and Keightley, 2009).

This method, assumes that there are two types of nucleotide sites: i) sites at which all mutations are neutral and ii) sites at which some of the mutations are subject to selection (positive or negative). Also it is assumed that any new adaptive mutation in a population would not be detected in the polymorphic phase but only in the divergent one (as the advantageous mutations would fix rapidly in a population), and that the DFE can be represented with a gamma distribution (Figure 1.3).

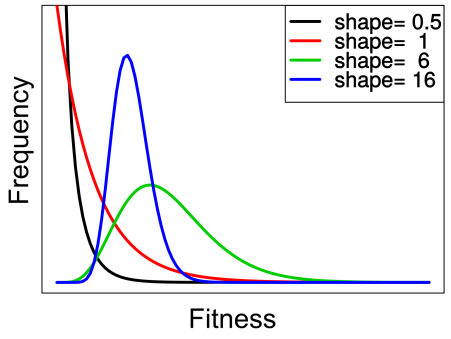

Figure 1.3: Example of different Distribution of Fitness Effects (DFE) represented by a gamma distribution. Many distributions can be represented by modifying the shape parameter of a gamma distribution, from a leptokurtic (shape parameter less than 1) to an exponential (shape parameter equal to 1) or a skewed normal distribution (shape greater than 1).

The divergence at the neutral sites is then proportional to the mutation rate per site and the predicted divergence at the selected sites (in the absence of advantageous

mutations) is proportional to the product of the mutation rate together with the average fixation probability of a selected mutation. This probability of fixation is is inferred based on the DFE and other parameters estimated from the polymorphism data analysis (Eyre-Walker and Keightley, 2009). The difference between the observed and predicted divergence therefore estimates the divergence due to adaptive substitutions. Using this method Eyre-Walker and collaborators estimated that in *Drosophila* genes approximately 50% of amino acid substitutions and approximately 20% of substitutions in introns are adaptive (Eyre-Walker and Keightley, 2009).

Messer and Petrov performed molecular evolution simulations to test if the estimates of different tests, like the MKT and the more sophisticated DFE-alpha, are accurate under different realistic gene-structure and selection scenarios (Messer and Petrov, 2013), specially in the presence of genetic draft (stochastic effects generated by recurrent selective sweeps at closely linked sites) and background selection (interference among linked sites by lightly deleterious polymorphisms). They found that in the presence of slightly deleterious mutations, MKT estimates of α are severely underestimated and that DFE-alpha is very accurate to calculate α even in the presence of genetic draft, background selection or demography changes (Messer and Petrov, 2013).

Methods lilke the DFE-alpha would be ideal to analyse intra-specific variation in a natural population at a genomic level. In the last years, different population-genomic projects have sequenced, in different species, the genome of many individuals of a population (or a set of populations) (The 1000 Genomes Project Consortium, 2010; Mackay et al., 2012; Pool et al., 2012; Wallberg et al., 2014), providing a valuable resource of genomic polymorphism data at the population level. One of these projects is the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a publicly available tool for molecular population genomic analyses, briefly described in the following subsection.

1.2.3 The *Drosophila melanogaster* Genetic Reference Panel

DGRP consists of 192 inbred strains derived from a single outbred *Drosophila melanogaster* population. The inbred lines were constructed from collected mated females from a Raleigh (North Carolina, USA) population, followed by 20 generations of full-sibling inbreeding of their progeny (Mackay et al., 2012). 168 inbred lines were then sequenced using Illumina (129 lines), 454 sequencing (10 lines) or both (29 lines). Therefore, the DGRP contains a representative sample of naturally segregating genetic variation.

Mackay et al., 2012 used the DGRP sequence data in combination with genome data from *Drosophila simulans* and *Drosophila yakuba* to analyse polymorphism and divergence, the recombination landscape, and infer the action of natural selection on an unprecedented genome-wide scale. They found that the patterns of polymorphism differ by autosomal chromosome region, and between the X chromosome and autosomes, contrary to the divergence patterns. Using version of the MKT test, they estimated that on average 25% of the fixed sites between *D. melanogaster* and *D. yakuba* are adaptive (24% non-synonymous, 30% in introns and 7% in UTR sites) (Mackay et al., 2012).

1.3 *Drosophila* as a model organism

The fruit fly, *Drosophila melanogaster*, has been a great valuable tool for biological research. Its use as a model system dates back to the beginning of the 20th century.

In 1908, Thomas H. Morgan started to grow flies in large quantities to study gene mutations. At that time, the gene concept was an abstract one, as the nature and location of the genes was still disputed. The main advantages of using flies were their rapid generation time and that they were easy to culture and cheap to maintain (Arias, 2008). In his lab at the University of Columbia, Morgan found a fly with white eyes (the wild-type eye color is red), which became a subject of his research for many years. Eventually, he discovered that the allele of the gene, that he called *white*, was located on a sex chromosome, demonstrating for the first time the sex-linkage of genes (Morgan, 1919). Morgan's students also demonstrated that mutations were inducible with X-rays and introduced the use of "balancer" chromosomes to keep stable stocks of mutants (Arias, 2008). The research carried out in Morgan's lab laid the basis modern genetics, and its fly room became a central node in the genetics research, establishing *Drosophila* as a organism model.

However, *Drosophila*'s development was difficult to study, as the embryos were not large enough to experimentally manipulate them, and not transparent enough to visualize with a microscope (Gilbert, 2014). Molecular biology techniques allowed finally to study fly genes and their effect on embryogenesis, unravelling some of the mysteries of *Drosophila*'s development. Also, histological methods (which consisted in following back to the blastoderm stage the location of larval organ precursors) and cell ablation methods (killing cells in the blastoderm and correlate its position with the position of the defects detected later) were used to create a fate map of the *Drosophila* blastoderm (Campos-Ortega and Hartenstein, 1985) (see Fig. 1.4 in Box 1).

1.3.1 *D. melanogaster* life cycle

Drosophila melanogaster is a holometabolous insect, which means that it goes through a complete metamorphosis, i.e., the larva and the adult forms are very different. The entire life cycle is usually not longer than 10 days. Its embryonic development is very fast, the larva hatches in less than 24 hours at 25°C. The larva grows and passes through two moults (in 4 days it increases 200-fold its weight) before becoming a resting stage called a pupa in which the body is remoulded to form the adult (Stocker and Gallant, 2008). Much of the adult body is formed from the imaginal discs and the abdominal histoblasts which are only present as undifferentiated buds in the larva.

Developmental stages

In Table 1, a brief summary of the embryonic development of *D. melanogaster* is shown. For a comprehensive lecture, see (Campos-Ortega and Hartenstein, 1985; Roberts, 1998; Gilbert, 2014). The staging system shown in Table 1, correspond to the 16-stage system proposed by Roberts (1998), with approximate developmental timings at 22 °C. The numbering of the stages shown are similar to the one proposed by Campos-Ortega and Hartenstein (1985), except that the latter add a stage 17 to the fully differentiated embryo. The 16-stage system is used by the BDGP (Tomancak et al., 2002). Therefore, Table 1 can serve as a reference when mentioning specific developmental stages in this work.

Table 1. Embryonic stages of *D. melanogaster* and morphological criteria for identifying approximate ages (from Roberts, 1998)

Stage	Developmental time	Morphologic features and main developmental events
1	0 to 15min	**Freshly laid egg.** Homogeneous cytoplasm
2	15min to 1h 20min	**Early cleavage.** A cap of clear cytoplasm becomes visible at the posterior pole
3	1h 20min to 1h 30min	**Pole cell formation.** Surface cytoplasmic layer becomes thicker and inhomogeneous
4	1h 30min to 2h 30min	**Syncytial blastoderm.** Nuclei divide four or more times. Cortical cytoplasm becomes clearly delimited from the underlaying yolk
5	2h 30min to 3h 15min	**Cell formation.** Cell membranes move down between adjacent nuclei, separating cells
6	3h 15min to 3h 35 min	**Early gastrulation.** Ventral furrow formation along the ventral midline of the embryo
7	3h 35 min to 3h 45min	**Midgut invaginations.** Cephaplic furrow has deepened and is visible from the side
8	3h 45min to 4h 30min	**Germ band extension.** Germ band extends along the dorsal side until the posterior midgut invagination reaches the head region at 65% length
9	4h 30min to 5h 10min	**Stomodeal plate formation.** Cephalic furrow no longer visible.
10	5h 10min to 6h 50min	**Stomodeal invagination.** Anterior midgut anlage moves posteriorly. Ectodermal segmentation becomes apparent as regularly spaced
11	6h 50min to 9h	**Three-layered germ band**. Segmentation is clearly visible. Due to neuroblast proliferation, the dense yolky regions gradually disappear from the head
12	9h to 10h 30 min	**Germ band retraction.** Yolk sac extends to the dorsal side. Anterior and posterior midgut anlagen form visible projections which gradually approach each other
13	10h 30min to 11h 30min	**Shortened embryo.** Germ band completely contracted. Anterior and posterior midgut have fused laterally. The head bends dorsally. Dorsal head ridge formation
14	11h 30min to 13h	**Head involution and dorsal closure.** The germ band stretches anteriorly. Hindgut grows antero-dorsally . Yolk sac is covered by the serosa in the dorsal middle region
15	13h to 15h	**Dorsal closure complete.** Subsequently constrictions divide the midgut into three regularly spaced subdivisions
16	15h until the end of embryonic development	**Condensation of CNS.** Conversion of the sac-like gut into a long convoluted gut. Muscular movements begin in the gut and somatic musculature.

Box1. Fate maps and gene expression maps

Fatemap The "fate map" is a very important concept in developmental biology. Its name refers to the practice of cartography (or map making), i.e., contructing two-dimensional (2D) representations of a usually three-dimensional (3D) space. In a fate map the prospective fate is mapped onto the 2D representation of usually an early embryo (Gilbert, 2007).

The first fate maps were constructed by tracking cell lineages to identify cell fate only by observation. In 1905, Conklin tracked the cell lineage of the tunicate embryo, providing the first fate map (Conklin, 1905). In the 1980's José A. Campos-Ortega and Volker Hartenstein combined labelling techniques (injecting horseradish peroxidase) and histological methods to create a very precise fate map of the *D. melanogaster* blastoderm (Campos-Ortega and Hartenstein, 1985), which is still considered a standard modern reference. (see Figure 1.4).

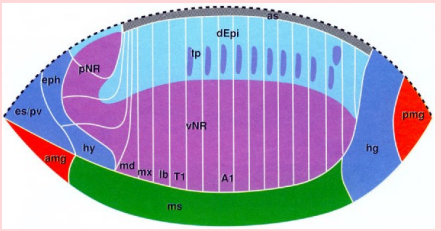

Figure 1.4: Fate map of the *Drosophila melanogaster* blastoderm The fate map is projected onto a planimetric reconstruction of the blastoderm. A1 Abdominal segment 1; amg anterior midgut rudiment (endoderm); as amnioserosa; dEpi dorsal epidermis; eph epipharynx; es esophagus; hg hindgut; hy hypopharynx; lb labium; md mandible; ms mesoderm; mx maxilla; pmg posterior midgut rudiment (endoderm); pNR procephalic neurogenic region; pv proventriculus; vNR ventral neurogenic region; T1 thoracic segment 1; tp tracheal placodes. Diagram from Hartenstein (1993)

Gene expression maps Techniques such as mRNA in situ hybridization allow to map gene expression patterns directly on the embryo, allowing the creation of "gene expression maps". In situ hybridization is based on labelled probes that are complementary to the mRNA (or DNA) that is wanted to map (Gall and Pardue, 1969). The probe accumulates then only where the mRNA of interest is found. Another technique to map gene expression is the use of a reporter gene. A reporter gene, which codes for a protein that can be easily identified (like the green fluorescence protein or beta-galactosidase), is linked to the regulatory region of the gene of interest so the reporter gene is going to be expressed where the gene of interest is expressed. Gene expression maps can also be used to create (or refine) fate maps (Gilbert, 2007). For example, if a gene is know to be expressed only in mesoderm precursors, mapping their gene expression in the early embryo will reveal where such mesodermal precursors are located.

Importantly, fate maps and gene expression maps do not necessarily have to coincide totally. Fate maps inform about which cells in the early embryo will give rise to different cell types or tissues, even when at such early stage the cells can be genetically equivalent.

1.3.2 Gene expression databases of *D. melanogaster*

Berkeley Drosophila Genome Project

The Berkeley Drosophila Genome Project (BDGP) is actually comprised of many projects, whose goals include 1) to complete the high quality sequence of the euchromatic genome of *Drosophila melanogaster* and to generate and maintain biological annotations of this sequence; 2) to produce gene disruptions using P element-mediated mutagenesis; 3) to develop informatics tools that support the experimental process and identify features of DNA sequence; and 4) to characterize the sequence and spatial and temporal expression of cDNAs.

The BDGP insitu project has produced a high-throughput database of mRNA expression in different embryonic stages of *D. melanogaster*, that can be used to complement and extend microarrays or RNAseq analyses (Tomancak et al., 2002). BDGP divides the first 16 stages of embryogenesis into six stage ranges (stages 1-3, 4-6, 7-8, 9-10, 11-12 and 13-16).

A brief description of the hybridization protocol follows, for details see (Tomancak et al., 2002). For the hybridization, they used a set of cDNA clones from the Drosophila Gene Collection (Stapleton et al., 2002), to produce a digoxigenin-labeled antisense RNA probe (Tomancak et al., 2002). Hybridization is carried out in fixed *Drosophila* embryos in 96-well plates. Successful hybridization plates are mounted on slides to document the expression pattern of each gene with high-resolution digital photographs. Then each image is assigned to one of six developmental stage ranges (Weiszmann et al., 2009). Finally, images and annotation data are stored in a modified version of Gene Ontology database. The entire dataset is available to browse or can be download from its webpage (http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl).

Databases like BDGP are suitable for computational image analysis, as the protocols used to produce the images are standardized (Tomancak et al., 2002) and the images can be aligned to an anatomical view (e.g., dorsal, lateral) (Kumar et al., 2011). An example of the power of using a computational image analysis approach is the work of Frise and collaborators. Frise et al. (2010) analysed the spatial expression pattern of 1800 genes (from the BDGP database) in the blastoderm stage of *Drosophila*, projecting them onto a virtual representation of the embryo made of ca. 300 triangles. After clustering the triangles based on their expression similarity, they produced a co-expression map that resembled the fate map shown in Figure 1.4 (see Box 1 for a brief discussion of the relation between fate map and expression map).

Flyexpress

The FlyExpress database (http://www.flyexpress.net/) contains a digitalized library of computationally filtered and standardized images from the high-throughput databases of mRNA expression Fly-FISH and BDGP, and from and peer-reviewed publications. It contains an image-matching search engine that can be used to search for many genes with similar or overlapping patterns of expression in the developing embryo.

The high-throughput databases from which FlyExpress extracts and computationally filter gene expression data differ in the hybridization protocol they use, the number of stages and the staging system, making direct comparisons between them difficult. In contrast with the BDGP database (described in the previous subsection), Fly-FISH uses fluorescence in-situ hybridization probes (Lécuyer et al., 2007) a 17-stage system

(compared to a 16-stage system in BDGP) and five stage ranges (compared to six in BDGP). FlyExpress uses a semi-automated pipeline to standardize and align embryos, separating the multi- embryo images of BDGP into single images and discarding partial embryo images (Konikoff et al., 2012). After that, images are assigned to one of three anatomical views: dorsal, ventral or lateral. Therefore, the expression pattern of a gene at a specific stage and view could be represented in FlyExpress by more than one in-situ image in more than one anatomical view.

In this work, from the images available in FlyExpress, I downloaded only those from BDGP, since BDGP uses more stage ranges than FlyFISH and these represent better the whole embryogenesis of *D. melanogaster*. In the Fly-FISH database is focused specially on the early stages, as the last eight developmental stages are contained in one stage range (stages 10-17). I used the FlyExpress database, instead of the BDGP directly, because the standardization protocol used by FlyExpress produces images with embryos in the same orientation and with a cleared background that are more suitable for image computational analysis.

1.4 The Hourglass model in *Drosophila*

In the 19th century, Karl Ernst von Baer stated in his "laws" that within a group of animals the general characteristics appear earlier in development, while the most special appear in late development (see Box 1; von Baer, 1828). This would lead to low morphological variation at early development, gradually increasing as development proceeds. Other authors (Medawar, 1954; Slack et al., 1993; Duboule, 1994; Raff, 1996) proposed an alternative pattern in which there is great variation in early and late development, while the mid-development would show less variation. This pattern of variation (or conservation) has been called 'phylotypic egg-timer' (Duboule, 1994) and 'developmental hourglass' (Raff, 1996).

Duboule's concept of 'phylotypic egg-timer' was based on the concept of 'phylotypic stage' of Sander (1983), who coined this term to describe the convergence into a conserved segmented germ band stage in insects from very divergent early development (Sander, 1996). In vertebrates, there has been controversy around what should be the phylotypic stage (Ballard, 1981; Slack et al., 1993; Duboule, 1994). Richardson (1995) argued that indeed there is no single conserved stage in vertebrate's development and instead he proposed the term 'phylotypic period' instead. Initially, two explanations for the hourglass model were proposed. Denis Duboule, after observing that the expression of the Hox genes seemed to coincide with the phylotypic stage, he considered that this could not be a coincidence and proposed that the activation of the Hox genes was the cause for the morphological invariance (Duboule, 1994). In contrast, Rudolf A. Raff proposed that the phylotypic stage was the result of complex interaction between developmental modules at this stage (Raff, 1996).

There is an ongoing discussion about whether the hourglass model (HG), the von Baer law or some other pattern fits the divergence among developmental stages in phylogeny (Richardson et al., 1997; Poe and Wake, 2004; Kalinka and Tomancak, 2012). Also, it is not clear if the HG, that seems to fit well in vertebrates and arthropods, would apply to other phyla (Raff, 1996; Salazar-Ciudad, 2010). For example, it is known that the HG model does not apply to spiralians, at least at the morphological level, as many members of this phyla exhibit an early equal cleavage pattern (Henry, 2002).

1.4 The Hourglass model in *Drosophila*

Recently, the HG have received support from different gene expression studies. Kalinka et al. (2010) used micro-arrays for six *Drosophila* species and quantified expression divergence at different developmental stages. They found that gene expression was most conserved during the extended germ-band stage (considered the phylotypic period) and that the non-synonymous divergence per site (Dn) correlated with their divergence measures. They also proposed that the HG pattern is a product of natural selection that acts to conserve patterns of gene expression during mid-embryogenesis (Kalinka et al., 2010). Also, the HG model has been shown to be reflected in the age of the transcriptome. Domazet-Lošo and Tautz (2010) found that when analysing the age of the transcriptome at different stages in the Zebra fish (*Danio rerio*) development (analyzing gene-specific expression data with a phylostatigraphic method), mid-embryonic stages show the older transcriptome (Domazet-Lošo and Tautz, 2010). In another analysis using Zebra fish, it was shown that the mid-development conservation included that of regulatory regions, with sequences of regulatory regions being most conserved for genes expressed in mid-development (Piasecka et al., 2013).

Studies that have measured the conservation of genes at the DNA sequence level also seem to support the HG model. Davis et al. (2005) assessed whether proteins expressed at different times during *D. melanogaster* development varied systematically in their rates of evolution (comparing with *D. pseudoobscura*) and found that proteins expressed early in development and particularly during mid-late embryonic development evolve slower. This suggests, according to the authors, that embryonic stages from 12 to 22 hours are highly conserved between *D. melanogaster* and *D. pseudoobscura*, which is consistent with the HG. In a similar study, Mensch et al. (2013) calculated the dN/dS ratio for more than 2,000 genes among six *Drosophila* species, separating genes in three categories: maternal genes (genes whose products are left by the mother in the egg), genes expressed in early development and genes expressed in late development. They found that maternal genes and lately expressed zygotic genes show higher dN/dS ratios (i.e., are less conserved) than early expressed zygotic genes. Finally, it has also been found that genes expressed in the adult have higher dN/dS ratios than genes expressed in the pupa and those of the pupa have higher dN/dS ratios that those expressed in the embryo (Artieri et al., 2009). Some limitations of these last studies is that they classify the genes in a few broad temporal categories that do not permit to precisely determine the temporal dynamics of conservation and that are based only in divergence data (dN/dS ratios between two species). A study that integrates polymorphism data from natural populations would improve the evolutionary interpretation of these patterns, as it would allow to estimate what proportion of the dN are adaptive (as explained in section 1.2.2). Measuring adaptation is specially relevant as some authors have argued that the HG is caused by different selection pressures in early and late development (Slack et al., 1993; Kalinka and Tomancak, 2012; Wray, 2000).

Box2. Haeckel, von Baer and the *Naturphilosophie*

In the 19th century, important contributions to embryology were made by advocates of *Naturphilosophie*, a philosophical movement based in Kant and Goethe's ideas, aimed to classify nature into categories or classes. Among their classification efforts, they classified embryological phenomena and draw analogies between embryos of different taxonomic groups (Horder, 2010; Ghiselin, 2005).

The first pattern to be recognized, when comparing developmental trajectories of different species, was the Meckel-Serres law, which proposed that embryos followed a linear succession following the *scala naturae* (a hierarchy of all beings arranged in order of 'perfection', with the man at the top). According to this view, influenced by the *Naturphilosophie*, the embryonic development of a higher organism would be a succession of adult forms of lower organisms (Russell, 1916; Amundson, 2005).

Karl Ernst von Baer K. E. von Baer, a German-Estonian naturalist considered the father of comparative embryology (Russell, 1916), refuted the Meckel-Serres law and formulated his own, known as von Baer's laws (von Baer, 1828). Von Baer's first law state that the more general characteristics of a large animal group (e.g., notochord in chordates) develop before special characteristics (e.g., fur in mammals), while his fourth law state that the embryo of a "higher" animal never resembles the adult of another animal form, but only his embryo.

Importantly, von Baer's views were not evolutionary. The resemblance between developmental trajectories of different species was for him only a reflection of their relationship in the Natural System (Amundson, 2005). Ironically, Darwin used and reinterpreted von Baer's observations on embryonic stages in different species to support common ancestry and therefore, evolution (Darwin, 1859).

Ernst Haeckel Ernst Haeckel supported Darwinism and, in what is known as Haeckel's "Biogenetic Law", said that development (or ontogeny) is a brief summary of the slow and long phylogeny (Haeckel, 1874). In his view, a "higher" organism would pass through a series of conserved developmental stages that represent ancestral forms (this view is known as the "recapitulation theory"). However, in contrast with the Meckel-Serres law, he recognized that this recapitulation was almost never complete, due to evolutionary modifications in development.

> *"The falsification of the original course of development is based to a great extent on a gradually occurring displacement of the phenomena, which has been effected slowly over many millennia, by adapting to the changed conditions of embryonic existence. This displacement can affect both their location and time of appearance. Those former we call heterotopy, the latter heterochrony." (Haeckel, 1903).*

Haeckel's views were more complex than usually acknowledged (Richardson and Keuck, 2002). In fact, he said that it was not that all the mammalian eggs were the same, it was just that with the available tools was impossible to detect the subtle, individual differences, "which are to be found only in the molecular structure" (Haeckel, 1903).

Now is evident that none of von Baer's or Haeckel's hypothesis can be considered "laws", as they are not universal.

1.5 *Ciona* as a model organism

The ascidian *Ciona intestinalis*, a marine invertebrate animal, has a long history in developmental biology and evolutionary biology. Darwin highlighted the importance of the ascidians due to their close phylogenetic relationship to the vertebrates (Darwin, 2009). Also, it provided one of the first evidences of localized determinants of cell specification (Conklin, 1905). Although their adult form is a sessile filter feeder, its tadpole larva has characteristic features of the chordate group: a dorsal neural tube, a notochord surrounded by muscle and a ventral endodermal strand Satoh (2003).

Some features of *C. intestinalis* development that attracted the attention of developmental biologists more than a century ago (Kowalewski, 1866; Chabry, 1887) included: its easy to collect, it has a rapid embryonic development (it takes less than 20 hours from the fertilized egg to the larva), its invariant cell lineage and the already mentioned similitude between the ascidian larva and the vertebrate tadpole. More recently, the almost transparent body, which facilitate many genetic techniques, and the sequencing of the *C. intestinalis* genome (Dehal et al., 2002) are partly responsible for the re-emergence of *C. intestinalis* as model organism in developmental biology (Levin et al., 2012).

The sequencing of *C. intestinalis* genome have facilitated the study of gene expression data during its life cycle (Azumi et al., 2007). Relevant efforts have been made to describe the spatial expression patterns of individual genes during embryogenesis (Satou et al., 2001; Fujiwara et al., 2002; Kusakabe et al., 2002; Imai et al., 2004; Miwata et al., 2006), making this an invaluable resource to investigate the spatio-temporal dynamics of gene expression. Taking advantage of the ascidian invariant cleavage pattern and well described lineage analysis (Conklin, 1905; Nishida, 1987), the spatial expression of many genes have been described at the single cell level up to the early gastrula stage. This allowed Imai et al. (2006) to determine the distribution of most of transcription factors and signaling molecules at single-cell resolution for every cell of the *C. intestinalis* early embryo, which they used then to deduce regulatory networks in the early embryo (Imai et al., 2006).

1.5.1 *Ciona intestinalis* life cycle

Ascidians, or tunicates (named after the "tunic" or thick cover in the adult form), are sessile animals that attach to rocks and shells and filter plankton and other nutrients from seawater (Satoh, 2014). During embryogenesis, ascidians show morphogenetic movements during gastrulation and neurulation similar to vertebrates and both share common genetic regulators of cell specification (Satoh, 2003). Its embryonic development is bilaterally symmetric, with invariant cell-lineage and tightly regulated cell division rates (Stolfi and Brown, 2015). As mentioned before, its larval form resembles a (simplified version of the) vertebrate tadpole. The larva is commonly divided in "trunk" (anterior part) and "tail" (posterior part). The trunk contains the anterior central nervous system (CNS), peripheral nervous system (PNS), and undifferentiated mesoderm and endoderm. The tail is composed of caudal CNS and PNS, notochord, muscle the endodermal strand (Stolfi and Brown, 2015). Embryonic development can be divided in cleavage, gastrula, neurula and tailbud stages (Hotta et al., 2007). In Table 2, these embryonic stages are briefly described for *C. intestinalis*, with the developmental time at 18°C, based on (Hotta et al., 2007). Table 2 can be used as a reference when mentioning specific developmental stages in *C. intestinalis* in this work.

1.5.2 The ANISEED database

The ANISEED database version 2015 integrates expression data from large-scale in situ hybridization studies with embryo anatomical data of ascidians (Brozovic et al., 2016). The 2015 version represents a considerably improvement from the ANISEED version 2010 (Tassy et al., 2010). Previously, there were ontologies for each stage, with equivalent terms having different IDs in each stage, making the comparison between stages more difficult. The data accessibility has also improved considerably, as the new database version contains all ISH data for *C. intestinalis* in a single parsable XML file, more amenable to computational analysis.

This database includes 27,707 *Ciona intestinalis* gene expression profiles by in situ hybridisation for approximately 4500 genes acquired from more than 200 manually curated articles (Brozovic et al., 2016). The expression data is represented using an ontology-based anatomic description of the embryos. The ANISEED database also includes expression data from the Ghost database (Satou et al., 2005), which contains the spatial expression patterns of more than one thousand cDNA clones by whole-mount in situ hybridization at different developmental stages.

The ANISEED database also includes biometry data (e.g., volume, surface/volume) and 3D embryo models (at a single-cell resolution) of ascidian embryos until the early gastrula (Tassy et al., 2006). Importantly, combining the gene expression and 3D embyo models at a single cell resolution it is possible to reconstruct the gene expression pattern in 3D, as I did here.

Table 2. Embryonic stages of *C. intestinalis* and main morphological characteristics (based on Hotta et al., 2007)

Stage	Description	Time after fertilization	Morphological characteristics
1	**Zygote**	0min to 55min	From the fertilisation event up to the end of the first mitotic cycle
2-5	**Early cleavage**	55min to 3h	Five mitotic divisions, until the 32-cell stage. First and second cleavages separate the left and right halves, and the anterior and posterior halves, respectively.
6-9	**Late cleavage**	3h to 4.5h	Very small B7.6 cell pair in the posterior end. Asymmetric divisions in the vegetal hemisphere. Embryo flattens on its vegetal side. Vegetal cells take a columnar shape
10-13	**Gastrula**	1.5h to 6.3h	Invagination and migration of endodermal and mesodermal cells inside the embryo. At the early gastrula, the vegetal side of the embryo takes a horseshoe shape. Embryo starts elongating anteriorly
14-16	**Neurula**	6.3h to 8.5h	The embryo, with an oval shape, continues elongating. Notochord precursors intercalation and convergence. Neural tube formation and closure (starting from the posterior side)
17-20	**Initial and early taibud**	8.5h to 10h	Clear separation between trunk and tail. Neuropore closure. Tail starts bending
21-22	**Mid tailbud**	10h to 11.9h	Intercalation of the notochord cells is completed. The tail bends ventrally so the embryo adopts a half-circle shape. Length of the tail twice as long as the trunk
23-25	**Late tailbud**	11.9h to 17.5h	Pigmentation of the otolith can be observed. Palps formation. Vacuolization of notochord cells. The tail is bent dorsally
26	**Hatching**	17.5h	The larva hatches. Head adopts an elongated rectangular shape

2 Aims of the study

In this work, I have analysed publicly accessible spatio-temporal gene expression data of two model organisms, *Drosophila melanogaster* and *Ciona intestinalis*, together with population genomics data of *D. melanogaster*. Using a statistical approach, I address these following questions, which have been selected for the great interest they have aroused in the scientific community since the early days of developmental and evolutionary biology:

I How do complexity and compartmentalization increase in the embryo during development?

II Are there differences in the pattern of compartmentalization and complexity increase when comparing different species (i.e., *D. melanogaster* and *C. intestinalis*)?

III Can adaptation be found in specific anatomical parts of the embryo or developmental stages?

IV Is the Hourglass model supported by evidence of natural selection when considering inter and intra-specific variation at the DNA sequence level?

3 Material and Methods

3.1 On the complexity measures used in this work

In the following paragraphs I will describe the three measures of complexity I used in here: relative area/volume, disparity and roughness. I consider them complexity measures because they inform about specific features in embryonic development that are intuitively associated to the increasing complexity of the organism. The relative area/volume of expression relates to the notion of the progressive compartmentalization of the embryo, disparity informs about how the different parts of the embryo become more different (at the genetic level) from each other, and the roughness measure relates to the notion of genes being expressed in progressively more complex spatial expression patterns (as defined by the shape of the gene expression pattern). For information on the statistical tests used (e.g., Kruskal Wallis, ANOVA, permutation test) see the corresponding study.

Compartmentalization

As mentioned before, the process of embryo compartmentalization, is expected to be largely reflected in the expression of the genes in progressively smaller areas. Therefore, a good measure of compartmentalization would be to measure the relative area of expression of all genes during development. Gene expression patterns are usually visualized as 2D images, which reflect the distribution of a gene product from a specific anatomical view (e.g., lateral, dorsal). Recently, methods like 3D imaging technique Optical Projection Tomography (Sharpe, 2003; Summerhurst et al., 2008) allow to record gene expression patterns in 3D. In the case of 2D images, the measure of compartmentalization I use in here is the relative area of the expression, i.e., the pixels with expression divided by all the pixels of the embryo image. This "relative area" measure ranges from 0 (no expression) to 1 (ubiquitous expression). In the case of a gene expression in 3D, the compartmentalization measure becomes the relative volume of expression, which also ranges from 0 to 1 and is calculated as the volume of the cells/tissues with expression divided by the whole embryo volume.

Disparity

The disparity measure is related to McShea's "number of part types" complexity measure. Ideally, the number of cell types, if this could be precisely known, would be a good measure of complexity during development. However, as mentioned before, there is no clear criteria to determine when (at the genetic level) a new cell type is formed during development. Instead of trying to determine the number of cell types during development, I decided to quantify how different at the gene expression level are the cells or regions in the embryo. In *D. melanogaster*, as it was not possible to have expression for each individual cell, I divided the 2D embryo in "regions" of approximately the same area using polar coordinates (Fig. 3.1A; see study I). In the case of *C. intestinalis*, I used expression data at a single cell level for the early stages and at tissue level for the tailbud stages (see study II).

To quantify this, I decided to use pearson's correlation as similarity measure between cells/tissues or regions. With this method, the expression of all the genes available in two cells can be compared, giving a value of 1 if all genes have exactly the same expression

(their expression is completely correlated), to -1 if all the genes show opposite gene expression (their expression is anti-correlated). An advantage of this method is the possibility to include the information of all available genes, diminishing a possible bias in the gene selection. I computed pairwise similarities between cells/regions as the Pearson correlations using the function *corSimMat* of the R package *apcluster* (Bodenhofer et al., 2011). In here, I was interested on quantifying the difference (i.e., disparity) in gene expression between cells, not on their similarity. Therefore, the disparity between two cells becomes: disparity = 1 - pearson's similarity. The disparity measure ranges from 0 to 2, with a 0 value when the gene expression between cells is exactly the same.

Synexpression Territories Using the Pearson's similarity matrix I performed a hierarchical clustering of cells/ regions using the function *hclust* of the R package *stats* (R Core Team, 2015) with the average method UPGMA and an euclidean distance function. The resulting dendrograms, with as many terminal branches as cell/regions analysed, were cut into a given number of clusters, called in here "synexpression territories". In *D. melanogaster* (study I and III) the dendrogram resulting from the analysis of the regions of all stages was cut into 40 synexpression territories. The territories are not exactly the same between study I and III, as in study III the clustering was done again with the 1199 genes dataset (see section 3.2.1). In *C. intestinalis* (study II), because the information in the early stages is at the cell level while in the tailbud is at the tissue level, we performed two separate synexpression territory analyses one with the 32, 64 and 112 cells stages (n = 1550 genes), and another one with the early, mid and late tailbud stages (n = 820 genes). The dendrograms resulting from the early and tailbud stages were cut in 24 and 10 "synexpression territories".

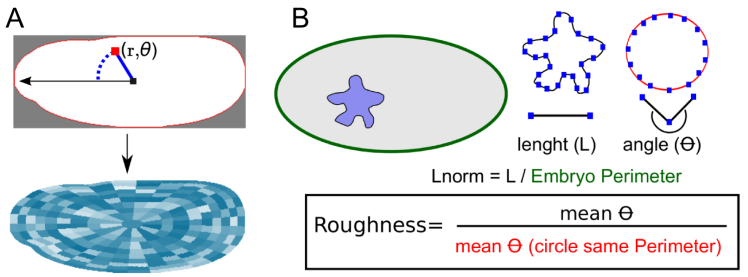

Figure 3.1: Polar regions and 2D roughness measure. A) The embryo of each stage was divided in 257 regions using polar coordinates. The embryo for stage 11-12 is shown with the 257 regions in a random color. B) A schematic embryo (gray) with a gene expression pattern in blue. Roughness is the mean major angle (θ) between each node (at every L length pixels in the contour) and its two immediate neighbours, normalized by the mean angle of a circle of the same perimeter.

Roughness

The roughness measure analyses the complexity of the shape of a gene expression pattern. In the case of 2D images, the shape of the gene expression pattern is extracted as a closed outline formed by the boundaries of gene expression. For 3D patterns, the shape of the expression pattern is the 3D external surface of the union of the cells that are expressing such gene.

Therefore, a gene expression pattern reflects necessarily the spatial distribution of the cells expressing such gene. When analysing and comparing the shape of diverse gene expression patterns, i.e., the cells/tissues with expression are different between genes, there is an obvious impossibility to determine landmark points (whether around a 2D outline or 3D surface) that would establish a clear one-to-one correspondence between them. This could be done in the case of comparing the expression pattern of a single gene at a specific developmental stage between different individuals. Therefore, a landmark-free method (like outline methods for 2D or surface methods for 3D data) is best suited to deal with the type of data analysed in here.

There are practically no studies in the literature that have quantified and compared the shape of gene expression patterns in a systematic manner (one exception is the recent study of Martínez-Abadías et al., 2016). In here, I will consider that a gene expression pattern is complex based on the curvature of its 2D contour or 3D surface.

2D Roughness For 2D gene expression patterns, I used a "roughness measure", that is similar to the shape function $\phi^*(l)$ used in eigenshape analyses (Lohmann, 1983), as it measures how much the curvature of a closed outline deviates from the angles of a circle of the same perimeter (Fig. 3.1B; see study I). To calculate the roughness of a expression pattern I first selected points in the contour every L (length) pixels. Then, vectors between each node and the two immediate neighbour nodes in the contour are calculated and the biggest angle formed between them is measured. The roughness value is then the mean angle normalized by the mean angle of a circle of the same perimeter.

I selected the roughness measure instead of some other measure outline based method like Fourier analysis because the roughness value gives an intuitive descriptor of complexity, i.e., a value of 1 would be a simple "circle-like" shape, and a value greater than 1 would mean a higher curvature of the outline. McLellan and Endler (1998) compared various measures of spatial complexity applied to the outlines of the leaves of many tree species. They included a "margin roughness" measure that is very similar to the one I use here (the difference is that they does not normalize by the mean angle of the circle nor he uses different lengths of vectors) and found that there was no marked differences between the margin roughness and a Fourier analysis with up to 64 harmonics (both performed equally well). Other feature of the roughness measure is that allows to measure the complexity of shape at different spatial scales (varying the L length).

Dirichlet Normal Energy (DNE) In order to use a similar measure of curvature in 3D, I used the Dirichlet normal energy (DNE; described briefly in section 1.1.3) which quantifies the deviation of a surface from being planar (Fig. 3.2; see study II). Importantly, both measures are normalized to remove size and orientation effects.

To calculate the DNE, I used the Morphotester software version 1.1.2 (Winchester, 2016) available in the webpage "http://morphotester.apotropa.com/". For details see study II.

It is important to mention that the aim of this analysis is not to discern which mechanisms (e.g., cell-cell signalling or morphogenetic movements) are responsible for the changes in complexity of the shape of gene expression pattern, but to quantify how this happens during embryonic development.

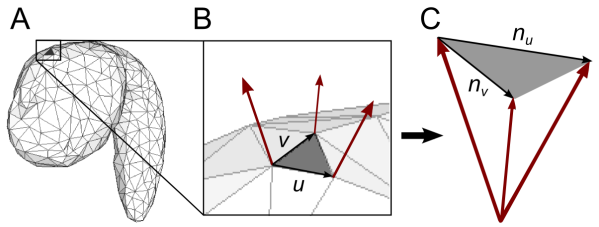

Figure 3.2: Dirichlet Normal Energy (DNE). A) A surface mesh representing a a mid-tailbud embryo in *C. intestinalis*. B) DNE calculates the energy value $e(p)$ of each polygon (like the one in grey) in the surface. The polygon is characterized by vectors u and v, which represent the edges of the polygon. Then, normal unit vectors are estimated as the normalized average of normal vectors of the triangle faces adjacent to each vertex (red arrows). C) If vertex normals are translated to a common origin point, their end points form a polygon with edge vectors nu and nv, which represent the spreading of nu and nv. In a simplistic way, DNE can be defined as the spreading of nu and nv relative to the spreading of u and v (Bunn et al., 2011; Winchester, 2016).

The relationship between these measures

The three different measures of complexity are informative of different and independent aspects of complexity and are not necessarily correlated. For example, a decrease in the area/volume of gene expression should not necessarily mean an increase in disparity, as the genes that are reducing their expression area could be restricted to the same part of the developing embryo. Only in the case of an embryo with all genes showing ubiquitous expression, there is a clear relationship between disparity and relative area/volume, as the relative area of expression and disparity would be 0 and 1 respectively. If there are however, many genes expressed in only a part of the embryo, these measures are not necessarily correlated.

This can be illustrated with a simple example shown in Fig. 3.3, in which there are different alternative gene expression scenarios of an imaginary embryo with six cells. In each scenario, the embryo expresses four genes in different relative areas (i.e., in a different number of cells). The mean relative area is 0.5 for all scenarios, but the mean disparity varies in a two-fold manner. In the scenario that shows the largest disparity, each cell expresses a unique combination of genes, while the scenario with the lowest disparity, 4 of 6 cells do not have a unique expression profile.

The roughness and disparity independence can be easily exemplified in the case of a blastula. Blastula is the name to define the multicellular aggregate stage that results from the subdivision (cleavage) of the zygote. The blastula shape topology and geometry is usually simple (Forgacs and Newman, 2005), typically consisting of a ball of cells with an interior cavity (called "blastocoel"). If in a spheric blastula, composed of also spheric cells (like that of a sea urchin) a large proportion of genes would be expressed ubiquitously and a small proportion of genes would be expressed in single cells, both roughness and disparity would be relatively low. However in the case of a large proportion of genes expressed in different single cells, and a low proportion of genes expressed ubiquitously, the disparity would be high, but the roughness would be very similar than in the previous case. This would be because the roughness quantifies the shape of the expression pattern, irrespective to size. Therefore the roughness of a gene expression in a single spheric cell would be practically the same that the roughness of a

gene expression in the whole spheric embryo.

The independence of the roughness measure with the size of the gene expression (i.e., relative area/expression) comes from the roughness normalization. The normalization of the 2D roughness consists of dividing the mean angle of a gene expression pattern by the mean angle of a circle with the same perimeter, and in the case of 3D roughness (i.e., DNE) it consists on transforming the 3D expression surface into a polygonal surface mesh with a determined number of polygons.

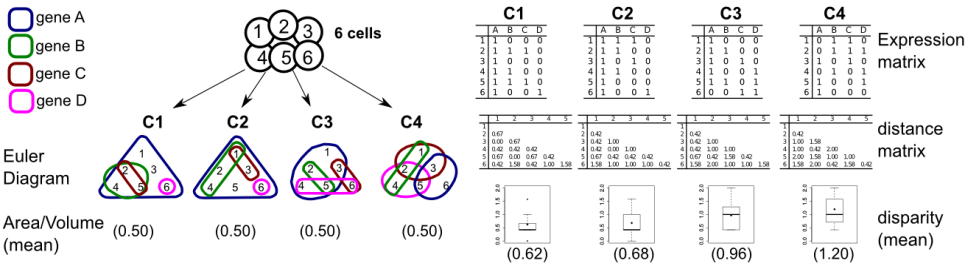

Figure 3.3: Relation between area/volume of expression and disparity measures. An embryo of six cells (top left) is shown expressing four different gene expression combinations (C1, C2, C3 and C4) of four genes (A, B, C and D). All combinations have a mean relative area/volume of 0.5. Each gene expression configuration is represented as an Euler diagram (representing the subset of the cells in which it is expressed in a color code shown at the top left) and as binary expression matrix (top right). The pairwise distance between the cells, calculated as 1-(pearson's correlation), is shown as a matrix. At the bottom right, a distribution plot of the pairwise distances of each combination and the mean disparity are shown below in parenthesis.

3.2 Data mining and handling

The work presented here is mainly based on the analysis of publicly available data contained in many databases, introduced in the Review of the Literature section. In the next paragraphs I will describe briefly how the data used in here was acquired and processed. For more details see the corresponding study.

3.2.1 In situ Hybridization data

D. melanogaster (study I and III)

Image acquisition and filtering. Images were systematically downloaded (with an ad hoc Perl script) from FlyExpress version 5.1 (Kumar et al., 2011) on February 2013. Only genes with laterally oriented images for the six stages used in BDGP (Tomancak et al., 2002) were considered.

Images were resized as in Konikoff et al. (2012) and the gene expression pattern was extracted using an adaptive threshold, based on the mean and variance of a grey-scale version of each image. Genes with ubiquitous expression in stages 1-3 and 4-6 were considered as entirely black images. To correct for small variations in the shape of the embryos, I morphometrically deformed each embryo to an stage ideal embryo shape. Finally, I applied a "smoothing filter" and eliminate isolated white/black pixels (see study I for details). I manually filtered images from the literature or directly from

BDGP of Transcription Factors or Growth Factor genes that did not have information in FlyExpress.

For study I, the resulting dataset contained 1218 genes with expression information in the six stages used in BDGP. For study III, obsolete or repeated genes were removed from the 1218 genes, after checking for gene annotation updates with the *biomaRt* package (Durinck et al., 2009), leaving a dataset of 1199 genes.

Main spatio-temporal expression profiles (study I). I performed a time series cluster analysis (with the STEM software; Ernst and Bar-Joseph, 2006) using the relative area of expression to know which are the most common spatio-temporal profiles. The resulting clusters were analysed with a GO term analysis with the same software.

Anatomical terms (study III). In addition to the whole-mount in situ mRNA hybridization images, the BDGP database contains, for each gene, the list of the embryonic anatomical structures in which such gene is expressed (Tomancak et al., 2007). Each gene expression is described by one or several of those of anatomical terms by an expert. This information was retrieved from the BDGP downloads page (http://insitu.fruitfly.org/ insitu/html/ downloads.html/), which contains the annotations of almost 8,000 genes. We removed genes with "no staining" as anatomical term in any stage, leaving a total of 5762 genes.

C. intestinalis (study II)

I downloaded the in situ hybridization data (ish.zip file) from the download section of the ANISEED database on 28th of December 2015. The expression data for the first three stages is at the cell level, while in the tailbud stages is at the tissues or specific regions of the embryo level. I extracted the information of the 32 cells, 64 cells, 112 cells, early tailbud, mid tailbud and late tailbud stages. Only expression data from experiments reported to have Wild type phenotype, "public" publication status, with in situ hybridization as experiment design and whose probe was assigned to a Kyoto Hoya (KH) (Satou et al., 2008) gene model. I excluded data from experiments whose image characterization was reported as "not sure" or too broadly as "part of whole embryo".

The number of genes analyzed is n=745 for the 32-cell stage, n=758 for the 64-cell stage, n=809 for the 112-cell stage, n=1082 for the early tailbud, n=1092 for the mid tailbud and n=887 for the late tailbud. The gene expression as text-based annotation was transformed into a 3D expression pattern using 3D embryo models (see below) with the Meshlab software version v1.3.3_64bit (Meshlab Visual Computing Lab ISTI-CNR; see study II for details).

Transcription factors and signalling genes I used the comprehensive list of TFs (http:// ghost.zool.kyoto-u.ac.jp/ TF_KH.html) and SIGs (http:// ghost.zool.kyoto-u.ac.jp/ ST_KH.html) deposited in the Ghost database (last access in July 2015).

This list is based mainly in Imai et al. (2004), who determined the expression profiles of 389 transcription factors (TFs) and 118 signaling molecules (SIGs) genes from the egg to mid-tailbud embryos. TFs are categorized in nine gene families: basic helix-loop-helix (bHLH), homeodomain (HD), Fox, ETS, bZIP, nuclear receptor (NR), HMG, T-box transcription factors or as "other TFs" (mainly with diverse Zinc finger genes).

The SIGs genes consist of genes of receptor tyrosine kinase pathways including ligands such as FGFs and intracellular signalling molecules such as MAPK, Notch, Wnt, TGFβ, Hedgehog and genes in the JAK/STAT pathways.

3D embryo models I downloaded, from the ANISEED database, 3D embryo models (at a single-cell resolution) for the 32-cell, 64-cell and 112-cell stages. Also for these stages, I downloaded files (biometry.zip folder; see study II) with a quantitative description of the geometry of individual blastomeres, including the volume of each blastomere relative to the whole embryo (Tassy et al., 2006) used in the relative volume analysis. For the tailbud stages I used a 3D model of *C. intestinalis* mid tailbud (stage 22) anatomy at a single cell resolution (Nakamura et al., 2012), downloaded as a file "3DVMTE_THratio1.86.wrl" from http://chordate.bpni.bio .keio.ac.jp/3DVMTE/. 3D embryo models for early and late tailbud were not available, so I used the 3D mid tailbud for all the tailbud stages. In these stages the main morphogenetic process is the tail elongation by cell intercalation (Hotta et al., 2007), so the differences between these stages are largely restricted to tail length and width and should not affect largely the relative volume measure. From this file, I manually extracted the information of different tissues into separate 3D files and processed them using diverse filters of the Meshlab software version v1.3.3 (Meshlab Visual Computing Lab ISTI-CNR; see study II).

3.2.2 Transcriptomics and population genomic data

modENCODE (study III and IV). Gene expression levels in reads per kilobase per million mapped reads (RPKM) units for 30 developmental stages were retrieved from Gelbart et al. (2013), who analyzed RNA-seq throughput data from the modENCODE project (Graveley et al., 2011).

For using RNA-seq data to compare expression between samples, a normalization step was performed to adjust for varying sequencing depths and other potential technical effects across replicates (see study III)

DGRP (study III and IV). The population genomic data comes from 168 inbred lines of *D. melanogaster* sequenced in the Freeze 1.0 of the Drosophila Genetic Reference Panel (DGRP) project (Mackay et al., 2012). The DGRP population was created collecting gravid females from a single population of Raleigh, North Carolina (USA), and following the full-sibling inbreeding approach during 20 generations to obtain full homozygous individuals. DGRP lines showing high values of residual heterozygosity (>9%) that were observed to be associated with large polymorphic inversions (Huang et al., 2014) were not included.

Testes and immune genes (study III). To discard the possibility that the adaptation patterns are due to an excess of male-biased genes, testes specific genes or immune-related genes, known of being under positive adaptation (Civetta and Singh, 1995; Swanson et al., 2001; Artieri et al., 2009; Obbard et al., 2009), genes related to these functions were removed (for details see Methods study III).

Maternal, maternal-zygotic and zygotic genes (study III). A list of maternal, semi-maternal and zygotic genes was obtained using data from Thomsen et al. (2010),

who performed microarray analyes of unfertilized eggs and the early zygote embryos (for details see Methods study III).

3.3 Estimating adaptation with DFE-alpha

To estimate adaptation during *D. melanogaster* embryogenesis, the DFE-alpha method and software were used (see section 1.2.2; Eyre-Walker and Keightley, 2009), which infer adaptation combining polymorphism and divergence data.

The DFE-alpha software (DFE-alpha, Eyre-Walker and Keightley, 2009; see below) requires that all sites to have been sampled in the same number of chromosomes. Therefore, the original DGRP dataset was reduced to from 168 to 128 isogenic lines by randomly sampling the polymorphisms at each site without replacement. Residual heterozygous sites and sites with no quality value were excluded from the analysis. This software estimate several parameters (e.g., α and ω_α) from a set of genes as estimates based on single genes can be affected by the lack of segregating (divergent) sites. Therefore, in each analysis a group of genes was randomly sampled (bootstrap with replacement) (see studies III and IV). As neutral reference the positions 8-30 of short introns ($\leq$ 65 bp) were used (as in Heyn et al., 2014). For validation, 4-fold degenerate sites were also used.

The release 5 of the Berkeley Drosophila Genome Project was used as the reference genome (http://www.fruitfly.org/sequence/release5genomic.shtml/). The divergence statistics were estimated from a multiple genomic alignment between DGRP lines and *D. yakuba* BDGP 5 coordinates (from http://popdrowser.uab.cat; Ràmia et al., 2012). The number of sites and substitutions and the folded site frequency spectrum (SFS) were computed using an ad hoc Perl script.

Ortholog genes between *D. yakuba* and *D. melanogaster* were obtained from FlyBase (http://flybase.org/). *D. yakuba* was used as outgroup species as, due to the time since their divergence, there is less chance of ancestral polymorphism contributing to divergence, diminishing the effect of low divergence affecting the estimates of adaptive evolution (Keightley and Eyre-Walker, 2012).

3.4 Transcriptome age and genomic determinants

3.4.1 Transcriptome age

Gene phylogenetic age

A phylogenetic age, or phylostratum (PS), to each gene was assigned using the phylostratigraphic maps of *D. melanogaster* (from Drost, 2014; Drost et al., 2015). The PS assigned to each gene is based on the phylogenetic level at which ortholog genes are found.

I downloaded the PS dataset on May 2015 (available from http://dx.doi.org/ 10.6084/m9.figshare.1244948/). For study III, the number of analysable genes for the spatio-temporal and anatomical term analyses were 555 and 2722 genes, respectively (genes with PS values and analysable with the DFE-alpha method, see above).

Region phylogenetic age (study III)

The Transcriptome Age Index (TAI) is defined as the weighted arithmetic mean of phylostrata, using gene expression intensities as weights (Domazet-Lošo and Tautz, 2010). In here, I calculated the TAI for each region and territory of the embryo in a developmental stage, using the relative area of expression of a gene in a region or territory as weights. Therefore, for each region and territory j, the TAI was calculated as:

$$TAI_j = \frac{\sum_{i=1}^{n} ps_i A_{ij}}{\sum_{i=1}^{n} A_{ij}}$$

where ps_i denotes the PS of gene i , A_{ij} is the relative area of gene i in the region or territory j, and n the number of genes expressed in such region or territory. A relatively low value of TAI_j represents a high mean evolutionary age of the transcriptome in the region or territory j, and conversely. The TAI was calculated using the *myTAI* R package (Drost, 2014).

3.4.2 Genomic determinants

The following genomic features, called in here "genomic determinants" were obtained using coding exons and short introns annotations for *D. melanogaster*, obtained from FlyBase release 5.50.

Intron length. Average distance, in base pairs (bp), between the exons of a gene.

Intergenic distance. Average number of bp between two adjacent genes.

Gene size. Length of the coding region of a gene.

Messenger complexity. Number of transcripts divided by the number of exons.

Number of transcripts and exons. Number of different transcripts and exons of a gene, respectively.

Codon bias. Measured as the Frequency of optimal codons (Fop). Was estimated using CodonW (Peden, 1999; http://codonw.sourceforge.net/). This index is estimated as the ratio of optimal codons to synonymous codons. Its values range between 0, where no optimal codons are used, and 1, where only optimal codons are used.

Expression bias. Proportion of development stages in which a gene is expressed. Based on (Yanai et al., 2005) and (Larracuente et al., 2008), we estimated the expression bias, τ as:

$$\tau = \frac{\sum_{j=1}^{n} 1 - \log S_j / \log S_{max}}{n-1}$$

where S is the logarithm of the RPKM and n is the number of developmental stages. τ ranges from 0 to 1, with values close to 0 indicating broadly expressed genes and values close to 1 indicating genes with highly biased expression.

Expression level. Estimated as the logarithm of the maximum expression in RPKM units.

Recombination levels. Recombination rates estimates at 100 kb non-overlapping windows, crossing-over events (from Comeron et al., 2012).

4 Results and Discussion

4.1 Comparative study between *Drosophila* and *Ciona* (I and II)

4.1.1 Compartmentalization

As the development of *Ciona* and *Drosophila* are very different and it would be impossible to compare them stage-by-stage, I focused here in three major developmental periods: pre-gastrula, gastrula, and post-gastrula stages. These periods are easily recognizable in both species facilitating the comparative analysis.

I found that in both species, the relative area or volume decreased in a non-linear way (see Fig. 4.1 and Fig. 4.2). However, the timing of the major decrease was different. In *Drosophila* the major decrease occurred at very early development, from maternal to early gastrula stage (Fig. 4.1). Practically half of the genes follow this decrease pattern: 46% of the genes were characterized as having a non-linear decrease in their relative area. In contrast, in *Ciona* the volume of expression decreases mostly after gastrulation (between the 112-cell and the early tailbud stage). However less dramatic, I found significant differences between the 32-cell and 64-cell stages, and between the 64-cell and 112-cell stages.

Main spatio-temporal profiles in *Drosophila*

Using a time series cluster analys, I found the eight main spatio-temporal profiles of gene expression in the embryonic development of *Drosophila* (study I, Fig. 5). As expected, the most common profile (n=297 genes) follows the global profile of non-linear decrease in the first stages. I also found both linear increase and decrease profiles and a "hill-like" profile (initial increase and further decrease with the higher values at stage 7-8) The linear decrease profile (n=167 genes) was enriched with "mitotic cell cycle" (GO:0000278), "RNA processing" (GO:0006396) and "chromatin modification" (GO:0016568) GOterm genes, highlighting biological processes that first are present in the whole embryo and become more and more restricted in space as development proceeds. The "mitotic cell cycle" term, for example, most likely relates to the fast mitotic cycles in the earliest embryo stages. During stage 1-3 nine fast and synchronic

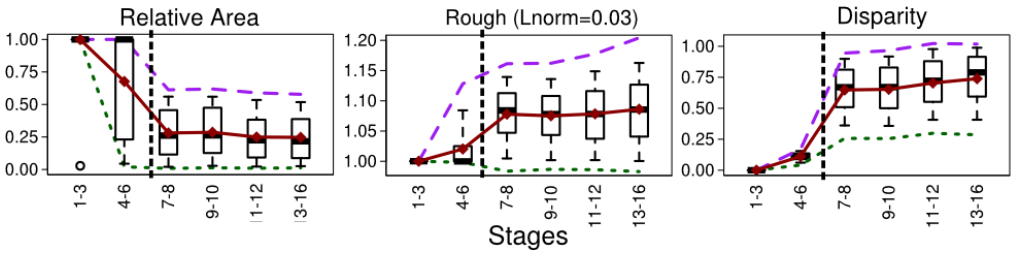

Figure 4.1: Measures in *Drosophila*.Distribution plot of the relative area of expression (left), roughness (center) and disparity (right) for all genes in each stage. Diamonds represent the mean, boxes the Inter Quartile Range (IQR). Whiskers 10 and 90 percentiles. Dashed line represents the max values and dotted line the min values (mean of the last and first decile, respectively). Stages on the x-axis, vertical dashed line represents gastrulation entry.

mitotic divisions take place in the entire embryo, then in stage 4-6 mitotic divisions 10-13 occur more slowly, almost synchronically. The 14th cycle, zygotically controlled, is long and of different durations in the embryo.

With a temporal co-expression cluster analysis using microarray data through the life cycle of *D. melanogaster*, Arbeitman et al. (2002) found that most cell cycle genes were expressed at high levels during the first 12h, but only a few are expressed at high level thereafter. My analysis is consistent with this, as I found that the profile of linear decrease (I, Fig. 5A) is enriched with such genes. In this sense, this study is complementary to Arbeitman et al., and adds the spatial dimension to their temporal expression profiles.

4.1.2 Disparity

As the relative area (or volume) of expression informs on how genes are expressed in progressively smaller regions in the embryo, the disparity measure can inform about how different regions of the embryo express increasingly different combinations of genes. My results show that in each species, the global disparity pattern is similar to the relative area or volume patterns. Therefore, in *Drosophila* the disparity increases mostly in the transition from the maternal to early gastrula and in *Ciona* this major change occurs after gastrulation.

It is important to notice that these measures should not necessarily correlate (see section 3.1). In *Ciona* I found an example of a case when there is no perfect correspondence between the relative volume and the disparity of expression: disparity increased significantly between early to mid-tailbud stages but no significant differences between the relative volume of expression of these stages were found (II, Fig. 3A). This means that, on average, genes are expressed in a similar number of tissues in these stages, but in the mid tailbud the combination of genes expressed in these tissues are more different between each other.

This shows that the disparity measure is useful specially when is complemented with the relative area (or volume) measure to describe the compartmentalization of the embryo.

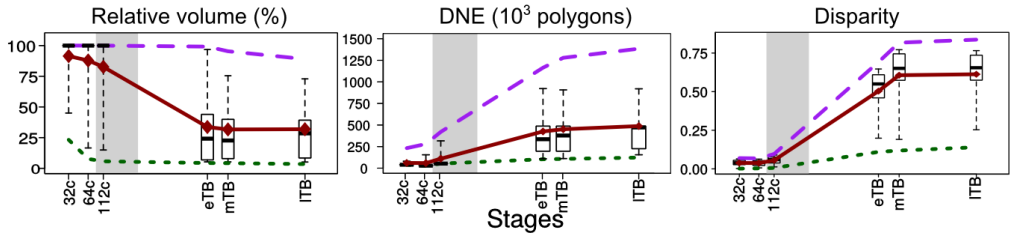

Figure 4.2: Measures in *Ciona*. Distribution plot of the relative volume of expression (left), DNE (center) and disparity (right) for all genes in each stage. Diamonds represent the mean, boxes the IQR. Whiskers 10 and 90 percentiles. Dashed line represents the max values and dotted line the min values (mean of the last and first decile, respectively). Stages on the X-axis (s32c, 32-cells; s64c, 64-cells; s112c, 112-cells; eTB, early tailbud; mTB, mid tailbud; lTB, late tailbud). Grey area represents gastrulation period.

4.1.3 The leading role of TFs and GFs (and other signalling molecules)

Using a GOterm analysis in *Drosophila*, I found that TFs (GO:0003700) and GFs (GO:0008083) showed smaller relative area of expression that the rest of genes in the blastoderm stage (Fig. 4.1). The TFs are also expressed in smaller areas than the rest of the genes in all subsequent stages, while the GFs are expressed in smaller areas at the blastoderm (stage 4-6) and extended germ band stages (stage 9-10 and 11-12) (I, Fig.4). In the blastoderm stage the disparity of the regions based only on the TFs is much greater than the one based on all the genes (KW pvalue < 0.001, see study I) confirming that these genes account for a large portion of the diversity of gene expression patterns in the blastoderm stage.

These results are consistent with a previous study of TFs expression during *Drosophila* embryogenesis done by Hammonds et al. (2013). They made an extensive analysis of TFs expression using manual annotation of gene expression based on an anatomical controlled vocabulary and classifying every gene as ubiquitous, patterned, ubiquitous-patterned, or maternal (from the BDGP database; Tomancak et al., 2007). They found that the fraction of TFs expressed in a restricted pattern (assigned to a tissue) was higher, when compared to other genes, in all zygotic stages with the exception of the stage 13-16. The results I show for stages 4-6, 7-8, 9-10 and 11-12 are consistent with Hammonds et al., as the higher proportion of the TF genes showing a restricted or tissue-specific expression pattern would imply that TFs are expressed in smaller areas in the embryo. For the 13-16 stage, contrary to these authors, I showed that the TFs are highly compartmentalized. This might indicate a limitation of the annotation method used by Hammonds et al., to capture the high spatial compartmentalization of the TFs in this stage.

In *Ciona*, I performed a similar analysis using the categorization of TFs and signaling molecules (SIGs) made by Imai et al. (2004). SIGs consist of genes of receptor tyrosine kinase (RTK) pathways such as FGFs and intracellular signalling molecules such as MAPK, Notch, Wnt, TGFβ, Hedgehog and genes in the JAK/STAT pathways (Imai et al., 2004).

As expected, TFs volume of expression decreased faster than non-TFs. The TFs showed lower volume of expression in the 64-cell and 112-cell stages (II, Fig. 3B). The results are similar for maternal and zygotic genes (maternal/zygotic classification based on Matsuoka et al., 2013; II,Fig. S1). I then compared TF families and found that six TF families showed lower relative volume in the early gastrula (BZIP, T-box, bHLH, HMG, Nuclear Receptor, and 'Other-TFs') but only T-box genes showed a lower relative volume from the 32-cell stage until gastrula (II,Fig. S2).

The results obtained for the T-box gene family (conserved in metazoan and several non-metazoan lineages (Sebé-Pedrós et al., 2013) are consistent with the known important role these genes have in diverse metazoan species early cell fate specification (reviewed in: Papaioannou, 2014; Showell et al., 2004. Examples of T-box genes in *Ciona* are Tbx6 and *brachyury*, crucial for muscle tissue formation (Mitani et al., 1999; Nishida, 2005) and for notochord specification (Yasuo and Satoh, 1998), respectively. I also found that the SIGs showed significant lower relative volume of expression than the rest of the genes in the 32-cell, 64-cell, and 112-cell stages (II, Fig. 3B). Specifically, in the 64-cell stage RTK-MAPK, Wnt and TGFβ families showed significant higher disparity in the 64 cells stage, suggesting a predominant role of these pathways in the patterning of the embryo at this stage. This is consistent with known short range induction events

by nodal and various FGFs, which are part of the TGFβ and RTK-MAPK signalling pathways, respectively (Lemaire et al., 2008).

In general, the fact that in these two species that display a very different development TFs and GFs (or SIGs in the case of *Ciona*) are more compartmentalized than the rest of the genes precisely in the stage before entering gastrulation, is consistent with these genes having a special role in pattern formation and compartmentalization. Thus, these results confirm the leading role of TFs and GFs in driving pattern formation and compartmentalization in the early embryo.

4.1.4 2D and 3D roughness analyses

The results show that both 2D and 3D roughness (measured with DNE; see section 3.1) increase in a non-linear way during development. As with the compartmentalization and disparity, the difference between species is when the major change occurs.

In *Drosophila*, the major change is found in the transition from the blastoderm to the early gastrula (Fig. 4.1). When analysing the maximal values (mean of the last decile) it can be seen that they increase initially in the pre-gastrula, reach a stationary phase at mid-embryogenesis and finally increase in the last stages. The maximal values are informative about the overall morphological spatial complexity of the embryo in a given stage. When comparing roughness at different spatial scales (I, Methods), I found that in the last three stages the roughness values are significantly higher at smaller spatial scales (see study I for more detail; Fig. S2 study I).

In *Ciona*, the 3D roughness increase throughout development (II, Fig. 5), with the major change between the 112-cell and the early tailbud (Fig. 4.2). The max (mean of the last decile) values increase substantially already between the 64 and 112 cells stages (with 1000 and 10000 polygonal faces), while the min values (mean of the first decile) remain practically constant during development, showing that the most complex patterns in each stage get increase their DNE value but there is always a proportion of very simple expression pattern. Also, I found that at low spatial scales (1000 and 10000 polygons per mesh; II, Fig. 5) the mean DNE of the late tailbud is higher than at the mid tailbud (one-way ANOVA pvals < 0.05).

In summary, this results show that the complexity of distribution in space of cells/ tissues expressing a gene increases through development, and that these complexity (measured with the 2D and 3D roughness) increase in both *Ciona* and *Drosophila* in a similar way than the other two measures, compartmentalization and disparity. Both measures not only inform about the overall imbrication or convolution of the shape of a gene expression pattern, but also do it at different spatial scales. By analysing the 2D and 3D roughness at different scales, I found compelling evidence that complexity may be increasing not only through development but also that it does at finer spatial scales over time.

4.1.5 Synexpression territories

In both species I used a clustering algorithm to produce dendrogram representing the relative degrees of similarities between all regions of different stages at the same time (Fig. 4.3 and Fig. 4.4). I will refer to the regions that clustered together as "synexpression territories" (STs).

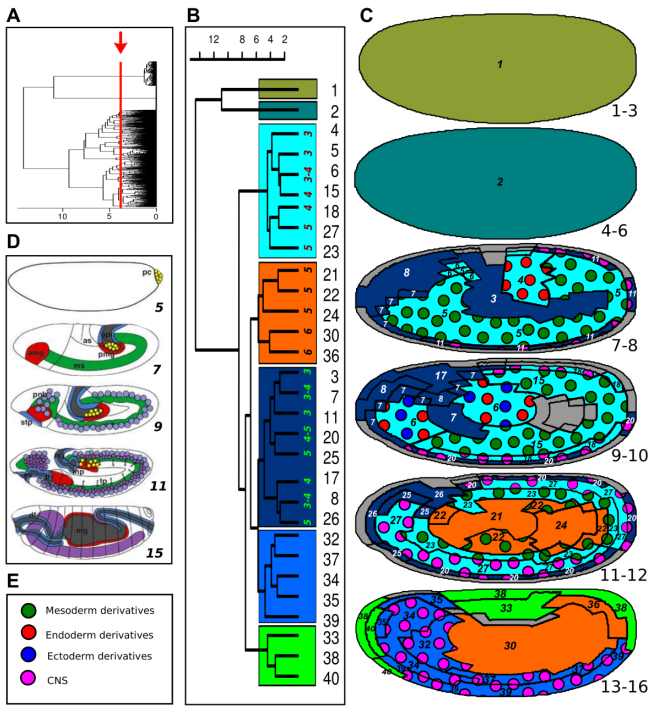

Figure 4.3: Synexpression territories (ST). (A) Dendrogram produced by hierarchical clustering on a similarity matrix (pearson's correlation) of all the embryo regions of the six stages. Red line shows the cut-off to produce 40 STs. (B) Dendrogram reconstructed using only territories with at least 50 genes with a minimum specificity (I, methods). The coloured boxes show the main branches of the dendrogram. The number indicated inside the boxes represent the stages each ST corresponds to (3 is stage 7-8, 4 is stage 9-10, 5 is stage 11-12 and 6 is stage 13-16). The ST number is at the right. (C) STs mapped onto the embryo. Gray regions have less than 50 genes expressed. Background color refers to which 'meta-territory' (in B) each ST is part of. Coloured circles represent GOterm enrichment of a specific tissue/germ layer derivative (shown in E). Stages in the lower-left part of each embryo. From stage 7-8, the ST number (as in B) is indicated. (D) Hartenstein's embryo schemes (Hartenstein, 1993) with their respective stages in the left upper part. (E) Colour code of specific tissue/germ layer derivative used in C.)

Drosophila

In *Drosophila*, after cutting the dendrogram at a specific threshold and filtering out STs with less than 50 genes expressed with a minimum specificity (see methods in I for a detailed description), 30 STs were selected for further analyses (Fig. 4.3 B).

Finally, I grouped the STs in eight 'meta-territories', as I wanted not only to see how the regions in the embryo formed different STs, but also how different STs cluster with each other, as this is informative of the degree of differentiation between stages. If STs cluster with other STs in the same stage, it would mean that the majority of genes change their expression in a similar way over time independently of where they are. If STs cluster with other STs in the same part of the embryo in successive stages, it would mean that this part of the embryo has expression dynamics independent from other parts of the embryo, which would be expected in already differentiated cells/tissues. The results show that stages 1-3 and 4-6 each one form a ST. If a cut-off is selected so

that stage 4-6 is divided in four sub-territories (I,Fig. S3) the embryo splits in four parts: anterior, posterior, dorsal and ventral. This correspond to a nearly Cartesian system one could expect from the two signalling systems known in the earliest patterning in *Drosophila* (the A/V and D/V signalling cascades; Gilbert, 2014). The STs seem to coincide with the known embryo fate map (see Fig. 4.3 D; Hartenstein, 1993) and many of them are enriched with GOterms that coincide with their expected fate. For example, in stage 7-8 (just after gastrulation) there is a ST that corresponds spatially with the germband and is enriched with mesodermal GOterms (Fig. Fig. 4.3 C).

Two meta-territories appear in the last stage (light blue and green, Fig. 4.3 C), which suggests that the tissues/organs related to those STs differentiate quite late. One of these meta-territories is enriched with terms related to epidermis such as cuticle development ("chitin catabolic process" [GO:0006032] and "cuticle development" [GO:0042335] STs 33 and 38), which coincides with cuticle deposition by epithelial cells during stage 16 (Ostrowski et al., 2002). The other meta-territory corresponds spatially with the CNS of the embryo and is indeed enriched with CNS GO-terms. The CNS territory is enriched with GOterms like "dendrite morphogenesis" (GO:0048813) and "axon guidance" (GO:0007411).

This analysis is similar to the work of Frise et al. (2010) (mentioned in section 1.3.2), who created a representation of gene expression patterns in the *Drosophila* blastoderm and then, using a clustering algorithm, found that spatial clusters of co-expression resembled the known blastoderm fatemap (Fig. 1.4). In contrast with Frise et al., the objective in here was not to create a fatemap in the early embryo, but to quantitatively characterize the overall spatio-temporal dynamics of development and differentiation through the entire embryonic development (as mirrored in the spatio-temporal gene expression).

Ciona

In *Ciona*, because gene expression information in tailbud stages is based on tissues and not on individual cells as the early stages, I analysed the STs of these stages separately (II, Methods). If in the early stages, three "meta-territories" are formed, each one would correspond to one stage, i.e., STs in early stages cluster by stage. Thus, even if at the first three stages a high proportion of blastomeres express a nearly unique combination of transcriptional factors (Imai et al., 2006), the bulk change in gene expression is common to all blastomeres. Within each early stage, STs coincides very well with the know fate map (II, Fig 6A; II, Fig. S8), with some exceptions I will describe in the next subsection. In contrast, in tailbud stages practically all STs cluster by tissue/cell type, which indicates that the in early tailbud, most tissues are already quite differentiated. This is consistent with studies analysing these stages at the level of individual or small sets of genes (Corbo et al., 1997; Di Gregorio and Levine, 1999).

This analysis in the early stages is similar to the one made by Imai et al. (2006), who used the expression profile of 53 zygotically TFs in single cells in the 16, 32, 64, and 112-cell stages, to perform a hierarchical clustering (for each stage separately). It is different in two aspects: I performed the clustering using the blastomeres of different stages and my analysis is not restricted to TFs. As I said previously, using various stages is informative of the overall differentiation process and can be used to discern between differentiation scenarios, as the differences between early and tailbud stages I found here.

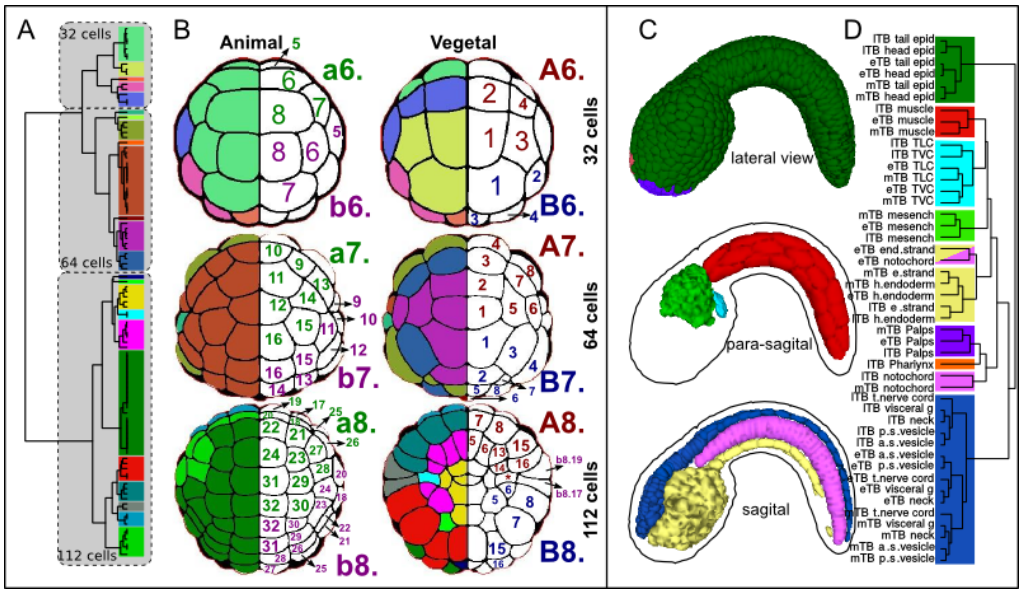

Figure 4.4: Ciona synexpression territories. (A) Dendrogram produced by hierarchical clustering of cells in 32-cell, 64-cell and 112-cell stages. Dashed boxes show that STs cluster by stage. Coloured boxes show the cut-off to produce 24 STs. (B) Names of cells (Conklin nomenclature; Conklin, 1905) indicated with a prefix shown at right. STs in the 32 cells, 64 cells and 112 cells stages (top, middle and bottom, respectively). Colour refers to which ST of the dendrogram (in A) each cell is part of. Animal view based on Nicol and Meinertzhagen (1988) and vegetal view based on Cole and Meinertzhagen (2004). The cell marked with a star (*) is the A7.6 cell, that in this analysis represents their descendant cells (A8.11 and A8.12). C) Dendrogram produced by hierarchical clustering of tissues in early, mid and late tailbud stages. The coloured boxes show the cutoff to produce 10 STs. (D) STs in the tailbud stages shown in a lateral, para-sagital and sagital views of a mid tailbud 3D embryo model (from Nakamura et al., 2012). Colour refers to which ST of the dendrogram (in C) each tissue is part of.

4.1.6 Coda

The difference in the timing of the major change on the complexity measures between species must relate to differences in their specific development. The earlier compartmentalization of *Drosophila* is most probably due to its derived early development, namely, the syncytial blastoderm. During the blastoderm stage, approximately 4,000 cell nuclei can "communicate" with each other only by TFs (Jaeger, 2011). The direct cross regulation of gene expression facilitates a rapid and highly dynamic process which seems to be responsible for the early spatial restriction of a great proportion of developmental genes. It could be then expected that these early increase in complexity in *Drosophila* would be shared by all insects with a syncytial blastoderm stage. Also, it could be that the early increase in complexity might be affected by the number of cell divisions that occur until the blastoderm is cellularized. It is known that *Drosophila* cellularizes relatively late (so there is more time for patterning within the syncytial bastoderm). In contrast, in the desert locust (*Schistocerca gregaria*) cellularization occurs very early, even before the formation of the blastoderm (Ho et al., 1997).

In contrast, *Ciona*'s early embryonic patterning is based on maternal determinants and signalling events mostly between neighbouring cells (Lemaire, 2009), which act in a

combinatorial way (Hudson et al., 2007) to establish a unique TF combination in more than half of the blastomere pairs before gastrulation (Imai et al., 2006) determining most of their fates. Thus, even when in *Ciona* most of the cell fates are already determined (by the specific combination of a fraction of TFs) and the embryo can be said to be already highly compartmentalized, this is not evident at the global level of gene expression, which I am measuring here. Therefore, the "delay" of compartmentalization observed in *Ciona* could be explained by the relatively slower process of signal transduction (as in *Ciona*) compared to the gap gene network (in *Drosophila*).

Another main difference between species is that, based on the synexpression terriories analysis the differentiation process in *Drosophila* seems to continue throughout whole embryogenesis (as new STs were formed until the last stage I analysed) and different organs differentiate at different developmental times. In contrast, the *Ciona* embryo seems to be already genetically differentiated at the early tailbud (as the STs of all the tissues in the tailbud stages cluster together) so the last embryo stages consist only of moderate morphogenetic movements (mainly cell elongation; Hotta et al., 2007). Hence, the ST analysis is a valuable tool based on differential gene expression to get a global perspective on the local differentiation of the embryo.

4.2 Discrepancies between fate map and STs (II)

I found a few cases in *Ciona* in which cells with the same fate where contained in different STs. As explained in Box 1 (section 1.3.1), it would be expected that a fate map would largely coincide with a gene expression map. This analysis could not be made in *Drosophila* as the gene expression data is not a the single level resolution.

A lack of correspondence, as it was found in here, could be due to: 1) cells whose fate is disproportionally affected or determined by a small number of genes (as this analysis reflect quantitative differences at the level of hundreds of expressed genes but can not distinguish between the relative importance of each gene) or 2) cells that although having a restricted fate at a certain stage their differentiation is not complete (at the level of gene expression). An example of the latter is a ST in the 112-cell stage (in magenta; 4.4 B; II, Fig. S8) that contains precursors of the notochord (A8.5, A8.6, A8.13, and A8.14, B8.6) and mesenchyme (B8.5) (Tokuoka et al., 2004). The latter come from a secondary notochord/mesenchyme bipotential cell (B7.3). It has been reported that the expression of Twist-like 1, necessary for mesenchyme differentiation, starts at this stage (Imai, 2003). This evidence, together with the inclusion of the mesenchyme cell in this otherwise exclusively notochord territory (primary and secondary), seems to indicate that the differentiation of cell pair B8.5 as mesenchyme is still incomplete at this stage.

Gene expression dynamics in cell-lineages

During *Ciona* early embryogenesis, I analysed the gene expression similarity between lineage-related cells i.e., between daughters cells and between mother/descendants cells (II, Fig. 8). In general, cells are more closely genetically to their sister cells than to their mother/descendants, which is also reflected in the clustering of STs by stages discussed before. I also found that at the 64-cell stage, cells that show more genes expressed differently than their ancestors are neural fated cells. This could be related with the change from unrestricted state of these cells at this stage (i.e., their descendants will

give rise to different cell fates) to a restricted state in the next stage (112-cell stage) (Imai et al., 2006). Therefore, it could be hypothesized that when a cell changes from a unrestricted to a restricted cell fate state, a major change in gene expression should be evident when following gene expression dynamics of its cell-lineage.

4.3 Adaptation in *Drosophila* embryogenesis (III and IV)

I combined the Synexpression Territories (STs) approach with genome-wide coding-region polymorphism data (from the DGRP database) and the coding-region divergence between *D. yakuba* and *D. melanogaster* in order to estimate the proportion of adaptive non-synonymous substitutions (ω_α) in the genes expressed in each ST (n=589 genes; III, Methods). Using this approach, I could chart a spatial map of natural selection acting on *Drosophila*'s embryo anatomy. I complemented this with a analysis using available annotation of gene expression (n=2,835 genes) using a controlled vocabulary of anatomical structures from the BDGP database (Tomancak et al., 2007). The results showed a few STs with significant higher or lower ω_α (permutation test; III, Methods).

4.3.1 STs or anatomical terms with high ω_α

STs 13 and 32 (ST number comes from the hierarchical clustering algorithm; see Fig. 4.5), which showed a higher ω_α, seem to correspond to the forming foregut and hindgut (stage 11-12) and to the CNS (stage 13-16) respectively. To explore if ST 32 high ω_α was indeed related to the CNS, I separated the genes CNS or not-CNS related. I found that both groups showed a high ω_α, which suggests that in addition to the CNS, another structure in the anterior region would be under positive selection. Using the anatomical terms approach, no anatomical terms related to the CNS were found to have high ω_α with the initial criteria. I therefore applied a more stringent criterion to consider genes as part of an anatomical term (before a gene could have a maximum of seven anatomical terms associated instead of a more stringent number of three) and found that "Embryonic brain" showed high ω_α (permutation test, $p = 0.046$). Also, with the anatomical terms approach, I found that genes associated with "Gonads", in the last stage, clearly showed evidence of adaptive evolution (III, Figure 2), which is consistent with previously reported high rates of adaptive substitution in the testes (Akashi, 1994; Civetta and Singh, 1995; Nuzhdin et al., 2004; Pröschel et al., 2006)

4.3.2 STs or anatomical terms with low ω_α

STs 20 and 29, which showed low ω_α (Fig. 4.5) seem to correspond to the forming midgut (stage 11-12) and to the forming larval digestive system (stage 13-16) respectively. When using the anatomical terms, low ω_α was found in many anatomical terms related to the digestive system in the last stage: "Embryonic midgut", "Embryonic salivary gland", "Embryonic hindgut", "Embryonic proventriculus". Also, combining three related anatomical terms, "Embryonic foregut", "Embryonic epipharynx" and "Embryonic hypopharynx", that separately did not have enough genes to be considered in the analysis, showed low ω_α.

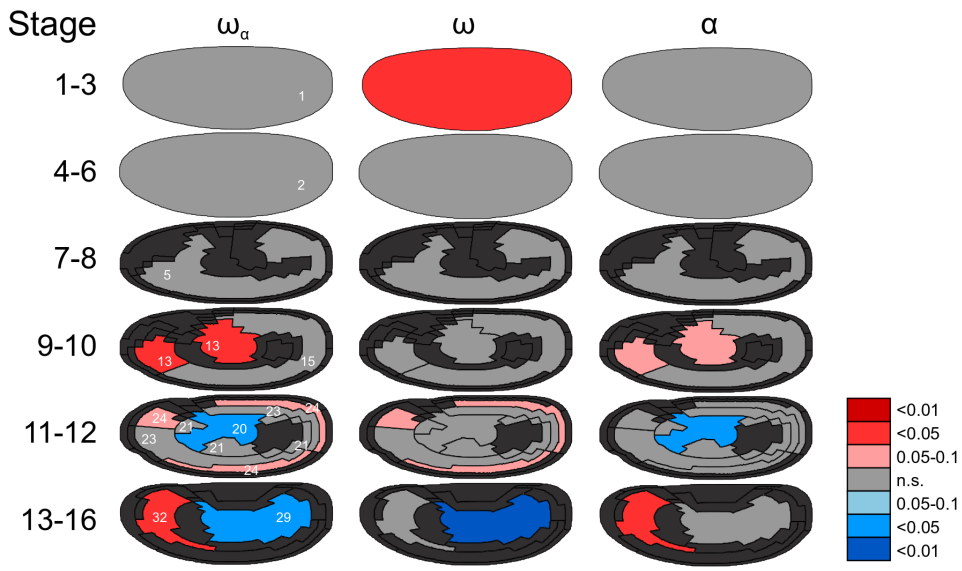

Figure 4.5: ω_α on embryonic territories over space and time. Territories drawn in red in the central column mark significantly high ω_α while those in blue mark significantly low ω_α in space in each of the 6 developmental stages (rows). Other columns depict α, the proportion of base substitutions fixed by natural selection, and ω, the rate non-synonymous substitutions relative to the mutation rate. Territories in dark gray are territories without enough specific genes to be analyzed. The statistical was calculated by a permutation test using all the genes analyzed (see Material and methods). Territory 13 in stage 9-10 (ω_α: 0.059, p = 0.045). Territory 20 from stage 11-12 (ω_α: 0.022, p = 0.048; α: 0.259, p = 0.028). Territory 24 from stage 11-10 (ω_α: 0.070, p = 0.061). Territory 29 from stage 13-16 (ω_α: 0.037, p = 0.047; ω: 0.074, p < 0.001). Territory 32 from stage 13-16 (ω_α: 0.068, p = 0.044; α: 0.71, p = 0.04).

The lack of adaptive change in the forming digestive system might reflect their relative enrichment in metabolic genes (Marianes and Spradling, 2013), as the coding regions of metabolic genes have been found to be more conserved than non-metabolic genes (Peregrín-Alvarez et al., 2009). Also, it has been shown that genes regulating primary metabolism processes follow an hourglass divergence pattern (Kalinka et al., 2010).

4.3.3 Transcriptome age index and other genomic determinants

Using the phylostratigraphic maps of *D. melanogaster* (see Methods; Drost et al., 2015), I found that STs with low ω_α express (on average) older genes (high TAI values; see Fig. 4.6). Similar results were found for anatomical structures (III, Fig. S2). Also, using a modified version of the Transcriptome Age Index (TAI) (see Methods; Domazet-Lošo and Tautz, 2010) applied to the polar regions and STs, I found that in stage 13-16 the mean phylogenetic age of the genes expressed in the endoderm is lower than in other germ-layers, specially compared to the ectoderm (Fig. 4.6). Similar TAI results between germ-layers were found by Domazet-Loso et al. (2007) but without comparisons between stages.

The correlation between adaptation and gene phylogenetic age is consistent with the expectation that older genes perform more essential functions than younger genes,

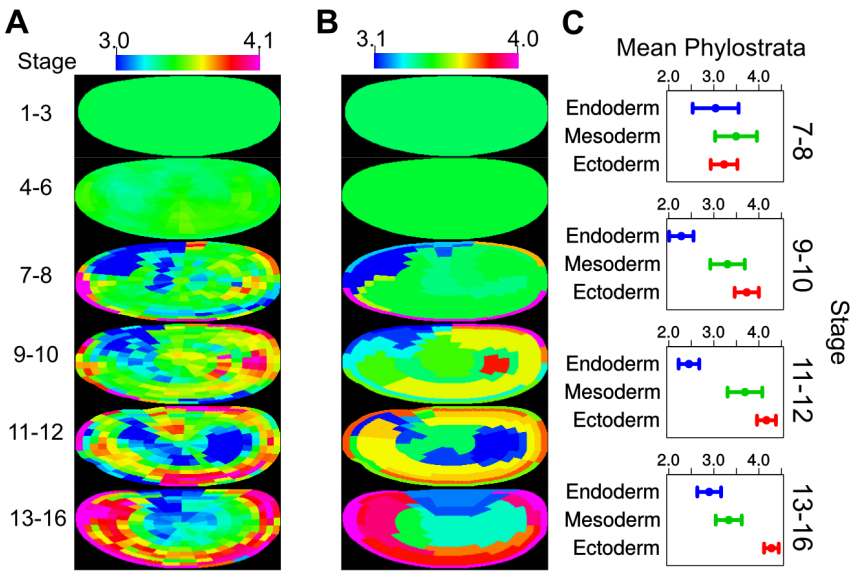

Figure 4.6: The center of the embryo expresses older genes. (A) Heatmaps showing the transcriptome age index (TAI) im polar regions (B) Heatmaps showing the TAI for STs. (C) Mean phylostrata of genes assigned to each germ layer. Circles represent the mean and whiskers the SEM.

and that as older genes would have been under selective pressure for longer time, they would be therefore more close to optimality (assuming that their function is conserved). Therefore, more opportunity for adaptive changes would be expectable in embryo regions with a greater proportion of younger genes.

I also found that embryo polar regions with high ω_α have low codon bias and that regions high codon bias show have high levels of gene expression (average RNA-seq levels per region; III, methods). The correlation between adaptation and codon bias (Sharp, 1991; Betancourt and Presgraves, 2002; Haerty et al., 2007), as the correlation between gene expression level and codon bias (Plotkin and Kudla, 2011) have been previously reported.

To clarify the relation between these three variables, I fitted a multivariate linear regression and found that embryo regions with high ω_α exhibit low codon bias relative to what would be expected from their gene expression levels (III, Fig 5). The negative correlation between codon bias and protein adaptation that I found would be expected given that, an adaptive aminoacid change in a protein would be probably different from a change that would increase codon usage efficiency (Hershberg and Petrov, 2008; Presnyak et al., 2015).

4.4 Adaptation through *Drosophila* life cycle (IV)

4.4.1 Temporal adaptation profile

The results showed adaptation in two different periods in the life cycle of *Drosophila*: 1) in the earliest 2 hours of the embryo development (ω_α, ω_d and ω show their highest

value at this stage) and 2) from the L3 larval stage onwards, specially in the pupal and adult male stages which exhibit the highest levels of adaptive change.

In between these stages of high adaptation, mid and late embryonic stages show high conservation. Similar results were found when considering after excluding immune system and testes genes (IV, Fig. S2) or when the mutation rate is estimated using the 4-fold degenerate sites (IV, Fig. S3).

The high adaptation rate in males is consistent with previous reports of higher adaptive substitutions in the genes expressed in males Pröschel et al., 2006; Haerty et al., 2007). In contrast, based in a hybrid mis-expression assay with *D. melanogaster* and *D. sechellia*, Artieri and Singh (2010) suggested a highly conserved pupal stage under strong stabilizing selection, which is contrary to the results shown in here, which indicate that, at least at the level of DNA coding sequence variation, pupal stages are among the least conserved.

As the morphology and other aspects of the phenotype of the larva and the adult arise primarily through the genetic, cellular and tissue interactions of embryonic and pupal development respectively, the adaptation in the larva or the adult morphology should be reflected in the genes expressed in embryonic development and pupal development, respectively. Therefore, the evidence that most embryonic development shows low rates of adaptive change while the larva and pupa stages show higher rates of adaptative change suggests that there has been more adaptive changes in the adult morphology than in the larva morphology (between *D. melanogaster* and *D. yakuba*).

4.4.2 Selective constraint in late embryogenesis

From hour 10 until 24 of embryogenesis show significant low ω_α and ω (see Fig 4.7), which would be consistent with the low rate of adaptive change seen in many anatomical structures in stage 13-16 seen in section 4.3.2 (as stage 13-16 of BDGP roughly maps to RNA-seq samples em10-12 hr, em12-14 hr, em14-16 hr, and em16-18 hr of modENCODE; Hammonds et al., 2013). Therefore, the two different approaches used in here (adaptation map in the embryo and adaptation through the life cycle) show that the proteins produced in late embryogenesis change less their aminoacid sequence (i.e., are more conserved). This phenomenon, of some proteins evolving slower, has been called "selective constraint" and has been linked to the higher degree of functionality of such proteins (Kimura, 1983). Most importantly, I could identify which specific anatomical structures expressed genes with a higher degree of conservation.

4.4.3 Results support the Hourglass model

The temporal pattern of adaptation (and conservation) are roughly consistent with the Hourglass model (HG), specially for the early and mid embryonic development. During the first 6 hours ω and ω_α are significantly high (permutation test), which is consistent with the expectations of the HG. The same parameters are significantly lower during mid-embryogenesis, which is also consistent with the higher conservation expected from the HG, as the phylotypic stage (the most conserved stage) in *Drosophila* has been suggested to be between the 6th and 10th hour (Drost et al., 2015). However, during late embryonic stages (from 10-12h to 22-24h) ω and ω_α are significantly lower (permutation test), which is not what is expected from the HG.

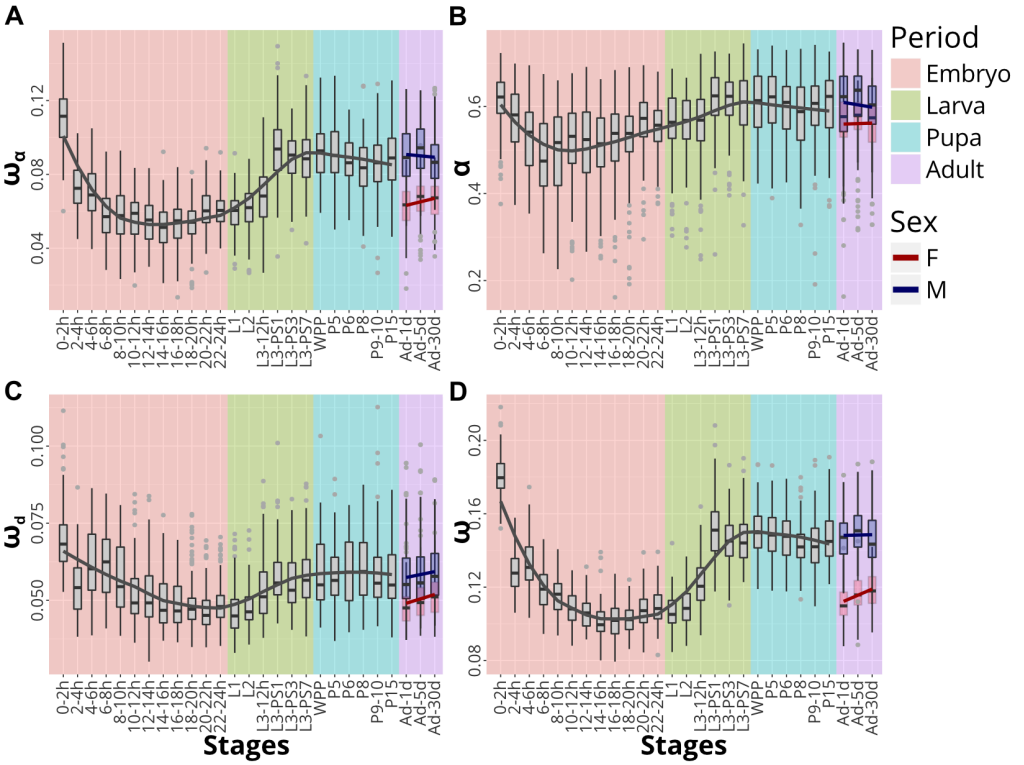

Figure 4.7: ω_α **(A),** α **(B),** ω_d **(C),** ω **(D) through the life cycle of** ***D. melanogaster*** Each time point represents 1,000 random samples of 350 genes (with replacement) expressed at a stage. Red line represents a LOESS regression. Female and male stages are fitted to a linear regression. There are 12 embryonic stages at 2hr intervals (from 0h to 24h). Larval stages at first instar (L1), second instar (L2) and third instar (L3). L3 stages are subdivided into the first 12 hours (L3-12h) and several puff stage (L3-PS1 to L3-PS7). WPP is the white pre-pupae stage. Pupal stages are phanerocephalic pupa, 15h (P5), 25.6 hours pupa (P6), yellow pharate, 50.4 hours (P8), amber eye-pharate, 74.6 hours (P9-10), green meconium pharate, 96 hours (P15). Adult stages are 1, 5 and 30 days after eclosion (Ad-1d, Ad-5d, Ad-30d).

In contrast with some previous studies (Davis et al., 2005; Kalinka et al., 2010) we do not find that the later stages of embryonic development are less conserved. It was found however, that a cluster composed of genes whose expression is high only in late development (done with a fuzzy clustering algorithm, cluster 8; see study IV), shows a significant ω and ω_α. In here, this group of genes have only a minor effect on the global pattern. It could be that, due to the different methodology used by these other studies, these genes would have a relatively higher effect on the pattern observed. It could also be that the differences are partly due to the different species used in the analyses.Davis et al. (2005) use D. pseudoobscura as outgroup species while Kalinka et al. (2010) use six different *Drosophila* species including *D. melanogaster* but not *D. yakuba*, the species used as outgroup in this work.

Despite these differences, the overall ω and ω_α pattern (Fig 4.7) is consistent with the HG model of embryonic development in *Drosophila* (Kalinka et al., 2010).

4.4.4 Correlation of adaptation with some genomic determinants

Many "genomic determinants" temporal profiles correlate either positively other negatively with ω_α (IV, Figs 2 and 3). Thus, messenger complexity (number of transcripts divided by the number of exons) correlates with ω_α (rank correlation; see study IV). On the contrary, gene size, number of exons, codon usage bias and number of transcripts per gene negatively correlate with ω_α (all with significant rank correlations; see study IV).

This is consistent with previous studies that have shown that small gene size has been correlates with ω (Duret and Mouchiroud, 1999; Comeron et al., 2012). It has also been suggested (Gellon and McGinnis, 1998), that developmental genes tend to have a complex gene structure with many exons and cis-regulatory elements and a complex regulation in space and time. Therefore, the correlations we observe between ω_α and some genomic determinants is likely to simply reflect the fact that during mid-embryonic development, genes have a more complex spatio-temporal regulation and a more complex regulatory structure (as measure by the messenger complexity measure).

Based on the results shown in here, it is suggested that these genomic determinants can serve as predictors of adaptive change during development and that the temporal pattern of the genomic determinants are simply a consequence of the complex spatio-temporal regulation of gene expression occurring in embryonic development (as suggested on more qualitative grounds Duboule and Wilkins, 1998).

An important difference between this analysis and previous ones is that the DFE-alpha method allows to differentiate finely between conservation (indicated by low ω), adaptive evolutionary substitution (high ω_α), non-adaptive substitution (high ω_D) and the proportion of adaptive versus non-adaptive substitution (high α). In a previous study, it was found that the 150 genes with the highest number of non-synonymous substitutions are expressed more strongly in larva and pupa than in embryo and that their highest level of expression is in male adults (Davis et al., 2005). The work presented here would also be consistent with Davis et al., although in the latter case conservation and positive selection cannot be distinguished.

5 Concluding Remarks

The study of organismal complexity during embryonic development presented here shows that there are commonalities and differences between *D. melanogaster* and *C. intestinalis*. Both species showed a non-linear increase in all complexity measures, while the most remarkable difference is the timing of the major change in complexity, which is earlier in *D. melanogaster* (around gastrulation). Another common pattern is the early increase in complexity when considering only transcription factors or growth factors (or other signalling molecules). This confirms the special role these genes have in early metazoan development. It could be therefore expected that, based on the evidence presented here, the same pattern when considering these type of genes should also be observed in other species.

One important result of this work is that within each species, the three complexity measures showed a similar pattern (even when it would not be necessarily the case; see section 3.1). This means that altogether, these measures (compartmentalization, disparity and roughness) are reflecting a global pattern of increase in complexity in each species. Therefore, it could be hypothesized that a similar increase in complexity would be found using alternative measures of complexity (e.g., spatial entropy). Further analysis would be required to test this hypothesis. Also, the Synexpression Territories analysis allowed to "reconstruct" the main embryonic differentiation events in both species in a consistent manner with the current knowledge of the development of these model organisms and without focusing in specific genes.

The elaboration of an adaptation map on the fruit fly embryo can be considered a proof of concept of how the combination of diverse fields like evolutionary developmental biology and population genomics, and new techniques such as the phylostratigraphy, can be useful to give a fresh view on an old problem. Using these maps, it was possible to visually identify that the center (internal part) of the embryo expresses a more conserved and older transcriptome, while the outside (external part) expresses phylogeneticaly younger and less conserved genes. This evidence seems to support the hypothesis of the antecedence of the endoderm with respect to the ectoderm (Hashimshony et al., 2014). It would be interesting to extend this adaptation mapping analysis for the entire development (until the adult stage) as it could be that in later stages, different structures or organs have been under positive or negative selection.

The estimation of adaptation over the entire life cycle of *D. melanogaster*, as presented here, supports the HG model of development. We find, as other analyses previously have, that the mid-embryogenesis is highly conserved. The work presented here is different from previous ones in that it uses a more complete spatio-temporal dataset and a method that uses inter and intra-specific DNA coding variation to estimate, with an unprecedented precision, the proportion of adaptive changes. Furthermore, as a result of this work is hypothesized that the hourglass model can be best predicted by various genomic features. However, further work is necessary to test this hypothesis.

The observed patterns of complexity and adaptation/conservation throughout the embryonic development of *D. melanogaster* might be intricately connected. The increase in spatial disparity of gene expression in late embryogenesis (Fig. 4.1) likely reflects the expression of genes with multiple spatial domains. Genes expressed in many different places and times during development likely require an elaborate genetic structure (reflected in their exons number, intron length, transcripts number) that could permit such complex spatio-temporal expression regulation. It could be then hypothesized that a mutation in such a gene would have high pleiotropic effects (as it would affect many

different parts of the embryo) which could result in stabilizing selection against mutational variation (Raff, 1996; Galis et al., 2002). The correlation between specific genetic features intuitively related to spatio-temporal regulation of gene expression and the high level of conservation at late embryogenesis seems to support this hypothesis.

In here, analysing publicly available databases, I have quantified how complexity and compartmentalization increase during development of two species (*D. melanogaster* and *C. intestinalis*) and estimated the rate of adaptive evolution over the entire embryo's anatomy and in the whole life cycle in *D. melanogaster*. Thus, the work presented here confirms that a statistical approach in developmental biology can provide valuable information on fundamental processes by describing their properties at a statistical level, and therefore allows to attain a global view that transcends the role of individual genes.

Future directions

The approach presented here could be applied to other model organisms for which gene expression databases, similar to the databases I analysed in here, are available (e.g., *mouse*, *Xenopus*). Also, it could be applied to model systems in developmental biology for which there is sufficient spatio-temporal gene expression data available, like the *Drosophila* wing imaginal disc or the vertebrate limb bud.

The emergence of new techniques, like "spatial transcriptomics" of tissue sections at single-cell resolution (Stahl et al., 2016) could make possible to have information, derived from a single experiment, of all the genes expressed in a 2D section of an embryo. The application of the measures presented here could be applied to data derived from this new technique in a straightforward manner, solving the limitation in resolution of the work presented here.

It is important to mention that this work has used differential gene expression in the embryo and its spatial distribution as a tool to investigate complexity. However, embryonic development can not be reduced to differential gene expression. Cellular behaviours and the physical properties of the cells and tissues have also a causal role in the developmental process. It would be interesting to be able to measure the differential apportionment to complexity increase of the different developmental mechanisms.

The increase of organismal complexity and the study of adaptation during development remain fascinating topics after many centuries, and still offer many open questions to be solved. The incredibly fast pace of data generation, the development of new techniques and sophisticated methods give hope to finally open the black box of development.

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