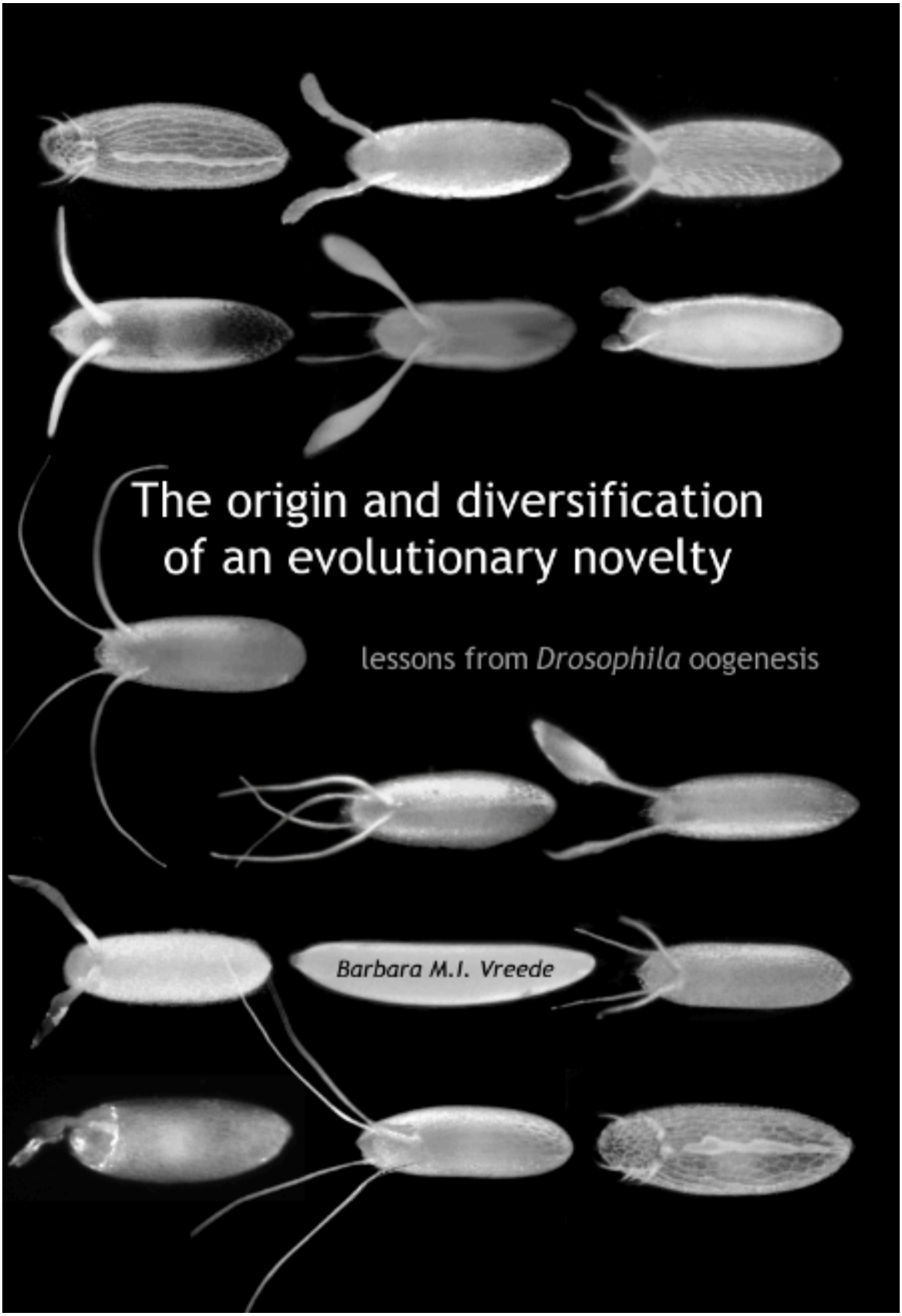
The origin and diversification of an evolutionary novelty
lessons from *Drosophila* oogenesis
Barbara M.I. Vreede

The origin and diversification of an evolutionary novelty: lessons from *Drosophila* oogenesis

Barbara M.I. Vreede

Successfully defended on November 30th, 2012 at the Instituto Gulbenkian de Ciência in Oeiras, Portugal, before a jury presided over by:

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Dr. J. Élio da Silva Sucena

Declaração / Declaration

Esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Janeiro de 2008 e Julho de 2012 no laboratório do Dr. Élio Sucena, Instituto Gulbenkian de Ciência em Oeiras, Portugal, no âmbito do Programa Gulbenkian de Doutoramento (edição 2007-2008). Parte do capítulo 2 está aceite para publicação no BMC EvoDevo como "Co-option of a coordinate system defined by the EGF and Dpp pathways in the evolution of a morphological novelty", B.M.I. Vreede, J.A. Lynch, S. Roth, e É. Sucena. O modelo computacional apresentado no capítulo 4 está integrado num manuscrito em preparação para submissão com autoria de A. Fauré, B.M.I. Vreede, É. Sucena, e C. Chaouiya.

This dissertation is the result of my own research, carried out between January 2008 and July 2012 in the laboratory of Dr. Élio Sucena, Instituto Gulbenkian de Ciência in Oeiras, Portugal, in the Gulbenkian Doctoral Programme (edition 2007-2008). A part of chapter 2 has been accepted for publication in BMC EvoDevo as "Co-option of a coordinate system defined by the EGF and Dpp pathways in the evolution of a morphological novelty", B.M.I. Vreede, J.A. Lynch, S. Roth, and É. Sucena. The computational model presented in chapter 4 is part of a manuscript in preparation, authored by A. Fauré, B.M.I. Vreede, É. Sucena, and C. Chaouiya.

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Dissertation presented to obtain the Ph.D degree in Evolutionary Biology

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Knowledge Creation

Voor oma, die ons verhalen vertelde,

en Boema, die als eerste de mijne las.

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This thesis would not exist without the following people: first, my father, whose idea it was that I should study biology. Without the encouragement of Bas Zwaan and Patrícia Beldade, I may not have continued my scientific career beyond graduation. It was Wallace Arthur's writing more than anything else that led me to evo-devo. Patrícia Beldade pointed me to the IGC, and Henrique Teotónio accepted me to the doctoral programme. Finally, Élio Sucena provided the fantastic idea of *Drosophila* oogenesis as the perfect model for a question I had long wanted to answer, and invited me to join his lab.

But many, many more were instrumental in the course of this work, and I would like to take this opportunity to thank them all. While I will try my best not to forget any, I will undoubtedly do so. If this is the case for you, please accept my apologies on top of my gratitude.

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Throughout the entire process of my PhD there have been several people who graciously shared unpublished data and expertise, to an unknown girl on the other side of an email address. Many things in this thesis are a direct result of these interactions, and I feel immensely grateful to those who took the time to help me. In particular I'd like to thank Andrew Jessup, who helped me keep the *Ceratitis* alive, Al Handler, who gave us access to the *Ceratitis* genome, and Giorgos Pyrowolakis, and Leonard Dobens, who shared unpublished data.

This thesis results in part from several important collaborations: with Adrien Fauré and Claudine Chaouiya we worked on modelling eggshell patterning, and with Vítor Barbosa and Triin Laos, we worked on the mutant *troya*. I am very grateful for the discussions that followed in these collaborations, about oogenesis and the gene networks that govern it, in sometimes excruciating detail.

Then, my visit to Cologne was a fantastic opportunity to learn more about oogenesis in many different species, and try to develop an RNAi protocol in *Cer-*

atitis—to no avail, but not for want of trying! Jeremy Lynch and Siegfried Roth in particular were immensely important in helping me interpret the data on *Ceratitis* oogenesis, and together with the rest of the Roth lab they made my time in Cologne a great experience.

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At the very end, I received some incredible (practical!) support from several people: Marc wrangled LaTeX; Filipe helped me straighten out ideas; Matt diligently proof-read the entire thesis (although time constraints meant not all his suggestions could be followed; any errors in the language are thus mine and mine alone!); Triin put out last-minute egglays; Olivier got me inaccessible papers; Cláudia dealt with printers and practicalities; Ot made sure I stuck to the cause; Élio had faith and a lot of books, and was an insurmountable wall between me and the bureaucracy monster. Furthermore, my work was read and commented on by Adrien, Claudine, Diogo, Élio, Filipe, Hanneke, Jeremy, Leila, Marc, Raquel, Siegfried, and Triin; Alex translated the summary into Portuguese, and Filipe checked if it was up to standard (it was!).

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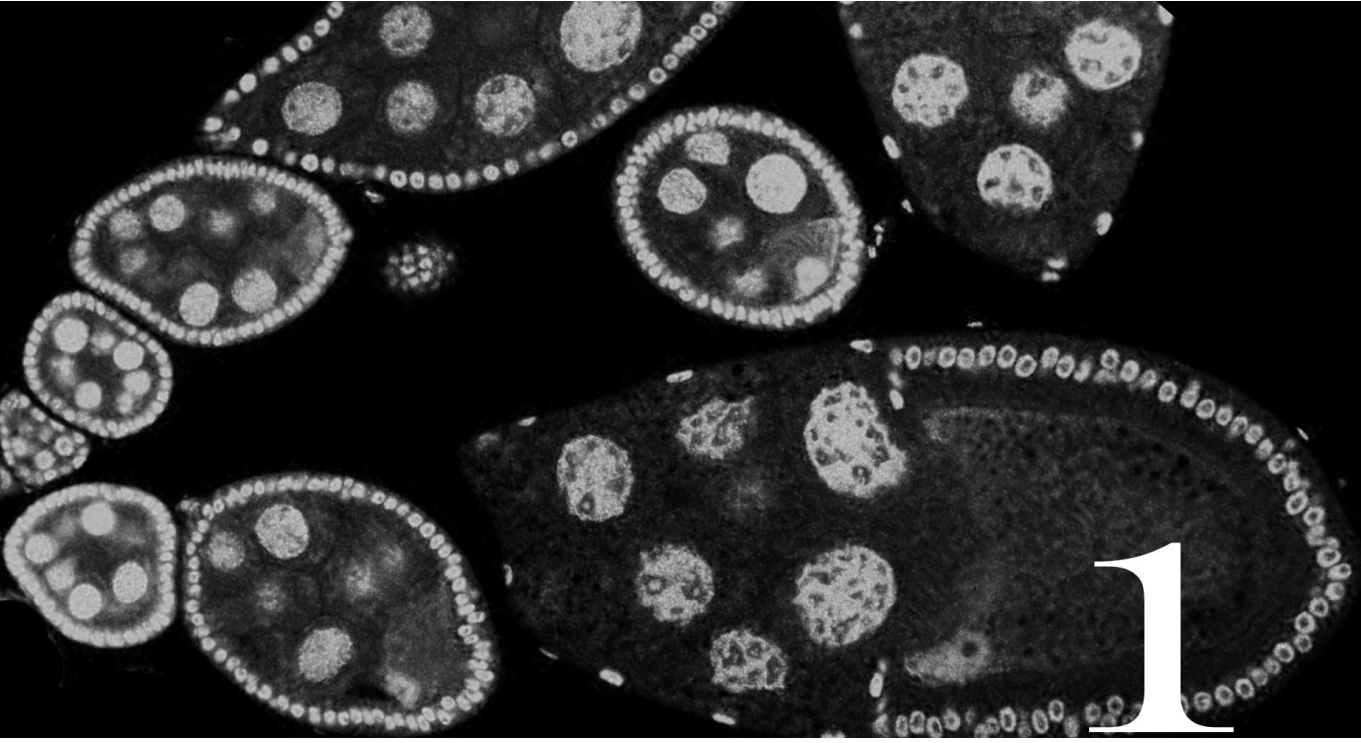

1

General introduction

Abstract

In any area of study, it is necessary to define the subject of investigation. In the case of evolutionary novelty this is a particularly difficult task, as a clear definition of the concept that is suitable across different levels of biological organization has yet to emerge. We proceed with a definition for morphological novelty proposed by Müller and Wagner (1991), and introduce the dorsal appendages on eggs of Drosophilidae as such a novelty. These structures are part of the eggshell, and help supply oxygen to the embryo. A wide variety of phenotypes can be found, which is thought to have a single origin in the common ancestor of Drosophilidae. To investigate both the evolutionary origin and subsequent diversification of dorsal egg appendages from a genetic and developmental viewpoint, we first review the current knowledge on the developmental background of the structure: oogenesis in *Drosophila melanogaster*. The *Drosophila* egg chamber consists of 16 germline cells, one of which is the oocyte, surrounded by a layer of somatic follicle cells. This follicle cell layer is responsible for depositing the eggshell. Demarcation of specific domains on this epithelium occurs via patterning governed by the activity of several signalling pathways, of which EGF and Dpp are the most important. Interestingly, these signalling pathways are pleiotropic during oogenesis, and also function for example in the definition of the main embryonic body axes (EGF), or are required for essential cell migration (Dpp). With this delineation of the playing field we introduce the research presented in this thesis, which will address both the origin and the diversification of this novel trait.

1.1 Evolutionary novelty: the origin of diversity

The concept of innovation seems both evolution's greatest triumph and its students' most difficult challenge. Biodiversity as it exists today is characterized by innovations in every lineage, acquired traits that may frequently have been the initial step in a species radiation, as they allow access to an as yet unexplored ecological niche. 'Innovation' as a concept has been applied to a wide range of cases across all levels of biological organization, including, but not limited to, behavioural performance (e.g. avian flight), developmental process (e.g. direct development of sea urchins), metabolism (e.g. the urea cycle), and morphology (e.g. vertebrate limbs). Its importance in evolution is thus paramount, and the issue of how novelties originate has been proposed many times as one of the major questions in evolutionary biology (e.g. Mayr, 1960; Wilkins, 2002). Historically, novelties in particular were the topic of a fierce debate after the appearance of the Origin of Species, between Darwin and Mivart. In the existence of complex 'useful structures', Mivart saw an obvious invalidation for the theory of evolution, as natural selection in his eyes could not account for the 'incipient stages' of these structures (reviewed by Mayr, 1960).

While novelty may no longer be problematic for the theory of evolution, it does provide a particular challenge to the biologist attempting to unambiguously and unbiasedly define the concept. Especially the fact that the concept can be applied to so many levels of biological organization is a problem, and leads to disagreement between biologists of different subdisciplines (Nitecki, 1990). This discussion is still ongoing, and even led some to question the validity of the concept itself, by asking: what, if anything is an evolutionary novelty? (Pigliucci, 2008)

1.1.1 What, if anything, is an evolutionary novelty?

Inevitably, the definitions of novelty currently in existence have been coloured by the author's particular discipline. Interestingly, within the diverse set of definitions it is possible to identify a dichotomy of approaches that can be traced back to the classical debate of form versus function. This debate of morphology against teleology was famously and extensively held between Geoffroy St.-Hilaire and Cuvier in the 1830s (Panchen, 2001), decades before the arrival of the Origin. And again, these opposing schools are reflected in the definitions of evolutionary novelty. A representative of 'function', this widely used definition of an evolutionary novelty comes from Mayr (1960):

> "any newly acquired structure or property that permits the performance of a new function."

This definition has the clear signature of an era in biology when the focus was not on generative mechanisms for biological diversity, but on selection and adaptive function. An increased focus on development and morphology is reflected in a second definition, published three decades later by Müller and Wagner (1991):

> "a structure that is neither homologous to any structure in the ancestral species nor homonomous[1] to any other structure of the same organism."

Both definitions have their merits, and their problems. Firstly, while Mayr's definition is broadly applicable to many levels of biological organization, Müller and Wagner focus on morphology alone. This makes the latter a more practical definition, as concepts identifiable as novelty in the morphological sense will be more readily comparable between each other. Importantly, this definition also distinguishes between true novelty and exaptations: a term coined by Gould and Vrba (1982) to describe structures that have diverged from their original function, and have been modified accordingly. Such structures can take dramatic forms, and include for example the narwhal's tooth, a large and highly specialized structure that originated from one of the ancestor's teeth. The exclusion of such traits as the narwhal's tooth has been a point of criticism for Müller and Wagner's definition (Moczek, 2008). A further problem is that it hinges on the concept of homology, which generates debate in and of itself. Or, in the words of Moczek (2008): according to Müller and Wagner, novelty begins where homology ends—but where *does* homology end?

However, without invoking the homology discussion at the present moment, there is one strong advantage to Müller and Wagner's definition. Namely, using a definition that depends on form rather than function is of particular use if the goal is to understand how novelties originate. A definition of novelty that emphasizes its function, such as proposed by Mayr (1960), does so as it aims to explain how natural selection favoured its propagation. However, as natural selection can only operate on traits that exist, natural selection cannot be held responsible for the origin of a novelty (Moczek, 2008). The advent of the field of evolutionary developmental biology (evo-devo) has come with a surge of studies on the origin of evolutionary novelty. As every change in the evolution of multicellular organisms inescapably has to start somewhere in development, the field of evo-devo is indeed exceptionally suited to address this issue. Moreover, it has been hypothesized that the process of development is not just the 'scene of the crime' when it comes to novel structures, but holds unique potential for innovation by its very nature (Müller, 1990). The argument made by Müller (1990) is that by redirecting the

[1] 'homonomy' is synonymous to 'serial homology'

development of intermediate stages of existing structures it is possible to generate quite dramatic novel features. Key to this is not just the intermediate stage, which provides the structural basis for the novelty, but the process of development itself. In a growing system, small changes in early stages can be magnified tremendously. Conversely, the coordination of the various components in a developing organism may buffer changes, effectively neutralizing underlying genetic variation. Accumulation of this variation beyond a certain threshold can be responsible for sudden dramatic changes in the morphology of the adult. Finally, Müller (1990) implicates the ectopic redeployment of existing developmental programs in generating dramatic morphological changes.

These are no strange concepts to the field of evo-devo. The latter point in particular has been shown to play a role in many current model systems of novelty, as genes or whole networks have been recruited in the development of novel traits. To illustrate this mechanism, we can look at two systems currently on the front line of the evo-devo research programme on evolutionary novelties: beetle horns, and butterfly wing patterns.

1.1.2 Examples of novelty: wing patterns and horns

The butterfly wing pattern is not just a textbook example of a morphological novelty, but a compelling case of a novel trait with a clear adaptive advantage. The immense variety that exists in pigment patterns between different butterfly species is exemplary for the power of novel traits in driving subsequent species radiation (Nijhout, 1991) (Fig. 1.1). The ecological relevance of wing patterns is well understood: elements on the wing may function for example in predator avoidance either by camouflage, or by diverting attention away from the butterfly's vulnerable body (Brakefield and Reitsma, 1991). Moreover, the wing patterns of *Heliconius* butterflies provide a classical example of Müllerian mimicry (Joron et al., 2006).

Butterfly wings are a complex innovation. A combination of scale-covered wings, pigmentation, and underlying spatial patterns had to evolve to give rise to the first actual wing pattern (Nijhout, 1991; Brakefield et al., 1996). Scales on the wing have their origin in sensory bristles, a structure existing on various body parts of all insects. A common genetic programme in their development as well as structural and developmental similarity underline this evolutionary relationship. For pigmentation, several genes known to function in pigment pathways have been redeployed to the wing scales (Carroll et al., 2005). Finally, in the underlying patterns, too, many known developmental regulators are recycled: the transcription factor Distal-less (Carroll et al., 1994), the receptor Notch (French and Brakefield, 2004), and the Hox gene *Antennapedia* (Saenko et al., 2011) have been shown to

be involved in one particular element, the eyespots of the butterfly *Bicyclus anynana*. Interestingly, a large degree of diversity exists in the genes generating the underlying patterns: no single patterning gene has yet been demonstrated to be required in all species examined thus far (Shirai et al., 2012).

The redeployment of old genes for novel functions is commonly referred to as 'gene co-option'. This mechanism is widely considered to be responsible for complex change of any kind, but clearly plays an important role in generating novelty. In another example of novelty, beetle horns (Fig. 1.1), the co-option of an appendage-forming genetic programme during prepupal head development has generated an outgrowth on the dorsal head, which can now be used as a weapon in combat (Moczek et al., 2006). Further, a gender-specific regulator has been incorporated in the pathway with the recruitment of the Hox gene *Sex combs reduced* (Wasik et al., 2010), generating sexual dimorphism in the trait.

These studies are extremely valuable in understanding the genetic and developmental mechanisms generating evolutionary novelty. However, they rely fundamentally on prior knowledge of genes, pathways, and networks in model systems like *Drosophila melanogaster*. Genomic tools are increasingly available for both systems(Beldade et al., 2007, 2009; Kijimoto et al., 2009), allowing the work to move beyond a *Drosophila*-inspired candidate gene approach. Still, the available tools at this time to explore genetic relationships are limited.

Investigating the origin of the novelty in question depends on comparative studies with outgroups, or, as phrased by Shubin et al. (2009): "It is not possible to identify what is new in evolution without understanding the old". The role of 'the old', or, species without the novel trait in question, is usually taken up by the classical models, irrespective of their phylogenetic distance and true basal position (Fig. 1.1). This may be problematic when those model systems are themselves very derived, as the case is with the classical model in development and genetics, *Drosophila melanogaster*. However, precisely this derived state could prove useful in the research programme on novelty, if we can identify and harness traits within this system that are themselves evolutionary novelties. Indeed, such a novelty can be found, in the dorsal-anterior appendages on the *Drosophila* eggshell.

1.2 The *Drosophila* eggshell as a new model system for evolutionary novelty

The model organism *Drosophila melanogaster* possesses many derived traits relative to most dipterans. This can prove problematic when the model is used as a backbone for exploring development in emerging or non-model systems. However, this characteristic makes *D. melanogaster* an exceptionally suitable hunting

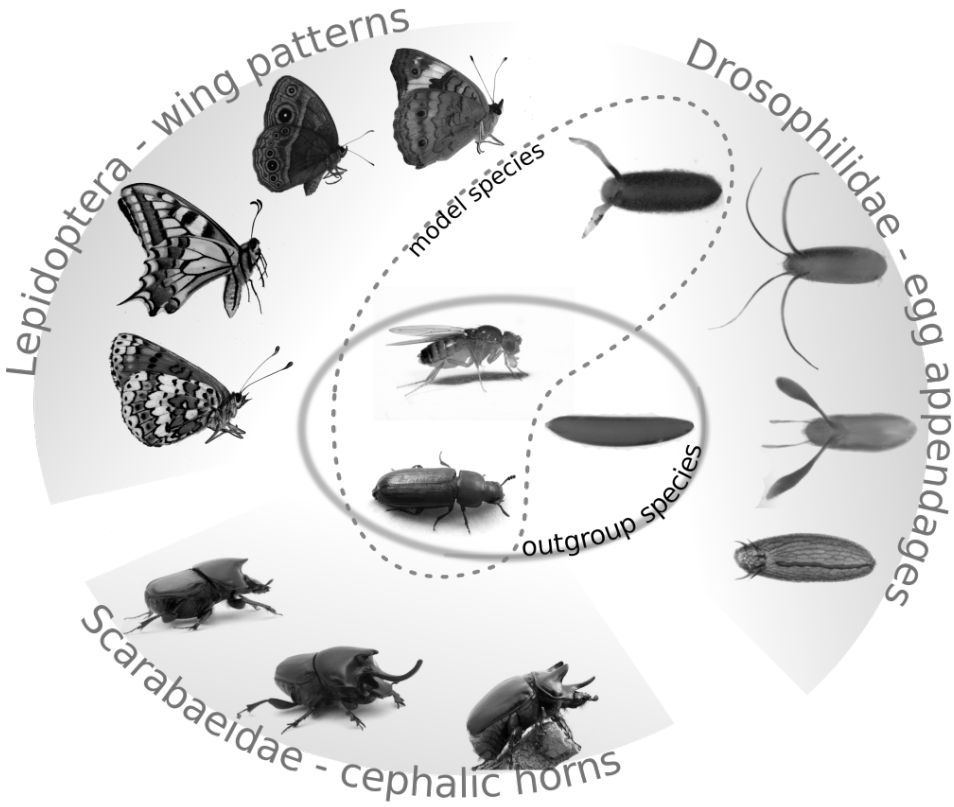

Figure 1.1: **Examples of evolutionary novelties in insects**. The central oval indicates species without the novel feature, used for comparative study. The dashed line groups classical model species *Drosophila melanogaster* and *Tribolium castaneum*, demonstrating that classical model species need not always be the outgroup comparison, as is the subject of this thesis. Wing patterns on Lepidoptera show immense diversity between species. Shown here are (left to right) the adults of *Melitae cinxia*, *Papilio machaon*, *Bicyclus anynana*, and *Junonia coenia*. To investigate the origin of this novelty, comparisons with the classical developmental model system *Drosophila melanogaster* (centre, top) are crucial. Similarly, studies on the head horns of Scarabaeidae (Coleoptera), benefit from comparison with the beetle model system of *Tribolium castaneum* (centre, left) as well as *Drosophila melanogaster*. Shown here are (left to right) *Onthophagus nigriventris* (minor and major male), and *Strategus*. Dorsal appendages on the eggs of Drosophilidae (Diptera) can be compared with eggs of *Ceratitis capitata* (centre, right), a Tephritid fly. Shown here are (top to bottom) eggs of *Drosophila melanogaster*, *Drosophila mojavensis*, *Zaprionus sepsoides*, and *Chymomyza pararufithorax*. Images of adult insects: copyright Suzanne Saenko (*Mc, Pm, Jc*), Patrícia Beldade (*Ba*), Darren Obbard (*Dm*), and Alex Wild (*Tc, On, S*), all used with permission.

ground for of evolutionary novelties. Indeed, the novel feature central to this thesis is found in *D. melanogaster*, and concerns the two appendages found on the dorsal-anterior side of Drosophilid eggs. These are large protrusions of the chorion, and promote the embryo's access to oxygen in the air, while managing the risk of dessication (Hinton, 1969, 1981). This trait has emerged in a common ancestor of the family Drosophilidae, and the phenotype has since diversified extensively (Okada, 1968). Most importantly for our purposes, the genetic and developmental basis of dorsal appendages is well understood, making it an excellent model system to investigate the generative mechanisms behind this evolutionary novelty.

Analysing the development of any trait is a powerful tool in determining its evolutionary origin, since every phenotype inevitably has to be formed by translating genes through development (Arthur, 2002). In this introductory chapter we will therefore discuss what is known about the ontogeny of dorsal appendages, which takes place during oogenesis. Of specific interest will be the fact that the pathways involved in forming the appendages and those that determine the main body axes of the future embryo are tightly linked. This will provide our research on the developmental basis of this innovation with a useful anchor, as the novel trait is developmentally connected to a highly important, likely conserved ontogenetic process.

To adequately introduce this model system, on the following pages a rough framework will be formed in which to view the processes discussed in this thesis, and with which to interpret our experimental results. The focus will initially be on the morphogenetic aspects of oogenesis, after which the genes and pathways are introduced that play a role in regulating these processes. Finally, we will discuss what is known about the patterning of the dorsal eggshell, which is the formative stage for the appendages we aim to study.

1.3 A framework to *D. melanogaster* oogenesis

1.3.1 Morphogenesis from germarium to eggshell

Drosophila ovaries consist of multiple strands of progressively ordered egg chambers, called ovarioles. Multiple developmental stages occur simultaneously in one ovariole: younger stages are located at anterior (Fig. 1.2 A), and the strands terminate in a completed egg at the posterior-most end, distally in the fly's abdomen (Spradling, 1993).

Oogenesis in *Drosophila* is meroistic, which means that the germline cells are not only fertilizable oocytes. Some germline descendants differentiate into nurse cells, which remain cytoplasmically connected to the oocyte to support its growth. Insects exhibit two modes of meroistic oogenesis: telotrophic, where the nurse

cells and the oocyte are encapsulated in separate follicles but remain connected through a nutritive cord; and polytrophic, where both nurse cells and oocyte form a single capsule as the oocyte matures (Heming, 2003). *Drosophila* oogenesis is an example of the latter type.

The egg chamber is formed in the germarium

The polytrophic *Drosophila* egg chamber is formed by 16 germline cells (one of which is the oocyte), encapsulated by a monolayer of ±1000 somatic follicle cells. The ontogenic basis of this structure is in the germarium, the anterior-most structure of the ovariole, from which each egg chamber is released (Fig. 1.2 B). Two germline stem cells are present at the anterior side of the germarium. Presumably, the asymmetric division of a stem cell generates a new stem cell and a cystoblast. The latter will undergo four consecutive mitoses with incomplete cytokinesis, forming a cyst with 16 interconnected cells. From this cyst, one cell is selected to become the oocyte. This selection is the first demonstration of asymmetry in the egg chamber, and will lay the foundation for future symmetry-breaking events. The selection of the oocyte and the precise regulation of early egg chamber development are complex processes that have been excellently reviewed by e.g. Deng and Lin (2001); Huynh and St Johnston (2004); Roth and Lynch (2009).

The oocyte will remain largely, though not entirely, transcriptionally silent (Mahowald and Tiefert, 1970, Raquel A. M. Santos and Vítor Barbosa, pers. comm.). Mostly, it is the nurse cells that provide mRNA and proteins through the cytoplasmic bridges (ring canals) that connect the cells, via a cytoplasmic structure called the fusome, and in many cases through transport via the microtubule network (Mahajan-Miklos and Cooley, 1994; Pokrywka and Stephenson, 1995). The minus end of the microtubule network is anchored at the posterior pole of the oocyte, and is vital for the oocyte's identity (reviewed in Deng and Lin, 2001; Huynh and St Johnston, 2004; Roth and Lynch, 2009).

Further in the germarium, the germline cyst is enveloped by a layer of somatic follicle cells (Fig. 1.2 B). These cells originate from two stem cells that lie on the border of the germarium regions 2a and b (Margolis and Spradling, 1995). Two subsets of the follicle cells cease division in region 2b, long before their neighbours do. These cells, originating from a single precursor, will form two groups of specialized follicle cells: (1) the stalk cells, connecting the different egg chambers, and (2) the polar cells (Ruohola et al., 1991; Margolis and Spradling, 1995; Tworoger et al., 1999). Two polar cells will end up on the anterior-most side of the egg chamber, while the other two localize to the posterior end (Brower et al., 1981). The posterior polar cells will play an important role in the polarization of the oocyte later on in oogenesis, while the anterior cells are required for a number

C
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Legend/abbreviations
BC - Border Cells
Cb - Cystoblast
CFC - Columnar Follicle Cells
CMFC - Centripetally Migrating Follicle Cells
DA - Dorsal Appendage
EnC - Endochorion
ExC - Exochorion
FC - Follicle Cell
FCp - Follicle Cell precursor
GSC - Germline Stem Cell
ICL - Inner Chorionic Layer
MP - Micropyle
Gurken
EGFr activity
JAK/STAT signal
Floor cells
Roof cells
NC - Nurse Cells
NFC - Nurse cell Follicle Cells
Oo - Oocyte
Op - Operculum
PC - Polar Cells
VM - Vitelline Membrane
A
germarium
st.6
st.8
st.9
st.10
st.12
NC
Oo
BC
NFC
CFC
CMFC
B
GSC
Cb
FCp
Oo
PC
region 1 2a 2b 3
A
V
D
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MP
Op
Floor
Roof
E
E''
Oo
VM
ICL
EnC
ExC
FC

of processes that shape the follicular epithelium, including the specification of an adjacent group of terminal anterior follicle cells (6-10 in total) known as border cells (Ruohola et al., 1991; Grammont and Irvine, 2001; Torres et al., 2003).

Oogenesis in 14 stages

At its encapsulation, the egg chamber will bud off from the germarium, remaining connected only through stalk cells that adhere to the polar cells of the successive egg chambers (Fig. 1.2A, B) (reviewed in Roth and Lynch, 2009). The egg chamber progresses through a total of 14 morphologically distinct stages that can be divided into the pre-vitellogenic stages (1-7) and the post-vitellogenic stages (8-14) (Fig. 1.2A). These stage groups are separated by the onset of vitelline membrane formation, which is the first component of the future eggshell that is synthesized in the egg chamber (Spradling, 1993). During the pre-vitellogenic stages, the egg chamber enlarges rapidly and the follicle cells undergo a number of divisions to enable further growth. Around stage 8 they stop dividing and switch to an endocycle to become polyploid (Brower et al., 1981).

Stage 7 sees a major reorganization of the microtubule network, when the posterior microtubule organizing centre (MTOC) in the oocyte disintegrates (see section 1.3.2), and microtubules now grow from the anterior-lateral cortex of the oocyte. This rearranges the oocyte's polarity entirely. At this stage, the oocyte nucleus moves from its previous position at the posterior pole to an anterior-lateral location (Guichet et al., 2001), pushed by the polymerizing microtubules (Zhao et al., 2012). This asymmetric localization of the nucleus at the anterior end

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Figure 1.2 *(preceding page)*: **An overview of oogenesis in *Drosophila melanogaster*.** (A) The ovariole consists of eggchambers of progressively later stages from anterior (left) to posterior (right). (B) The eggchambers are formed in the germarium. (C) Terminal follicle cells have been made competent by a JAK/STAT signal emanating from the polar follicle cells. Around stage 6, nucleus-associated Gurken activates EGFr in the adjacent follicle cells. (C′) Competent cells with activated EGFr take up posterior fate, and signal back to the oocyte, establishing anteroposterior polarity. (D) Around stage 8, the oocyte nucleus and associated Gurken have moved to the anterior cortex, and signal again to EGFr in adjacent follicle cells. (D′) Activated EGFr in the dorsal-anterior follicle cells will define embryonic dorsoventral polarity. (E) The appendage primordia are defined in stage 10, and consist of two groups of cells on either side of the midline that will form the roof of the tube (in purple), and a hinge bordering the anterior and central edge of this domain, with cells forming the floor (in green). (E′) The fully formed egg with an operculum, micropyle, and two dorsal appendages. (E″) A scheme of the multilayered eggshell, deposited between the oocyte and the follicle cells.

of the egg chamber constitutes the first event in the specification of the future dorsoventral axis, and the nucleus marks the future dorsal side of the egg (Fig. 1.2 D).

The follicle cell layer at stage 9 is rigourously restructured as most anterior follicle cells move posteriorly to cover the oocyte, and roughly 5% are stretched out into a squamous epithelium overlying the nurse cells. The other 95% form a columnar layer over the oocyte. At the boundary of the nurse cells and the oocyte, a designated group of follicle cells will move centripetally to cover the anterior part of the oocyte (these are referred to as the centripetally migrating follicle cells, or CMFC). This movement starts in early stage 10 egg chambers, and is complete by stage 10B (Spradling, 1993; Deng and Bownes, 1998). Concomitant with the restructuring of the outer follicle cell layer, the border cells (an anterior terminal cluster of specialized follicle cells) travel from the anterior-most end of the egg chamber, through the nurse cell cluster, to the anterior oocyte border (Fig. 1.2A). Subsequently, they move dorsally along the oocyte (Brower et al., 1981; Montell et al., 1992). These cells will later secrete the paracrystalline material that forms the pore of the micropyle, allowing sperm to enter the egg (Spradling, 1993).

Because of the constant content deposition by the nurse cells, the oocyte grows relatively faster than its supporting clones: at stage 10, the oocyte occupies half the egg chamber (Fig. 1.2A). During stages 10B-12 the nurse cells rapidly dump their cytoplasm into the oocyte, and in the final stages (13-14) they shrink and complete apoptosis (Chao and Nagoshi, 1999).

Formation of the vitelline membrane, chorion, and eggshell structures

The eggshell *in toto* is composed of the following layers: the vitelline membrane, the wax layer, the inner chorionic layer (ICL), the endochorion (itself consisting of an inner part, an outer part, and pillars in between), and the exochorion (Fig. 1.2E″) (Margaritis et al., 1980). The various components of the eggshell are secreted by the follicle cell layer, starting with the vitelline membrane: a solid layer that even without the chorion is able to maintain the integrity of the egg and support it during embryonic development. Synthesis of the membrane components starts at stage 8, but the vitelline membrane is not complete before 10B. At this stage, small vesicles containing the vitelline proteins fuse, and a single layer is formed between the oocyte and the follicular epithelium (Spradling, 1993). This breaks the communication between the follicular epithelium and the oocyte.

The chorionic layers require six major structural proteins, in addition to a number of minor components. The genes encoding these proteins are clustered: the X chromosome contains a cluster of proteins mostly important for ICL and endochorion synthesis, and another cluster on chromosome 3 encodes components

of the exochorion (Spradling, 1993). This clustering has been conserved in other dipterans (Konsolaki et al., 1990; Tolias et al., 1990).

Secretion of the chorionic proteins is rapid and profuse. Prior to protein synthesis, starting at stage 9, the chorion gene clusters undergo rapid amplification, which allows high-level expression of the genes and facilitates fast chorion protein production. Protein secretion occurs between stage 11 and the end of oogenesis, lasting no longer than five hours (Orr-Weaver, 1991; Spradling, 1993).

Some final touches are required to complete the eggshell: interaction between vitelline membrane components and chorionic proteins is necessary to further stabilize the eggshell and connect its layers. Final shape and hardness is attained when the shell is hydrated as the egg passes through the oviduct (Spradling, 1993).

During the prior eggshell patterning phase, subgroups of follicle cells have been defined that now form several specialized eggshell structures: the CMFC build both the operculum and the micropyle, which relate to hatching and fertilization respectively, while the border cells secrete the micropylar pore (Margaritis et al., 1980; Montell et al., 1992). While the formation of these structures is inextricably connected to the formation of the dorsal appendages, their precise development and morphology is beyond the scope of this thesis. However, an excellent review on micropyle and operculum development was written by Dobens and Raftery (2000).

Another subset of anterior-dorsal follicle cells will form the dorsal appendages. On either side of the midline is an appendage primordium (Fig. 1.2E), each consisting of two cell groups: floor cells and roof cells, which will form the floor and the roof of the appendage tube, respectively (Fig. 1.2E′) (Ward and Berg, 2005; Boyle et al., 2010).

The roof and floor cells are marked respectively by expression of *broad* (*br*), encoding a zinc-finger transcription factor, and *rhomboid* (*rho*), encoding a serine protease (Ruohola-Baker et al., 1993; Deng and Bownes, 1997; Ward and Berg, 2005). When forming the tubes, Br-cells constrict apically to form the roof, and Rho-cells elongate forming the floor. The appendage primordia are defined by stage 10B, and reorganization starts at stage 11, finalizing the tubes at stage 14 (Dorman et al., 2004). Within the *br*-expressing roof cells, two cell types can be distinguished based on their function during morphogenesis. The 'leading' roof consists of those cells adjacent to the floor cells in the epithelium, and is indispensable for the shape change that forms the appendage tube. The 'trailing' roof cells, meanwhile, merely follow (Boyle et al., 2010).

While the tube grows (which is emphatically only due to cell movement and reshaping, not to cell division), the chorion is deposited (Orr-Weaver, 1991). The eggshell of the dorsal appendages consists of endochorion and exochorion only

(Margaritis et al., 1980). Much more than in other areas of the eggshell, the outer endochorionic and exochorionic layers of the appendages contain pores, through which air can enter the meshwork of the endochorion (Spradling, 1993). This underlines the function of the egg appendages as respiratory structures (Hinton, 1969).

1.3.2 EGF signalling defines the main body axes of the future embryo

The eggshell with its dorsal structures is evidently polarized. The origin of this polarity is tightly connected to the establishment of the embryonic main body axes. Determining the anteroposterior and dorsoventral axes is of vital importance in the development of bilaterally symmetric animals, and is generally one of the first events in ontogenesis (Gerhardt and Kirschner, 1997; Gilbert, 2003). In insects, the symmetry-breaking events leading to the definition of the main body axes happen even prior to fertilization, during the development of the egg (Roth and Lynch, 2009). In fact, it is during the very first stages of oogenesis, when in the germarium the oocyte is selected from a group of 16 germ line cells, that the foundation is laid for embryonic polarization in *Drosophila melanogaster* (Huynh and St Johnston, 2004).

Embryonic anteroposterior axis determinants, specifically: mRNA of *bicoid* (*bcd*), *oskar* (*osk*), and *nanos* (*nos*), localize asymmetrically in the oocyte, and form gradients in the early embryo. Patterning of posterior embryonic regions then occurs downstream of *osk* and *nos*, while *bcd* defines anterior structures.

The cellular sublocalization of these mRNAs is microtubule-dependent, and is properly defined after a series of signalling events between the oocyte and the follicular epithelium. This interaction between the oocyte and the somatic follicle cells during oogenesis is essential in establishing polarity (reviewed in e.g. Roth and Schüpbach, 1994; Roth and Lynch, 2009).

The key in this interaction is the EGF signalling pathway (González-Reyes and St Johnston, 1994; González-Reyes et al., 1995). mRNA encoding the TGFα homolog Grk, an EGFr ligand, is present in the oocyte, and can be detected as early as stage 2B of the germarium. Once the egg chamber is fully formed, the mRNA and the oocyte nucleus localize to the posterior cortex of the oocyte (Neuman-Silberberg and Schüpbach, 1993). The Grk protein is translated in the oocyte nucleus, and, during later stages (8-10), the oocyte nucleus is also partly responsible for the transcription of the *grk* gene (González-Reyes and St Johnston, 1994; Saunders and Cohen, 1999; Cooperstock and Lipshitz, 2001). EGFr expressed in the follicular epithelium will respond twice to Grk in the oocyte. These signalling events define anteroposterior and dorsoventral polarity, respectively (Fig. 1.2C-D′).

Anteroposterior polarity

In addition to the location-specific Grk signal, prior differentiation of the follicle cells also determines their response. During stages 6-7, a combination of Notch and JAK/STAT signalling—originating from the germline cyst and polar cells, respectively—specifies a group of cells on either terminus of the follicular epithelium to adopt terminal cell fate (Grammont and Irvine, 2002; Xi et al., 2003). These cells are now competent to respond to the first EGF signalling event, which occurs between the oocyte and the terminal follicle cells at stage 7 (Fig. 1.2C). Grk in the oocyte activates EGFr in the adjacent terminal follicle cells, which now take up posterior fate (Peri et al., 1999). The posterior follicle cells now signal back to the oocyte, with a signal of yet unknown nature (Fig. 1.2C′).

Up until this point, microtubules have formed a direct transport system for mRNAs from the nurse cells to the posterior pole at the oocyte MTOC (Cooley and Theurkauf, 1994). This backsignalling event, however, breaks down the posterior MTOC, and microtubules rearrange and rebuild in the oocyte, changing mRNA sublocalization with it. The mRNAs required for embryonic anteroposterior polarity now take up their final location: *bcd* at the anterior cortex, and *nos* and *osk* at the posterior pole (Becalska and Gavis, 2009). Upon rearrangement of the cytoskeleton the nucleus moves to dorsal-anterior, as does the associated *grk* (Neuman-Silberberg and Schüpbach, 1993, 1996; Cooperstock and Lipshitz, 2001). Both *grk* mRNA and the Grk protein now border the dorsal-anterior cortex of the oocyte, the protein co-localizing with membrane associated F-actin (Neuman-Silberberg and Schüpbach, 1996).

Dorsoventral polarity

The second EGF signal, determining dorsoventral polarity, starts when Grk activates EGFr in the overlying dorsal-anterior follicle cells (Fig. 1.2D). Downstream of activated EGFr, expression of *mirror* (*mirr*) is up-regulated; a gene in the Iroquois complex that codes for a homeobox-containing transcription factor (McNeill et al., 1997; Jordan et al., 2000; Zhao et al., 2000). Mirr represses the gene *pipe* (*pip*), restricting its expression to the ventral side of the egg chamber, which leaves an asymmetric distribution of Pip protein in the perivitelline space by the end of oogenesis (Stein et al., 1991; Nilson and Schüpbach, 1998). Precise levels of EGFr activation are required for these patterns to be correctly established, which is achieved by the inhibitory activity of Cbl (Pai et al., 2000).

Pip essentially transfers dorsoventral polarity from the eggshell to the embryo. Together with Windbeutel (Wnd) and Nudel (Nud), the genes for which are also expressed in the follicular epithelium, it initiates a signalling cascade during early embryonic development by proteolytic cleavage of the Toll-ligand Spätzle (Nilson

and Schüpbach, 1998). Toll activation then causes the degradation of Cactus (Cact) in a polarized fashion in the embryo. As Cact prevents the transcription factor Dorsal (Dl) from entering the blastoderm nuclei, Toll receptor signalling ultimately results in a gradient of nuclear localization of Dl in the blastoderm stage embryo. This gradient subsequently defines the embryonic germ layers, as the transcription of several key regulators depends on the nuclear concentration of Dl (Gilbert, 2003; Moussian and Roth, 2005).

The EGF signalling cascade in dorsoventral polarity

While the cascade between *pip* expression and embryonic germ layer specification is extremely well known, the link between EGFr activation and *pip* expression is less straightforward. EGFr is a class I Receptor Tyrosine Kinase (RTK), a family of surface-bound receptors that function via intracellular kinase activity. In EGF signalling, ligand-binding induces phosphorylation of the cytoplasmic domain of the receptor, in turn leading to phosphorylation of Ras1. This initiates a signal transduction cascade via Raf and MEK/MAPKK (Mitogen Activated Protein Kinase Kinase) to MAPK (Mitogen Activated Protein Kinase), which once phosphorylated can target a number of transcription factors (Alberts et al., 2002, p. 871-879) (Fig. 1.3A).

One such transcription factor, which has been shown to be involved in dorsoventral patterning, is the HMG-box containing Capicua (Cic) (Goff et al., 2001; Atkey et al., 2006). Activated MAPK targets a highly conserved motive in the Cic protein, and its phosphorylated form is redistributed to the cytoplasm, which interferes with its transcription factor activity (Astigarraga et al., 2007; Ajuria et al., 2011). Cic usually functions as a repressor, and has been shown to repress *mirr*, which in turn represses *pip* (Andreu et al., 2012). However, early (stage 10A) visible expression of *mirr* is unaffected in a *cic* mutant, while stage 9-10a *pip* expression is completely absent (Goff et al., 2001). Despite these observations, recent experiments by Andreu et al. (2012) have shown that the supporting function of Cic on *pip* expression does depend on its repression of *mirr*: when the Mirr-responsive element in the *pip* regulatory region is removed, *cic* mutation no longer affects *pip* expression. Conversely, when *mirr* mutant clones are generated in a *cic* mutant background, *pip* is de-repressed. These experiments strongly suggest that Cic links EGFr activation to *pip* repression, via Mirr. We will discuss these findings further in relation to our own results in the Medfly in chapter **??**.

1.3.3 Dpp signalling establishes anterior eggshell structures

In the follicular epithelium, Dpp signalling is crucial in patterning anterior eggshell domains. Dpp, a BMP ligand of the TGFβ superfamily, binds to a combination of type I and type II receptors (Ruberte et al., 1995), which phosphorylates the receptor-associated Mad (Mothers Against Dpp, a Smad1/Smad5 homolog). The phosphorylated Mad (pMad) now dissociates from the receptor, and binds to its cofactor Medea (Med), a Smad4 homolog (Wisotzkey et al., 1998). The complex moves into the nucleus, and directly drives transcription of target genes (Alberts et al., 2002, p.888) (Fig. 1.3B).

Interference with this signalling cascade can occur in several ways. Firstly, the homolog of the inhibitory Smad7, Daughters against Dpp (Dad), can bind the type I receptor to prevent Mad from associating and being phosphorylated. Secondly, the Chordin homolog Short Gastrulation (Sog) can prevent signalling by binding the Dpp ligand itself (Alberts et al., 2002, p.888). Lastly, Brinker (Brk), an intracellular negative regulator of the pathway, blocks expression of Dpp target genes. Brk has been proposed to be an effector of low-level Dpp signalling, as the gene itself is a negative target of the Dpp signal; and, through repressing *brk*, Dpp signalling alleviates this repression on its low-level targets (Jazwinska et al., 1999).

At stage 8, Dpp signalling starts with the expression of the ligand *dpp* in a subset of anterior follicle cells (Twombly et al., 1996). Dpp protein diffuses to more posterior follicle cells, forming a morphogen gradient. It acts through type I receptors Saxophone (Sax) and Thickveins (Tkv) in the follicular epithelium to activate the pathway in a graded manner (Shravage et al., 2007). As the inward movement of the CMFC starts, *dpp* is expressed in the leading edge of these cells, and disrupted Dpp signalling has been associated with defects in this migration (Twombly et al., 1996). High levels of Dpp pathway activity are associated with operculum formation, thus, high Dpp activity suppresses dorsal appendage (DA) fate (Twombly et al., 1996; Dobens et al., 2000). Importantly, both the micropyle and the operculum are formed by a subset of the CMFC population, emphasizing the importance of Dpp signalling for the establishment of both these anterior structures (Spradling, 1993).

The expression and activity of Dpp persist through stages 9 and 10, and can be detected as late as stage 11, although the pattern is dynamic (Niepielko et al., 2011). Because expression of the receptor gene *tkv* is up-regulated in the appendage primordia, the dorsal Dpp activity gradient shifts to two patches corresponding to the appendage primordia in stages beyond 10B. The suppressive effect of Dpp on DA fate is thought to be involved in temporal regulation of Br, which will be elaborated on next (Yakoby et al., 2008b).

In summary, in the follicular epithelium two signalling pathways are active

A
Spi
Spi
Spi
Rho
Kek1
EGFr
Grk
Aos
Sty
Ras1
Raf
MEK
MAPK
Cbl
CF2
Pnt-P2
Cic
kek1
pnt
sty
aos
rho
P
nucleus
cytoplasm
B
Tkv
(R-I)
Put
(R-II)
Dpp
Dad
Med
Mad
Mad
Brk
dad
other targets
Med
Mad
brk
nucleus
cytoplasm

in regulating polarity (EGF), and cell migration (Dpp). How these pathways function in patterning the dorsal-anterior area of the epithelium specifically will be the subject of the following section.

1.4 Specification of the appendage primordia on the follicular epithelium

Pattern formation, a process that provides individual identities to cells or cell groups within a larger structure, is a classical case in developmental biology, and has played a prime part in theories of development. Who is not familiar with Lewis Wolpert's famous French flag analogy for the interpretation of a morphogen gradient, or the reaction-diffusion model that marked Alan Turing's legendary foray into the study of development (Roth, 2011)? Indeed, pattern formation both as a concept and a mechanism in development has been well explored. The many phenomena involved in this process, as well as the interactions between them, are capable of producing an endless variety of forms, which are often the basis for further processes shaping or colouring the organism in question.

The follicular epithelium of the developing *Drosophila* egg is no exception. At stage 10B of oogenesis, two groups of cells on either side of the midline have been designated with the dorsal appendage fate. These populations, the appendage primordia, consist of an anterior-midline cell row expressing high levels of *rho*, and a larger posterior-lateral patch with elevated levels of *br* expression. They will shape the floor and the roof of the appendage, respectively (Dorman et al.,

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Figure 1.3 *(preceding page)*: **The EGF and Dpp signalling pathways**. (A) A simplified schematic view of EGF signalling, depicting the membrane-bound EGFr; its activating ligands Spi and Grk; the inhibiting ligand Aos, and the membrane-bound inhibitor Kek; the intracellular signal transduction cascade via Ras1 through to MEK (MAPKK) and MAPK; intracellular inhibitors Cbl and Sty; CF2, Pnt-P2, and Cic, which are phosphorylation targets that translate the EGF signal to gene expression; and finally the protease Rho which cleaves Spi, preparing it to activate EGFr. The genes encoding many of these components are transcriptional targets of the pathway, such as *kek1*, *pnt*, *aos*, *sty*, and *rho*. (B) A simplified schematic view of Dpp signalling, depicting the ligand Dpp binding type I and type II receptors Tkv and Pnt; not shown here is type I receptor Sax; the intracellular signal transduction cascade, inhibited by Dad, which phosphorylates Mad, which binds to Med; the Med-Mad complex, which enters the cell and directly targets gene expression; and Brk, which represses Dpp target genes. *brk* is itself targeted by the pathway, as is the gene encoding the inhibitor Dad. Not shown is the extracellular inhibitor Sog.

2004; Ward and Berg, 2005) (Fig. 1.2E).

In the demarcation of these appendage primordia, several phenomena have been observed to act. In the following section we will describe in detail which genes are involved, and how they interact. Before delving into specifics, however, it is helpful to observe the processes from a distance.

First, global coordination of the epithelium occurs via the signalling activity of two previously described pathways: EGFr and Dpp. We can employ a useful simplification to describe their role in patterning the follicular epithelium: EGF signalling is responsible for providing positional information along the dorsoventral axis, while Dpp defines anteroposterior polarity in the epithelium. Indeed, early attempts at modelling follicle cell patterning have indicated that this is a useful approach of domain specification on the follicular epithelium (chapter 4 of this thesis). Importantly, this rough analysis does not simply stem from patterns of pathway activity, but is reinforced by the mutant phenotypes of elements of each pathway (section 1.4.1, below).

Both pathways also depend on (positive and negative) feedback loops to modulate their signalling activity to the appropriate levels. Here, pathway interactions take place, as the targets of one pathway can be involved in the feedback loop of the other. Moreover, we observe that the initial signals start to combine, as transcription factors are targeted by both pathways. Subsequently, these transcription factors will be crucial in specifying those cells that will take part in the appendage primordia. This phase will be discussed in section 1.4.2.

Finally, local organization comes into play. This is an important phenomenon in pattern formation responsible for the tight coordination between domains. In the eggshell patterning network we observe precise coordination between the domains of the operculum, the appendage floor cells, and the appendage roof cells. The details of this phenomenon will be elaborated on in section 1.4.3.

1.4.1 Elements of EGFr and Dpp signalling provide global coordination

Mutation of EGFr pathway elements affects dorsoventral polarity of the eggshell

The main role of the dorsal EGF signalling event is the regulation of dorsoventral polarity, but not just of the future embryo: the *Drosophila* eggshell shows clear dorsoventral polarity. As mutations in most known elements of the EGF pathway (Fig. 1.3A) largely affect eggshell dorsoventral polarity, we can conclude that the polarized eggshell is established downstream of EGF signalling. Mutations in EGFr and its ligand Grk ventralize the egg (Schüpbach, 1987), as does muta-

tion of Ras1, required for EGFr signal transduction (Brand and Perrimon, 1994; Schnorr and Berg, 1996). Conversely, ectopic activation of EGFr has dorsalizes the eggshell (Queenan et al., 1997), as does ectopic over-expression of EGFr ligands Grk (Ghiglione et al., 2002) and Spitz (Spi) (Sapir et al., 1998). When Cic, a transcription factor targeted for degradation by EGF signalling, is mutated, the eggshell is dorsalized (Goff et al., 2001). The same is true for three inhibitors of EGF signalling: Kekkon1 (Kek1) (Ghiglione et al., 1999), Sprouty (Sty) (Reich et al., 1999), and Cbl (Pai et al., 2000). Importantly, the respective strengths of the mutant phenotypes differ tremendously. While Sty loss-of-function results in a pronouncedly dorsalized eggshell (Reich et al., 1999), mutation of *kek1* only has a mildly dorsalizing effect, which slightly enlarges the space between the appendages (Ghiglione et al., 1999). Embryos from Kek1 mutant females develop normally (Musacchio and Perrimon, 1996).

Mutations in Dpp signalling affect the anterior portion of the eggshell

Conversely, manipulations of Dpp signalling (Fig. 1.3B) affect the anterior region of the eggshell, which is most clearly visible through its effects on dorsal-anterior structures. Overexpression of the *dpp* gene encoding the ligand severely enlarges the operculum, and depending on the severity of the phenotype, can either affect the shape and number of the dorsal appendages, or remove them altogether (Twombly et al., 1996; Deng and Bownes, 1997; Shravage et al., 2007). Eggs with reduced levels of Dpp receptors Sax and Tkv tend to be shorter, and show reduction in micropyle as well as operculum size (Twombly et al., 1996). Follicle cells mutant for Med produce an eggshell without operculum (Shravage et al., 2007). Loss-of-function of dSno, an antagonist of Dpp signalling, enlarges the operculum, and shifts the dorsal appendages toward posterior. Interestingly, the appendages on dSno mutant eggs are also slightly further apart, likely a testimony to the involvement of EGF signalling in the regulation of *dSno* (Shravage et al., 2007). Another antagonist of Dpp signalling is Brinker *brk*, the mutation of which, again, enlarges the operculum, frequently removing the dorsal appendages completely (Chen and Schüpbach, 2006).

1.4.2 Feedback loops amplify, specify, and connect Dpp and EGF signalling

While the reductionist approach of a simple coordinate system provides a fruitful first step in considering epithelial patterning, to fully understand the specification of the appendage primordia we need to know how the initial input is interpreted, and which interactions follow. To start, EGF signalling is under the control of

multiple feedback loops. Several inhibitors of EGF signalling are themselves EGFr targets (Fig. 1.3A), most notably the genes encoding RTK inhibitors Sty and Kek1 (Ghiglione et al., 1999; Reich et al., 1999), and the inhibitory ligand Aos (Golembo et al., 1996; Queenan et al., 1997). Additionally, a positive feedback loop amplifies the EGFr signal by targeting *rho*, which encodes a protease that cleaves Spi, an activating ligand of the pathway (Queenan et al., 1997). The precise transcription factors used to target the genes in both feedback loops are unknown. In the case of *rho*, early studies pointed at CF2, which is degraded after phosphorylation by activated MAPK (Hsu et al., 1996), while later research indicates Cic as a link between EGFr activation and *rho* expression (Astigarraga et al., 2007). The same study also shows the involvement of Cic in the regulation of *aos*. Additionally, both Mirr and Pnt have been suggested to be upstream of *rho* and *aos* (Morimoto et al., 1996; Jordan et al., 2000; Chang et al., 2003). However, none of the proposed links have been confirmed as direct regulatory interactions, and indirect evidence deserves to be treated with caution when feedback loops are involved.

Regarding the EGFr feedback loops, while EGFr activity dynamics via Rho (amplification) and Aos (inhibition) have long been the focus of study in eggshell patterning (Wasserman and Freeman, 1998), the importance of these elements has been called into question in the last few years. Most notably, a study by Boisclair Lachance et al. (2009) showed that their respective loss-of-function in follicle cells had no effect on the eggshell phenotype. Conversely, the role of Sty and Kek1 as factors in eggshell patterning was confirmed by the same study, and the importance of their influence on the quality of the EGFr activation gradient was emphasized by computational analyses (Zartman et al., 2011). In chapter 4 of this thesis, the role of these feedback loops will be discussed in more detail.

Similarly, antagonist activity regulates Dpp signalling to the appropriate levels in different cell populations (Fig. 1.3B). *brk* encodes a repressor of Dpp signalling necessary to facilitate *br* expression in the appendage primordia, and restrict operculum fate to the anterior-most follicle cells. EGF signalling up-regulates *brk* expression, while Dpp signalling negatively affects *brk* expression. Thus, the combined levels of Dpp and EGF signalling regulate the precise distribution of *brk* (Chen and Schüpbach, 2006). Additionally, EGF signalling targets *dSno*, which is another antagonist of the Dpp pathway, to be expressed in the posterior-lateral boundaries of the future appendage primordia (Shravage et al., 2007).

Furthermore, EGFr activity controls the expression of *pnt* (Morimoto et al., 1996). The *pnt* locus encodes two alternatively spliced proteins, each containing a different combination of domains: Pnt-P1 contains a DNA-binding ETS domain, and Pnt-P2 contains the same ETS plus a PNT domain (McQuilton et al., 2012). Both proteins are used during oogenesis, and have the same loss-of-function pheno-

type (Morimoto et al., 1996). However, they differ in the phenotype they generate when over-expressed: Pnt-P1 over-expression reduces the dorsal appendages in size or abolishes them altogether, while Pnt-P2 over-expression has no effect on the eggshell or the embryo (Morimoto et al., 1996). An explanation for this could be the fact that Pnt-P2 requires activation through phosphorylation by activated MAPK to be functional; thus, solely over-expressing the gene is insufficient for extending its activity over a larger area (O'Neill et al., 1994). A model proposing that expression of *pnt-p1* is under the control of activated Pnt-P2 (O'Neill et al., 1994; Morimoto et al., 1996) could explain the fact that both proteins have the exact same loss-of-function phenotype while differing in the effect of their over-expression. Furthermore, it would explain how Pnt is targeted by the EGFr pathway.

The transcription factor Mirr is a key element of the network. As described before (section 1.3.2), *mirr* is expressed in a large domain at the dorsal-anterior end of the epithelium, and has been shown to be under the repressive control of Cic (Goff et al., 2001; Atkey et al., 2006). However, up-regulation of *mirr* in *cic* mutants is limited to anterior regions, indicating the control of another anterior factor, proposed to be Dpp signalling[2], on *mirr* expression (Goff et al., 2001).

Ultimately, however, the most relevant outcome of the interactions between Dpp and EGF signalling lies in the localization of the transcription factor Br to the appendage primordia. It has recently been shown that the *br* expression pattern is driven by two regulatory elements: *brE* ('early') and *brL* ('late'). *brE* drives early ubiquitous *br* expression, and down-regulates *br* in the dorsal-anterior epithelium starting at stage 10A. By contrast, *brL* drives the expression of *br* in the two anterior dorsolateral patches corresponding to the appendage primordia (Fuchs et al., 2012). Both elements respond directly to Mirr: *brE* is down-regulated, and *brL* is up-regulated in the dorsal-anterior domain governed by this transcription factor. Additionally, *brL* is repressed by Pnt in the midline, and by Dpp signalling in the anterior-most cell rows, which generates the characteristic lateral-anterior patches that specify the appendage roof cells (Yakoby et al., 2008b; Fuchs et al., 2012).

1.4.3 Demarcation of domains via local interaction

In the patterning of the follicular epithelium, a clear separation between operculum identity and the appendage primordia has been proven crucial in the proper development of both structures (Dobens and Raftery, 2000). The boundary be-

[2]Some debate exists over the precise role of the Dpp pathway in *br* expression, but this is beyond the scope of this introduction. However, this issue will be addressed in detail in chapters 2 and 4.

tween operculum and appendage is defined by the expression of *bunched* (*bun*), a homolog of the mammalian transcription factor TSC-22. Expression of *bun* depends on EGFr activation, while high levels of Dpp signalling repress *bun* (Dobens et al., 1997, 2000). In turn, Bun is necessary to repress operculum fate (Dobens et al., 2000).

The mechanism through which Bun carries out this repression depends on the Notch signalling pathway. Elevated Notch signalling during stage 10 of oogenesis is associated with the centripetal movement of follicle cells belonging to the future operculum (Dobens et al., 2000). Notch is a membrane-bound receptor responding to membrane-bound ligands in adjacent cells, and activity of the pathway in follicle cells up-regulates expression of *notch*, as well as its ligand genes *delta* and *serrate*. Bun antagonizes Notch activity, likely by repressing *ser* in a cell-autonomous fashion, down-regulating Notch signalling in adjacent cells (Dobens et al., 2005). Bun is thus responsible for restricting operculum fate and centripetal migration to the anterior-most cells, and maintaining columnar cell fate in the future appendage primordia.

Interestingly, Notch signalling has also been shown to be responsible for the coordination between floor and roof cells. Cells at the floor-roof boundary with high Notch levels express the floor marker *rho*, whereas cells with lower Notch express *br* (Ward and Berg, 2005; Ward et al., 2006). Notch is necessary to maintain the boundary between the two cell types: here, the absence of Notch leads to ectopic expression of *br*, at the expense of *rho* (Ward et al., 2006). More specifically, however, Notch signalling appears to be responsible for the single row of *rho* expressing cells bordering the Br domain. Clonal analysis reveals that ectopic *rho* expression occurs in those cells neighbouring a Notch null clone, though only in regions that were not too distant from the wildtype *rho* hinge (Ward et al., 2006). The precise mechanism for this regulation has not been elucidated.

Finally, reciprocal inhibition between Mirr and Bun after the initiation of *mirr* expression may be responsible for the stable demarcation of the posterior border of the appendage primordia. After establishing the border between CMFC and columnar follicle cells, *bun* expression is down-regulated in the dorsal-anterior follicle cells, and its expression pattern now borders exactly on the *mirr* domain (Leonard Dobens, pers. comm., Raftery and Dobens, 2012). While these are preliminary and unpublished results, it is interesting to observe again that the gene *bun* is used in border specification, mimicking its earlier role during the establishment of the anterior border.

While some of the elements of pattern formation discussed here are clearly associated to the future dorsal appendages (like the late expression of *br*), for others this may be less clear. One of the aims of this thesis is to determine

which of the steps of eggshell patterning are part of an ancestral property of the epithelium, and which have been co-opted in the evolution of dorsal appendages. Furthermore, we want to explore at which stage(s) in the hierarchy of pattern formation the foundations are laid for phenotypic variation.

1.5 Varying the eggshell phenotype

As described in the previous sections, there is a wealth of data on the genetic and developmental mechanisms underlying dorsal appendage formation. This makes it an exceptionally suitable system for evolutionary developmental research. In this thesis we will address both the putative origin of this feature by comparing oogenesis between species with and without appendages, but also address the many different phenotypes that exist in dorsal appendage morphology between species of Drosophilidae.

1.5.1 Variation in dorsal appendage number, shape, and size

An extensive variety of eggshell phenotypes exists within the family Drosophilidae (Okada, 1968). *Drosophila melanogaster* belongs to the *Sophophora* subgenus, in which all species studied thus far lay eggs with two appendages, though size and shape may differ. Outside of this subgenus, however, species may be found with one (e.g. *Microdrosophila urashimae*) three (e.g. *D. phalerata*), four (e.g. *D. virilis*), or even up to 12 appendages (e.g. *Chymomyza* sp.). Some appendages are extraordinarily short (e.g. *Chymomyza* sp. and *D. phalerata*), while others can be up to four times as long as the egg (e.g. *Microdrosophila urashimae*). Another feature can be seen at the tip: *D. melanogaster* egg appendages are dilated distally to resemble a paddle, while many species' appendages end in a narrow point (e.g. *D. mojavensis*). Variation in shape and size can also exist between anterior and posterior appendages of species with more than two egg filaments (e.g. *Zaprionus sepsoides*) (Okada, 1968). Finally, some *Chymomyza* species lay eggs that are not entirely symmetrical, and differ in appendage number between left and right (chapter 4).

It has been suggested that the eggs of the last common ancestor of the *Drosophila* genus carried four appendages (Kagesawa et al., 2008). The two-appendage eggshell of *D. melanogaster* and others in the subgenus *Sophophora* would thus be derived phenotype. Interestingly, both in the *Drosophila* and *Zaprionus* subgenera, independent convergent evolution of the two-appendage state can be found (*D. melanica* and *Z. davidi*, respectively) (chapter 4 fig. 4.1). Most research into the developmental basis of variation in oogenesis has focused on the two-versus-four appendage phenotypes, often using *D. virilis* as a model for the four appendage

state (e.g. James and Berg, 2003; Nakamura et al., 2007), though several studies have gone beyond that, and explored epithelial patterning in other species of the *Drosophila* genus (e.g. Kagesawa et al., 2008). This research has demonstrated the limits of the predictive power of gene expression on the follicular epithelium regarding future phenotypes: indeed, while it is possible to distinguish four appendage primordia in stage 12 patterns of Br, earlier (stage 10B, 11) patterns, where the cells of the appendage primordia are already defined, are only partly predictive of the number of appendages on the future eggshell (James and Berg, 2003).

Chapter 4 of this thesis further explores the developmental basis of this variation. A more detailed review of the literature can also be found in the introduction of that chapter.

1.5.2 *Ceratitis capitata*, a tephritid dipteran with appendageless eggs

One of the aims of this thesis is to understand the evolutionary origin of a novel morphology—the dorsal egg appendages. To tackle this, we needed to find an appropriate model species that could serve as an outgroup of the Drosophilidae, and a representative of an ancestral, appendage-less state. The Tephritid fly *Ceratitis capitata*, perhaps better known as the Mediterranean fruitfly (or Medfly), serves our purpose excellently. Not only does this fly indeed lack eggshell appendages, but it is also a model system for ecological research as it constitutes an agricultural pest. The benefits of this status include a genome project (Handler et al., 2012), and the availability and ongoing development of tools for transgenesis (e.g. Loukeris et al., 1995). Not least, the available expertise on *C. capitata* husbandry greatly facilitates the use of this species as a laboratory model.

The last common ancestor for *D. melanogaster* and *C. capitata* was at the base of the Schizophora, a group in the order Diptera that radiated some 65 million years ago (Wiegmann et al., 2011). While after this radiation the Drosophilidae acquired elaborate eggshell structures, the principal elements of the chorion remained unchanged. Chorionic gene clustering and amplification, as well as their sequence identity, are strongly conserved between Drosophilidae and *C. capitata* (Tolias et al., 1990; Konsolaki et al., 1990). While not much is known about oogenesis in this species, the data on the chorion genes, as well as the fact that axis specification during oogenesis appears conserved even far beyond Diptera (Lynch et al., 2010), are good indications for the suitability of *C. capitata* for our intended comparison.

1.6 This thesis: objectives and outline

Much is known about the genetics and the development of drosophila oogenesis. Unfortunately, this subject has only marginally been examined in an evolutionary context. So far, the focus has been on the evolution of axis specification (e.g. Lynch et al., 2010) or variation in appendage phenotypes (e.g. Kagesawa et al., 2008). This thesis aims to establish the eggshell structures themselves as a model for morphological innovation, and explore what it can teach us about both the origin and subsequent diversification of novelty.

It is clear that an intimate link exists between the formation of the dorsal eggshell appendages and important developmental events required for the successful completion of oogenesis. As presented earlier, both the EGF and the Dpp pathways are upstream of both appendage primordia specification, and an array of functions vital for further development, most important of which is the definition of the embryonic main body axes. While it seems likely that the initial signalling events of both pathways are operational elements of oogenesis regardless of the formation of eggshell features, and thus precede this innovation evolutionarily, this has not been confirmed experimentally. Further, it is unclear which elements of the targeted network of transcription factors and other genes that eventually establish the appendage primordia are constituents of an ancestral regulatory network, and which have been co-opted concomitant with the evolution of dorsal appendages.

These questions will be addressed in chapter 2, with a comparison of oogenesis between *C. capitata* and *D. melanogaster*. This is the first step in analysing which developmental elements correlate with the formation of appendages, and understanding the developmental background from which they evolved. The aim of this chapter is also to identify candidate regulators that have played a role in the evolution of the epithelial patterning network.

The relation between polarity and eggshell patterning will be further explored in chapter 3. Here we will briefly introduce a mutation in *D. melanogaster*, which partially uncouples the ancestral feature of dorsoventral axis formation from the novel trait of eggshell patterning. Research on this mutant is in full swing at the moment, and this chapter should not be considered anything but preliminary. However, we chose to include this work despite its preliminary state for the simple reason that the severed connection between novel and ancestral traits in this mutant sheds an interesting light on genetic modularity and developmental robustness. Furthermore, our preliminary results diverge from the transcription factor-centred view of gene networks that is so prominent in current evolutionary developmental biology.

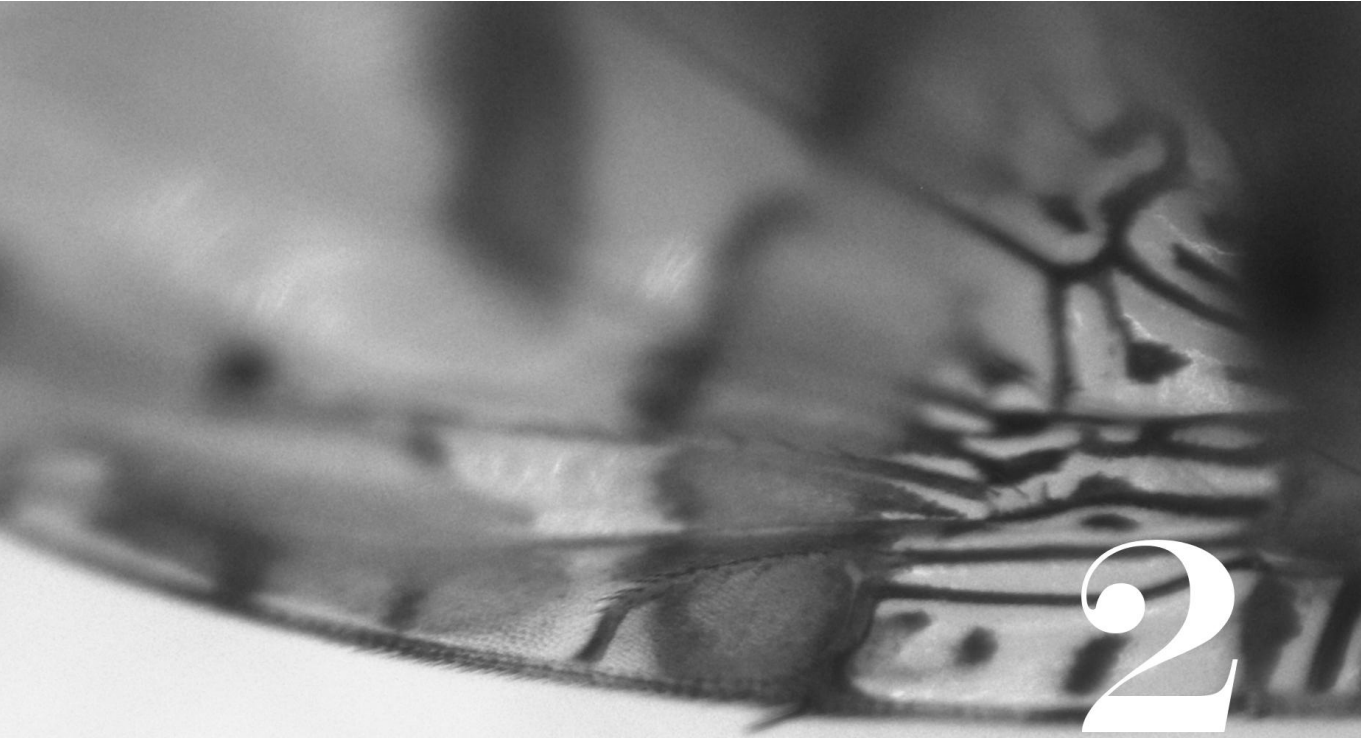
Chapter 4 will deal with the diversity in Drosophilid eggshell phenotypes, and use the combined approach of a computational model and laboratory results to in-

vestigate which part of the patterning network is responsible for variation between species. This chapter will also contain an elaborate review of current computational models of epithelial patterning during oogenesis, as well as a discussion on one specific element of the network: which genes are responsible for determining the posterior border of the appendage primordia?

Finally, in chapter 5, our results, as well as the available information on dorsal appendage formation, will be discussed in the broader context of novelty. Here, we also look at possibilities for future research, and consider further questions that can be asked about evolutionary novelty with the help of this model system.

Acknowledgements

I am extremely grateful to Leonard Dobens and Laurel Raftery for sharing unpublished data and answering many inquisitive e-mails, and to Alex Wild, Darren Obbard, Patrícia Beldade, and Suzanne Saenko for providing the beautiful insect photos used in Fig. 1.1. Thanks also Raquel Santos, Diogo Manoel, and Élio Sucena for reading and commenting on this introduction, and to Matthew Child for editing and proofreading.



2

Co-option of a coordinate system defined by the EGF and Dpp pathways in the evolution of a morphological novelty

Abstract

Morphological innovation is an elusive and fascinating concept in evolutionary biology. A novel structure may open up an array of possibilities for adaptation, and thus is fundamental to the evolution of complex multicellular life. We propose the respiratory appendages on the dorsal-anterior side of the *Drosophila* eggshell as a new model system for morphological novelty. To study the co-option of genetic pathways in the evolution of this novelty we have compared oogenesis and eggshell patterning in *Drosophila melanogaster* with *Ceratitis capitata*, a dipteran whose eggs do not bear dorsal appendages. During the final stages of oogenesis, the appendages are formed by specific groups of cells in the follicular epithelium of the egg chamber. These cells are defined via signalling activity of the Dpp and EGF pathways, and we find that both pathways are active in *C. capitata* oogenesis. The transcription factor gene *mirror* is expressed downstream of EGFr activation in a dorsolateral domain in the *D. melanogaster* egg chamber, but could not be detected during *C. capitata* oogenesis. In *D. melanogaster*, Mirror regulates the expression two important genes: *broad*, which defines the appendage primordia, and *pipe*, involved in embryonic dorsoventral polarity. In *C. capitata*, *broad* remains expressed ubiquitously throughout the follicular epithelium, and is not restricted to the appendage primordia. Interestingly *pipe* expression did not differ between the two species. Our analysis identifies both *broad* and *mirror* as important nodes that have been redeployed in the *Drosophila* egg chamber patterning network in the evolution of this morphologically novel feature. Further, our results show how pre-existing signals can provide an epithelium with a spatial coordinate system, which can be co-opted for novel patterns.

2.1 Introduction

Classically, the concept of evolutionary novelty is that of a new trait, usually an anatomical or morphological one, that opens up the possibility of a wide adaptive radiation into new niches (Mayr, 1960). This definition places its emphasis on adaptation and is thus illustrative of the central role novel traits may have on shaping life on earth. Yet, it is a restrictive definition in that it implies knowledge of the adaptive value of the trait, eliminating traits that have been phylogenetically validated as novelties but lack ecological context.

Moreover, this definition disregards the ontogenic aspects of the new trait particularly of novel morphologies, the most prevalent type of novelty reported. In this light, and in the confined context of this chapter, we will adopt the definition of morphological novelty proposed by Müller and Wagner (1991) that to a great extent circumvents the limitations described above by placing the concept in a more fecund ground for an evo-devo research program: "a morphological novelty is a structure that is neither homologous to any structure in the ancestral species nor homonomous to any other structure of the same organism".

At the mechanistic level, one of the most important contributions of evo-devo to our understanding of the evolutionary process has been the refinement and experimental validation of the gene recruitment concept (co-option). In recent years many examples demonstrate that evolution largely relies on recycling old genes and pathways to generate novel patterns and morphologies (e.g. Brakefield et al., 1996; Moczek and Nagy, 2005). A rewiring of regulatory networks thus seems to be at the core of the dramatic evolutionary changes associated with novelty, and even beyond the novelty concept this has led to an increased effort to understand the evolution of whole networks (Abouheif and Wray, 2002). True network evolution, unfortunately, is difficult to analyse, as such an analysis hinges on the understanding of the network as a whole, and beyond the existence of its separate components. Such knowledge is rare in emerging models of evo-devo, but an operational standard in many classical genetic models like *Drosophila melanogaster* (Sánchez et al., 2008).

The classical model *Drosophila melanogaster* has often been criticized for being extremely derived, and therefore a poor reference in understanding the prototypical insect. Here, we turn this argument around and use *D. melanogaster* as a source of novelty by identifying a novel morphological feature acquired in the evolution of the Drosophilidae family: the egg dorsal appendages (Fig. 2.2B). The formation of these dorsal-anterior chorionic filaments during *Drosophila* oogenesis provides an excellent model system for the study of many developmental mechanisms, such as epithelial patterning (Yakoby et al., 2008a; Lembong et al., 2009), and tube formation (Berg, 2005, 2008; Boyle et al., 2010).

Most eggs of Drosophilidae bear dorsal appendages, which are thought to have a single origin in their last common ancestor (Hinton, 1969). The appendages are hollow tubes protruding from the dorsal-anterior end of the chorion, and facilitate oxygen supply to the immersed egg (Hinton, 1969, 1981). They display a striking diversity within the Drosophilidae family (Okada, 1968; Nakamura and Matsuno, 2003; Kagesawa et al., 2008), which makes them an interesting subject from an evolutionary perspective. The evolutionary advantage of respiratory appendages is emphasized by Hinton (1969): they allow the egg to increase its oxygen-absorbing surface without risking desiccation. Indeed, similar eggshell structures have evolved independently at least 11 more times within Diptera, and at seven more instances in other insects (Hinton, 1969, 1981). Nonetheless, and despite their assumed evolutionary advantage, their phylogenetic mapping across Diptera strongly suggests the independent evolution of these structures in different lineages.

In addition to the dorsal appendages, the *Drosophila* egg carries an operculum and a micropyle: structures relevant for hatching and fertilization, respectively (Fig. 2.2B). These structures are formed during the last stage of oogenesis by designated cells in the follicular epithelium that change shape prior to the deposition of chorionic proteins (Dorman et al., 2004; Berg, 2005). Specification of the appendage primordia occurs chiefly through activity of two main signalling pathways: EGF and Dpp (Peri and Roth, 2000; Berg, 2005).

2.1.1 EGF and Dpp signalling defines appendage primordia

Around stage 8 of *Drosophila* oogenesis, dorsal patterning is initiated when the TGF-α-like ligand Gurken localizes to the dorsal-anterior corner of the oocyte. Gurken associates with the oocyte nucleus, which is pushed by microtubules to a dorsal-anterior position (Zhao et al., 2012), breaking dorsoventral symmetry in the eggchamber (Neuman-Silberberg and Schüpbach, 1993). The Gurken signal then activates EGFr in the adjacent follicle cells, leading (directly and indirectly) to the expression of several transcriptional targets, among which are *mirror* (*mirr*) (Jordan et al., 2000; Zhao et al., 2000), *rhomboid* (*rho*) (Ruohola-Baker et al., 1993), and *pointed* (*pnt*) (Morimoto et al., 1996) (Fig. 2.1).

Meanwhile, Dpp signalling starts at stage 8 with the expression of *dpp* in a subset of anterior follicle cells (Twombly et al., 1996). Dpp protein diffuses to more posterior follicle cells, forming a morphogen gradient. It acts via the receptor Thickveins (Tkv) in the follicular epithelium to phosphorylate Mothers Against Dpp (Mad), activating the pathway in a graded manner (Shravage et al., 2007). Dpp also has been suggested to be required for the expression of *mirr* (Atkey et al., 2006), which starts at stage 10A in a wide dorsoanterior domain (Fig. 2.4A). Recent

work by Fuchs et al. (2012) shows how the transcription factor Mirr, regulated by both Dpp and EGF signalling activity, and the ETS domain transcription factor Pnt, expressed in a more narrow stripe along the midline, subsequently establish two groups of cells expressing *broad* (*br*) through two rounds of signalling. First, Mirr represses *br*, which has been expressed in all follicle cells up to this point, in a wide dorsoanterior region through the *brE* enhancer. Then, *br* expression is upregulated again by Mirr, but repressed by Pnt, through the *brL* enhancer (Fig. 2.1). The two resulting patches of Br-positive cells on either side of the midline are identified as 'roof cells': they will later constrict apically and shape the roof of the appendage tube (Ward and Berg, 2005). Adjacent to the Br-positive patches is a single L-shaped row of cells, bordering the anterior and the central edge of the roof domain. These cells express high levels of *rho*, and elongate directionally to form the floor of the tube (Ward and Berg, 2005). *rho* expression is regulated mainly by activation of the EGF pathway, which is highly dynamic throughout oogenesis, and shows the same L-shaped pattern at the definition of the floor cells (Nakamura et al., 2007). Rho itself is involved in the dynamic EGFr activation as it cleaves the EGFr ligand Spitz (Spi) into its active form, thereby providing a positive feedback loop for EGF signalling (Ruohola-Baker et al., 1993; Wasserman and Freeman, 1998; Urban et al., 2001) (Fig. 2.1).

Importantly, EGF signalling also determines the dorsoventral axis of the future embryo (Queenan et al., 1997). Via Mirr, *pipe* (*pip*) expression is restricted to the ventral side of the egg chamber (Fig. 2.1), leaving an asymmetric distribution of Pip protein in the perivitelline space at the end of oogenesis (Peri et al., 2002; Technau et al., 2011; Andreu et al., 2012; Fuchs et al., 2012). Pip is upstream of a proteolytic cascade in the embryo, leading to the well-known gradient of nuclear Dorsal that regulates the germ layers of the early embryo (Moussian and Roth, 2005).

Dpp, too, is required for processes other than the specification of the appendage primordia. As the inward movement of the centripetally migrating follicle cells starts (Fig. 2.2A), *dpp* is expressed in the leading edge of these cells, and disrupted Dpp signalling has been associated with defects in this migration (Twombly et al., 1996). Dpp is required furthermore for the formation of the operculum (Twombly et al., 1996; Dobens et al., 2000).

In summary, EGF and Dpp signalling activity specify dorsoventral and anteroposterior polarity in the epithelium, respectively, and their signalling information is integrated by Br and Rho, which together specify the appendage primordia. In addition, both signalling pathways are crucial for proper egg formation and further embryonic development, linking the formation of secondary (novel) structures to essential (thus presumably ancestral) developmental events.

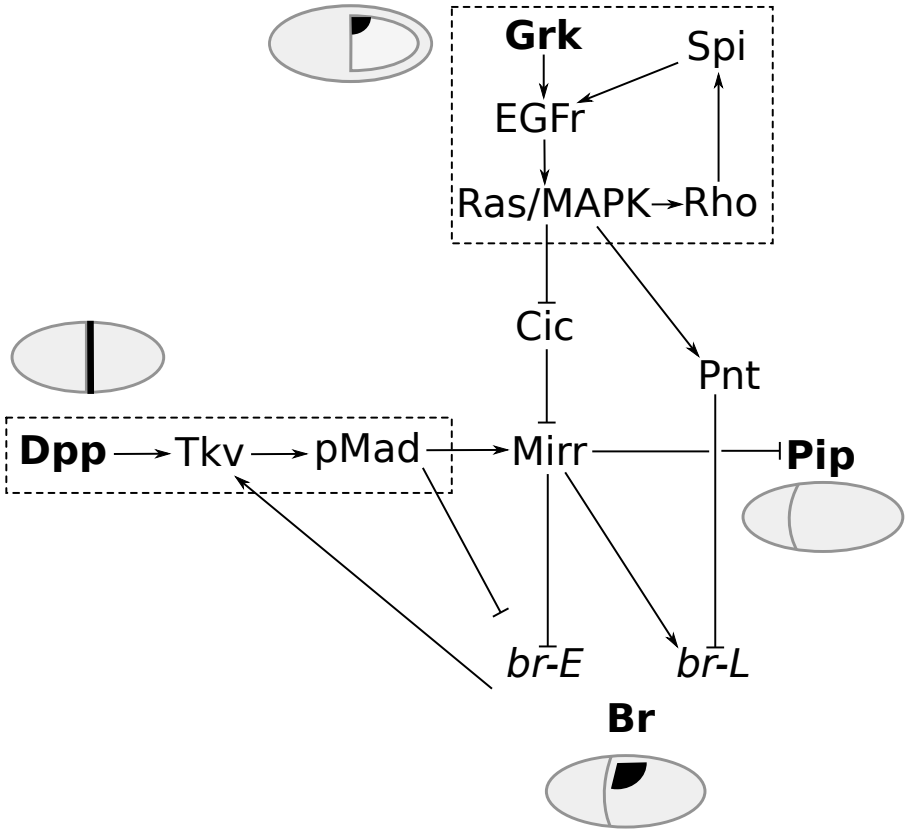


Figure 2.1: **A simplified representation of the genetic network underlying dorsoventral polarity (pip) and DA-formation (br) during *D. melanogaster* oogenesis.** Input comes from two main signalling pathways, EGF and Dpp, providing dorsoventral and anteroposterior information, respectively, and results in the specification of cell domains expressing *pip* and *br*.

2.1.2 *Ceratitis capitata*

Considering the relatively novel acquisition of elaborate eggshell structures, it is interesting to examine the underlying patterning network in the context of a fly species that does not possess these specialized structures. Tephritidae are estimated to be separated by about 65 million years of evolution from Drosophilidae (Wiegmann et al., 2011). For our comparison we chose a Tephritid fly that has been established as a laboratory organism: the Mediterranean fruit fly *Ceratitis capitata*. *C. capitata* is an agricultural pest, which has motivated widespread international research, including a genome project and the development of genetic tools (Loukeris et al., 1995; Zwiebel et al., 1995; Schetelig et al., 2009).

In this study, we have examined both EGF and Dpp signalling as well as their

downstream targets in *C. capitata* oogenesis, in order to understand the ancestral network patterning the follicular epithelium prior to the evolution of dorsal appendages. Determining which genes differ in their behaviour throughout oogenesis of appendage-bearing (*D. melanogaster*) and appendage-less (*C. capitata*) eggshells can help generate hypotheses on the co-option of genes and genetic network changes in the evolution of this novel feature. Our analysis points to a key role for the transcription factor Mirr, both in its regulation as in its transcriptional targets. Furthermore, the presence of both the EGF and the Dpp pathway in *C. capitata* oogenesis leads us to hypothesize that the positional information that these pathways provide to the ancestral follicular epithelium could have facilitated further downstream patterning required for developing the dorsal appendages.

2.2 Material and Methods

2.2.1 Fly maintenance

Our initial *Ceratitis capitata* culture was kindly (and repeatedly) provided by Andrew Jessup (IAEA Seibersdorf, Austria), originating from flies captured in Argentina. Adult flies were maintained on a diet of sugar and hydrolysed yeast protein, and larvae were reared on a mixture of bran, sugar and yeast. All stages were maintained at room temperature. *Drosophila melanogaster* Oregon R. was maintained on regular fly food at room temperature.

2.2.2 Cloning

Gene-specific sequences were isolated from *C. capitata* cDNA by PCR using degenerate primers (for *dpp*, *mirr*, *slbo*, *tkv*, and *wind*), as well as *C. capitata* specific primers (for *Cc-br*, *Cc-grk*, *Cc-nud*, and *Cc-pip*), designed using contigs from the *C. capitata* genome project kindly provided by the Medfly Whole Genome Sequencing Consortium (Handler et al., 2012). For *Cc-pip* two primer combinations were used, generating two separate probes for *in situ* hybridization. These probes were (1) against the common part of all *pip* isoforms, and (2) against *Cc-pip*-ST2, the homologue of *Dm-pip*-ST2 (isoform A). Corresponding probes were made for the positive controls in *D.melanogaster*.

2.2.3 Immunohistochemistry

Ovaries were dissected in cold PBS and fixed for 20 minutes at room temperature in 4% formaldehyde in PBTx (0.1% Triton-x100 in PBS). After fixation they were washed several times in PBTx-B (1% BSA in PBTx) at room temperature during

one hour. Antibody incubation was done overnight at 4°C. The rabbit anti-pMad antibody was kindly provided by the laboratory of Gines Morata, and was used at a concentration of 1:100 in PBTx-B. The anti-Fasciclin II (1D4) was used at a concentration of 1:50. The antibody was developed by C. Goodman, and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Secondary antibodies (Alexa fluor 488/546 goat-anti-rabbit or anti-mouse IgG (H+L), Molecular Probes) were used at a concentration of 1:2000, overnight at 4°C. Nuclear staining was done with Dapi (used at 1:1000 in PBTx) and Draq5 (Biostatus, used at 1:5000 in PBTx).

2.2.4 In situ hybridization

Ovaries were dissected in cold PBT (0.1% Tween-20 in PBS) and transferred to 4% paraformaldehyde in PBS, where they were fixed overnight. They were subsequently washed in PBS, dehydrated and stored in 100% MeOH at −20°C. The protocol for ISH was taken from Tautz and Pfeifle (1989) and modified for oogenesis. The main change concerned the adjustment of the proteinase K digestion to 10 minutes 50 ug/mL at room temperature.

Positive controls with embryos were done in the same well, thus following the exact same steps, as the ovaries, starting at the pre-hybridization step (i.e. the incubation in hybridization buffer at hybridization temperature). This was done because the proteinase K treatment for ovaries is much harsher than the one we used for embryos (10 minutes 50 ug/mL vs. no proteinase K at all).

2.3 Results

2.3.1 Assessing the suitability of *Ceratitis capitata* for comparison of oogenesis and egg formation

Orientation on the *Drosophila melanogaster* eggshell is simple, owing to the obvious eggshell structures on the dorsal-anterior end. By contrast, and the reason for which this system was chosen, the *C. capitata* eggshell carries no structures that can be identified as homologues of the operculum, or the dorsal appendages (Fig. 2.2B). Still, the egg is not entirely symmetrical, both over the anteroposterior axis and the dorsoventral axis. One end of the chorion shows markedly stronger imprints of (previously present) follicle cells than the other (Fig. 2.2B). Observations during both oogenesis (i.e. the orientation of a late stage egg chamber with chorion) and hatching, confirm that the 'imprinted' end of the egg is anterior. Orientation along the dorsoventral axis of the egg is more problematic. However,

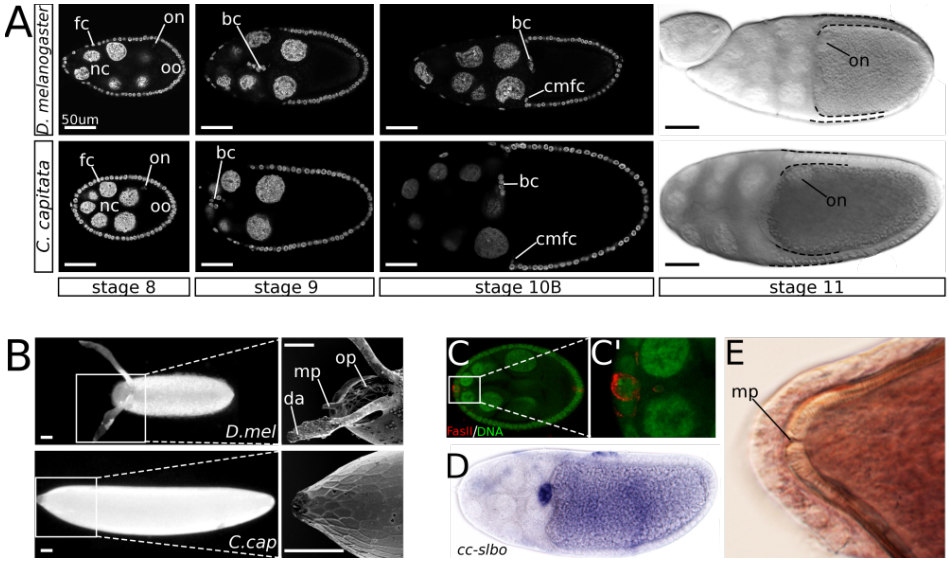


Figure 2.2: **Oogenesis and eggshell phenotypes of *D. melanogaster* and *C. capitata*.** Posterior is always to the right. The scalebar is always 50μm. (A) Stages of oogenesis were identifiable in *C. capitata* using criteria and developmental events described in *D. melanogaster*. At stage 8 of oogenesis, the oocyte nucleus (on) is localized asymmetrically in the oocyte (oo), which at this stage is of roughly equal size to the nurse cells (nc). At stage 9 the follicle cells (fc) start their migration to posterior, the anterior follicle cells stretching themselves over the nurse cells, and the posterior follicle cells forming a layer of columnar cells over the oocyte. At the same time, a cluster of border cells (bc) migrates in between the nurse cells to the anterior end of the oocyte. Late stage 10 sees the columnar follicle cells migrating centripetally (cmfc), in between the nurse cells and oocyte. Stage 11 shows a difference between *D. melanogaster* and *C. capitata* egg chambers in the relative thickness of the dorsal and ventral follicle cell layers. (B) Eggs of *D. melanogaster* and *C. capitata*, the former bearing obvious structures: dorsal appendages (da), operculum (op) and an outward micropyle (mp). (C) Fas-II staining of a stage 8 *C. capitata* egg chamber, identifying the polar cells, part of the border cell cluster. (D) *In situ* hybridization with a probe against *slbo* confirms the identity of the border cell cluster in the *C. capitata* egg chamber. (E) A small pore is visible in the newly formed eggshell of *C. capitata*, very likely a structure homologous to the micropyle (mp).

while we cannot say with absolute certainty which side is dorsal and which is ventral, it is clear that one is more convex than the other. As both late stage egg chambers (Fig. 2.2A,D) and early embryos are clearly more convex at the ventral side, it is a reasonable assumption that the convex side of the egg is ventral.

Aside from the egg morphology, one clear difference between oogenesis in the two species is size: the egg chamber of *C. capitata* is usually larger than the corresponding stage in *D. melanogaster* (Fig. 2.2A). To determine whether this is due to (or results in differences in) the number of cells in the follicular epithelium, we counted the columnar follicle cells in medial cross sections of stage 10 egg chambers of *D. melanogaster* (n=30) and the corresponding stage in *C. capitata* (n=30), as a proxy for the total number of cells in the follicular epithelium. We found no significant difference between the number of cells counted in *D. melanogaster* (52.57 $\pm$2.64) and *C. capitata* (52.83 $\pm$1.86). Thus, it is the size of the cells, not their number, that contributes to the difference in size between the egg chambers of the two species.

Crucially, in order to ensure a constructive comparative analysis of oogenesis between *D. melanogaster* and *C. capitata*, we needed to confirm that both systems are similar at a morphological level, and that they undergo the same morphogenetic changes. Indeed, *C. capitata* ovaries, like those of Drosophilidae, are meroistic polytrophic ovaries. The structure of the egg chambers as well as the progression of stages is nearly identical to that of *Drosophila* (Fig. 2.2A). Starting at mid-oogenesis, we can observe in both species the asymmetric localization of the oocyte nucleus (stage 8), as well as follicle cell migration (stage 9), and centripetal migration (stage 10B). Also visible is the dumping of nurse-cell content into the oocyte, as evidenced by the increasing size of the oocyte relative to the nurse cells, which disappear eventually. All these are important and stage-defining steps in *Drosophila* oogenesis. We will therefore from here on refer to the stages defined in *Drosophila* (Spradling, 1993) when describing *C. capitata* oogenesis.

In addition to the migration of the main body follicle cells, a cluster of anterior follicle cells can be seen to migrate in between the nurse cells at stage 9. Their migration ends at the posterior edge of the nurse cells, adjacent to the oocyte, where they are shortly joined by the centripetally migrating follicle cells. In *D. melanogaster* these cells are known as border cells, and can be identified by the expression of *slbo* (Montell et al., 1992), as well as with the polar-cell-specific label Fasciclin-II. Both markers confirmed the identity of the border cell cluster in *C. capitata* (Fig. 2.2C, D). Interestingly, as the border cells have been associated in *D. melanogaster* with the formation of the micropyle, no obvious external micropyle can be seen on the *C. capitata* egg (Fig. 2.2B). However, upon closer examination of the newly formed eggshell we found a pore-like structure on the anterior side of

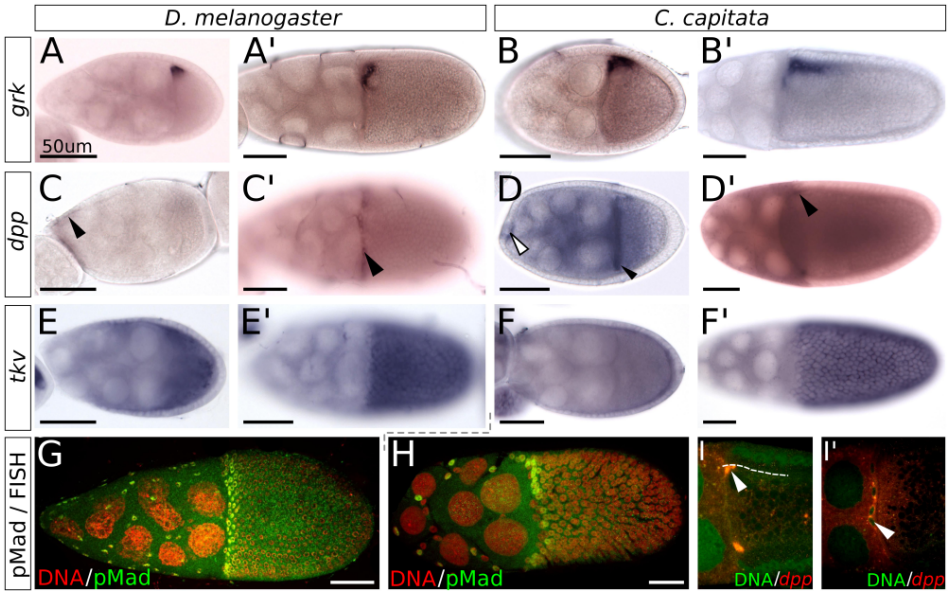


Figure 2.3: **Activity of Dpp and EGF pathways in *D. melanogaster* and *C. capitata* oogenesis.** Images A-F', I-I' are *in situ* hybridizations; G-H are immunostainings. Posterior is to the right, ventral is to the bottom. The scalebar is always 50μm. (A-B′) Transcript for the EGFr ligand **Grk** localizes to the dorsal-anterior corner of the oocyte, both in *D. melanogaster* (A, A′) and *C. capitata* (B, B′) egg chambers. A and B are stage 8; A' and B' are stage 10B-11 egg chambers. (C) Expression of *D. melanogaster* ***dpp*** starts in a subset of anterior follicle cells at stage 8 (arrowhead). (C′) At stage 10A, most of the anterior cells have become stretch cells, and *dpp* expression is only seen in a ring of anterior columnar follicle cells at the border between the nurse cels and oocyte (arrowhead). (D) Expression of *dpp* in *C. capitata* in the border cell cluster (empty arrowhead) of a stage 8 egg chamber, as well as the nurse cells. The transcript localizes anteriorly in the oocyte (black arrowhead). (D′) At stage 10, the transcript localizes at the anterior-outer edge of the oocyte, in a ring underneath the follicle cell layer (arrowhead), see also (I). (E-F′) Expression of ***tkv*** in all follicle cells of stage 9 (E, F) and early stage 10 (E′, F′) egg chambers of *D. melanogaster* and *C. capitata*. (G) In *D. melanogaster* stage 10A, activation of the Dpp pathway, visualized with **pMad**, occurs in the stretched follicle cells overlying the nurse cells, and a few rows of columnar follicle cells. (H) In *C. capitata* stage 10A, pMad shows Dpp activation also in the stretched follicle cells and a row of columnar follicle cells. (I) FISH of *Cc-dpp* in stage 10A of *C. capitata* shows the *dpp* transcript localizing just underneath the follicle cells (arrowhead; border of follicle cells indicated with dashed line), in a ring in the oocyte. (I′) FISH of *Cc-dpp* in a stage 11 *C. capitata* egg chamber shows expression in migrated follicle cells between the nurse cells and oocyte (arrowhead).

the eggshell, likely homologous to the micropyle pore (Fig. 2.2E). This is consistent with the observed border cell localization in *C. capitata*, as these cells are known to form the pore of the micropyle, but not the outwardly visible structure (Spradling, 1993).

2.3.2 Both EGF and Dpp pathways are active in *C. capitata* oogenesis

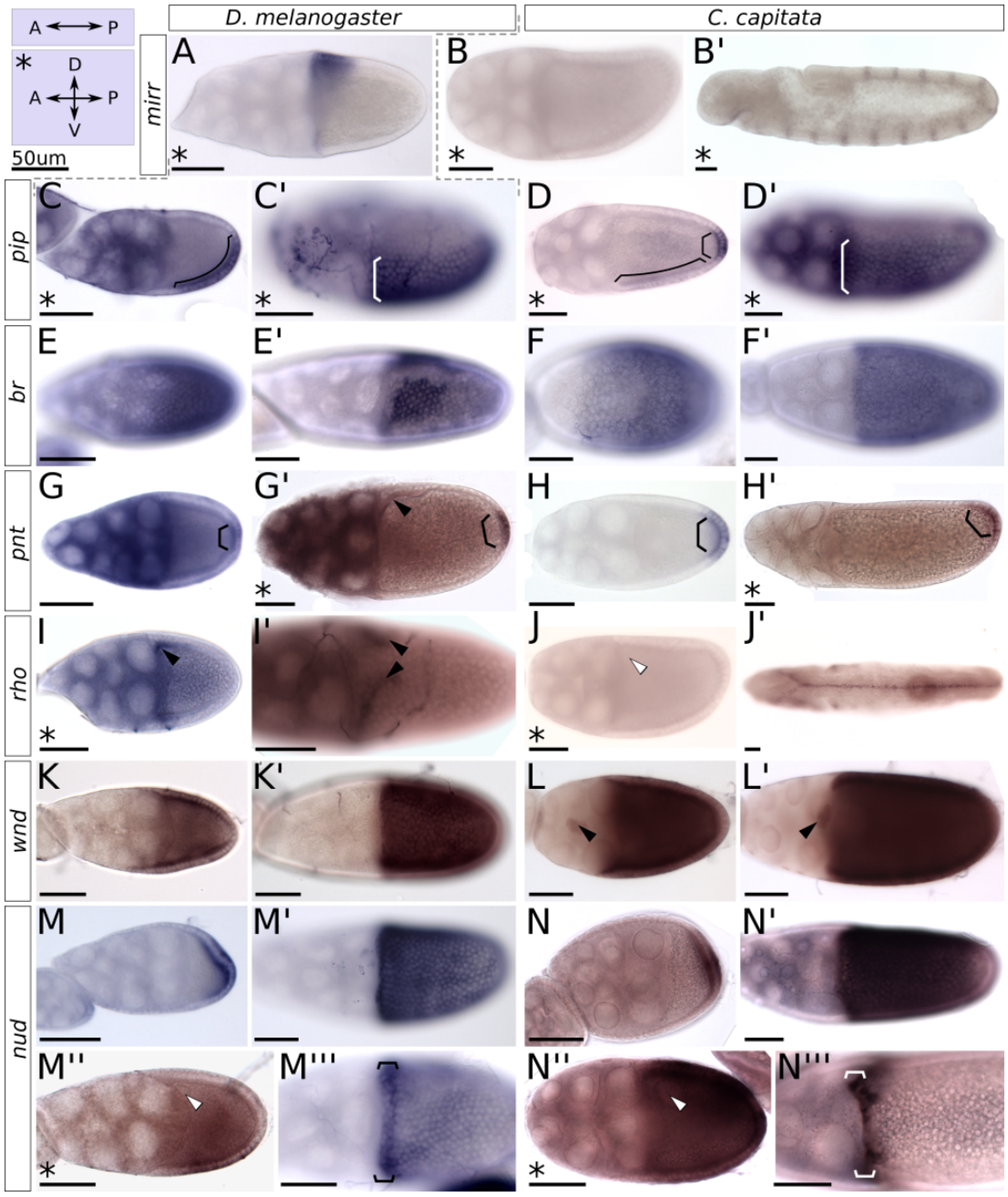
The initial activation of the dorsoventral patterning cascade in *D. melanogaster* oogenesis occurs through asymmetric localization of the ligand Gurken in the oocyte. When staining for the *grk* transcript in *C. capitata*, we found no difference in localization of the mRNA between the two species. In early stages of oogenesis in both species, the *grk* transcript is visible in the oocyte at the anterior cortex. Around stage 8 the pattern becomes restricted to the putative dorsoanterior side of the oocyte (Fig. 2.3A, B). The transcript disappears around stage 11.

While we were unable to obtain patterns of EGFr activation in *C. capitata* because of practical difficulties, it is unlikely that the activation of EGFr in the dorsal follicle cells is different in *C. capitata*: TGFα-EGF signalling is conserved in insects as distant as *Tribolium* and *Gryllus* (Lynch et al., 2010), functioning upstream of embryonic dorsoventral patterning even in drastically different systems of oogenesis. Indeed, we observed the dorsal repression of a known target of EGF signalling in *D. melanogaster*: the gene *pip* (Fig. 2.4D).

While EGF signalling is similar between the species, some differences were found in the Dpp pathway. In contrast with oogenesis in *D. melanogaster*, *Cc-dpp* is not expressed in the somatic follicle cells, but in the germline. Expression of *Cc-dpp* is first visible in the germarium. Once the eggchamber is formed, the *dpp* transcript is localized to the oocyte. When the oocyte increases in size, the mRNA accumulates at the anterior end of the oocyte, in a ring around the edge, adjacent to the follicle cells (Fig. 2.3D, I). Interestingly, this ring is reminiscent of the *D. melanogaster* pattern, where *dpp* is expressed in the stretched follicle cells as well as a few anterior rows of columnar follicle cells, resulting in a similar ring of *dpp* expression around the anterior end of the oocyte (Fig. 2.3C′). The main difference, of course, is that the transcript is located in different cell types.

One exception to the exclusive germline expression of *Cc-dpp* is the border cell cluster. This migrating group of anterior follicle cells is not known to express *dpp* in *D. melanogaster*, but is the only group of somatic cells during oogenesis to express *Cc-dpp*. Expression is visible around stage 8, when the cell cluster is defined (Fig. 2.3D, empty arrowhead), and persists through migration until the edge of the nurse cells is reached.

Another group of *Cc-dpp* expressing follicle cells was identified using fluorescent


D. melanogaster
C. capitata
A
P
D
V
50um
mirr
pip
br
pnt
rho
wnd
nud
A
B
B'
C
C'
D
D'
E
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F
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Figure 2.4 *(preceding page)*: **Expression of *mirr*, *pip*, *br*, *pnt*, *rho*, *wnd*, and *nud* in *D. melanogaster* and *C. capitata* oogenesis.** All images are *in situ* hybridizations; posterior is always to the right, and in the images marked with an asterisk, ventral is to the bottom. The scalebar is always 50μm. (A) ***mirr*** expression in a stage 10 egg chamber of *D. melanogaster*. (B) *mirr* expression in a stage 10 egg chamber of *C. capitata* could not be detected. (B′) A positive control for the *mirr* probe in a *C. capitata* embryo. (C) Expression of ***pip*** in a stage 9 egg chamber of *D. melanogaster*. *pip* is expressed in equal strength in ventral and posterior follicle cells. (C′) Stage 10B shows the final stabilized *pip* pattern. (D) Expression of *pip* in a stage 10A egg chamber of *C. capitata*. Two domains are visible: a ventral domain with weak expression, and a posterior domain with stronger expression. (D′) At stage 10B the pattern has stabilized and shows the same sharp on-off boundary between cells expressing and not-expressing *pip* as is seen in (C′). (E) Expression of ***br*** is visible in all follicle cells of the *D. melanogaster* stage 9 egg chamber. (E′) Stage 10B shows *br* expressed in the roof cells of the appendage primordia. (F) In *C. capitata*, a stage 9 egg chamber also shows all cells expressing *br*. (F′) A *C. capitata* stage 10B egg chamber shows how all follicle cells continue expressing *br*, and a pattern such as in *D. melanogaster* (E′) is not formed. (G) Expression of ***pnt*** in the *D. melanogaster* follicular epithelium is restricted to a posterior domain at stage 9. (G′) A stage 10B egg chamber, in which a dorsal expression domain (black arrowhead) can also be seen. (H) Expression of *pnt* in a stage 9 egg chamber of *C. capitata* shows the same posterior domain. (H′) A stage 11 egg chamber, where the additional dorsal domain seen in (G′) could not be detected. (I) Expression of ***rho*** in a stage 10A *D. melanogaster* egg chamber can be seen in a dorsal domain (black arrowhead). (I′) The late *rho* pattern, here in a stage 10B egg chamber, consists of two distinct hinges (black arrowheads) on the dorsal anterior end of the epithelium. (J) No *rho* expression could be seen in *C. capitata* egg chambers (white arrowhead marks the nucleus). (J′) A positive control for the *rho* probe in a *C. capitata* embryo (ventral view). (K) ***wnd*** expression in a stage 9 egg chamber of *D. melanogaster* can be seen uniformly throughout the follicular epithelium, also in a (K′) stage 10B egg chamber. (L) In *C. capitata*, the same uniform expression of *wnd* can be seen in the follicular epithelium of a stage 9 egg chamber, in addition to weak expression in the border cell cluster (black arrowhead). (L′) This pattern remains in stage 10B. (M) Expression of ***nud*** in *D. melanogaster* starts at stage 9 in a dorsal domain. (M′) at stage 10B, all columnar follicle cells can be seen expressing *nud*. (M″) Asymmetry in the expression pattern at stage 10A shows stronger expression in the ventral follicle cells (nucleus indicated by white arrowhead). (M‴) Anterior columnar follicle cells at stage 11 show higher levels of *nud* expression than more posteriorly located cells. (N) In *C. capitata*, the same early posterior expression can be seen at stage 8, (N′) also resolving into ubiquitous expression in the columnar follicle epithelium at stage 10B. (N″) Some asymmetry can be detected, with dorsal follicle cells expressing higher levels of *nud* than ventral (nucleus indicated by white arrowhead). (N‴) Anterior (centripetally migrating) columnar follicle cells express high levels of *nud* at stage 11, showing a sharp on-off boundary to posterior.

in situ hybridization (FISH): cells centrally located between the nurse cells and oocyte in late stage 11 (Fig. 2.3I′). However, this is a very preliminary result: due to a very small sample size we cannot say with certainty whether these cells are the border cells or part of the follicle cells that have centripetally migrated inwards. As the signal of *Cc-dpp* expression does not persist in the border cell cluster after migration is completed, this observation either indicates a new round of *Cc-dpp* expression in the border cells, or it points to conservation of *dpp* expression in the leading edge of centripetally migrating follicle cells.

While expression of the ligand may differ somewhat between the two species, downstream signalling is remarkably similar. The expression of the homolog of the Dpp pathway type I receptor *tkv* is not visibly different in *C. capitata* from *D. melanogaster*: *Cc-tkv* is expressed in the follicular epithelium (Fig. 2.3I-L), and disappears around stage 11-12. More importantly: activity of the pathway, shown through immunohistochemistry for the phosphorylated form of Mad (pMad), is initially not different between the two species, despite the altered localization of the *dpp* transcript.

The pattern of Dpp pathway activation starts being different between *C. capitata* and *D. melanogaster* around stage 10B-11. At this stage, expression of *Dm-tkv* becomes restricted to the Br-positive cells of the appendage primordia, naturally affecting pMad patterns (Yakoby et al., 2008b; Niepielko et al., 2011). These dynamics were not observed in *C. capitata*, where no Br-positive domains are formed (Fig. 2.4F′).

2.3.3 Patterning of the follicular epithelium downstream of EGF and Dpp

The dynamics of EGF and Dpp signalling, and the subsequent epithelial patterning, are key in defining the appendage primordia in *D. melanogaster* egg chambers. Therefore, identifying the point in the genetic network where *C. capitata* no longer resembles *D. melanogaster* is an important step in understanding the evolution of the dorsal appendages. This will provide a good indication of which genes have been co-opted into the patterning network that lead to a morphological change.

Our first candidate for co-option emerged upon the observation that no expression of *mirr* could be detected in *C. capitata* egg chambers (Fig. 2.4B). A positive control confirmed the proper identity and function of the probe against *Cc-mirr*: it showed clear expression in the *C. capitata* embryo, in a pattern reminiscent of the observed in *D. melanogaster* (Fig. 2.4B′) (McNeill et al., 1997). The same staining was done repeatedly (7 times in total), but never yielded any expression pattern of *mirr* in *C. capitata* ovaries. By contrast, the staining for *mirr* in *D. melanogaster* (and other *Drosophila* species) is extremely easy to achieve.

Mirr regulates the transcription of *br* in the characteristic group of cells that will give rise to the dorsal appendages (Fig. 2.4E′). Unsurprisingly, those *br*-positive groups do not appear on the *C. capitata* stage 10B follicular epithelium (Fig. 2.4F′), or during any other stage of oogenesis. Early expression of *br* could be seen uniformly in the follicular epithelium, as in *D. melanogaster*, but the late expression dynamics were not observed; instead, expression diminishes around stage 11 and has disappeared entirely by stage 12.

Preliminary results indicate that two other genes relevant for *D. melanogaster* epithelial patterning do not play a role in the *C. capitata* dorsal-anterior epithelium: expression of *pnt*, encoding the transcription factor responsible for the midline repression of *br*, could not be detected in the dorsal-anterior follicular epithelium of *C. capitata* (Fig. 2.4H′). A second known expression domain of *pnt* at the posterior pole of the egg chamber was clearly visible from an early stage (stage 8), providing a positive control for the *in situ* hybridization and the *pnt* probe (Fig. 2.4H). Transcription of the gene *rho* was also not detected in either the early dorsoanterior domain, or in the late hinge-shaped patterns adjacent to the *br* expressing domains (Peri and Roth, 2000) (Fig. 2.4I). A positive control in embryos confirmed the functionality of the probe (Fig. 2.4J′). However, as both early *rho* expression and the dorsoanterior domain of *pnt* are difficult to detect in *D. melanogaster* egg chambers as well (Fig. 2.4G,I), we cannot at this stage be completely certain of the absence of these genes in the dorsoanterior follicular epithelium of *C. capitata*.

2.3.4 Dorsoventral polarity

In *D. melanogaster*, the transcription factor Mirr is involved in patterning the dorsal-anterior epithelium, as well as regulating dorsoventral polarity. The absence of detectable *mirr* expression in *C. capitata* begs the question of how the embryonic dorsoventral axis is specified in this species. In *D. melanogaster*, Pipe is upstream of a genetic cascade conveying dorsoventral polarity to the embryo. Expression of the gene *pip* is restricted to ventral follicle cells through dorsal repression by Mirr. Interestingly, this same pattern of *pip* expression was observed in *C. capitata*: the transcript is expressed asymmetrically, and clearly localizes to the ventral follicular epithelium (Fig. 2.4D). Expression of *Cc-pip* starts at stage 8 in follicle cells at the posterior pole of the egg chamber. This posterior expression domain during stage 8 and 9 is well known in *D. melanogaster* (Peri et al., 2002; Andreu et al., 2012). During early stage 10, ventral follicle cells start expressing *Cc-pip* (Fig. 2.4D), and by late stage 10 expression in ventral and posterior follicle cells is of equal strength (Fig. 2.4D′). The pattern at this stage is identical to the expression pattern of *Dm-pip* (Fig. 2.4C′), including the sharp on-off boundary between dorsal and ventral

cells, not expressing and expressing *pip*, respectively. These results were obtained using two separate probes: one against the common part of all *pip* isoforms, and one specific to the homologue of isoform A (or *pipe-ST2*), confirming that the same isoform is used in *C. capitata* oogenesis as is known to function in *D. melanogaster* (Zhang et al., 2009).

Two other genes required for embryonic dorsoventral polarity (so-called "dorsal group" genes) are also expressed in the follicular epithelium: *windbeutel* (*wnd*), and *nudel* (*nud*). The transcript for *wnd* was detected, in both *C. capitata* and *D. melanogaster*, in all follicle cells overlying the oocyte. Expression starts around stage 8, and is last visible at stage 11. Interestingly, the border cell cluster in *C. capitata* also expresses *wnd*, a pattern not seen in *D. melanogaster* (Fig. 2.4K,L). Expression of *nud* in *C. capitata* can be seen from stage 8-11/12 (Fig. 2.4N). In *D. melanogaster*, *nud* is expressed by all follicle cells, though dorsoventral asymmetry in the expression pattern can sometimes be detected. In those egg chambers of *D. melanogaster* where expression was asymmetrical, ventral follicle cells expressed the highest levels of *nud* (Fig. 2.4M″), described also by Hong and Hashimoto (1995). Surprisingly, the asymmetry found in the expression pattern of *Cc-nud* was exactly the opposite: dorsal follicle cells express higher levels of the gene in *C. capitata* (Fig. 2.4N″). At stage 11, *nud* expression becomes largely restricted to anterior columnar follicle cells in both species, though this appears much more tightly regulated in *C. capitata*, where a clear on-off boundary of *nud* expression can be seen around the centripetally migrating follicle cells (Fig. 2.4N‴).

While the distribution of levels of *nud* expression may differ between the two species, all cells in the follicular epithelium express the gene. This is unsurprising, as Nud is an important and stably located component of the vitelline membrane, and the absence of the protein can lead to fragility in the eggshell (Hong and Hashimoto, 1995; LeMosy and Hashimoto, 2000). While Nud is required for the cascade that conveys dorsoventral polarity to the embryo, it is the asymmetric distribution of Pip, not Nud, that has been shown to be crucial for the correct formation of the embryonic Dorsal gradient (Nilson and Schüpbach, 1998). Thus, despite several minor differences in expression patterns, the conserved pattern of stage 10B *pip* expression, as well as the presence of both *nud* and *wnd* transcripts, suggest that indeed the specification of dorsoventral polarity is conserved between the species.

2.4 Discussion

2.4.1 Regulation of *mirr*

One of the most interesting and salient aspects of this model system is the intimate genetic link between the novel phenotype—the dorsal appendages—and an ancestral and vital feature of embryonic development—dorsoventral polarity. One element of the network draws specific attention: the transcription factor Mirror (Mirr). Mirr regulates both the expression of *pipe* (*pip*), the gene encoding a sulfotransferase that is pivotal in providing dorsoventral polarity to the embryo, and *broad* (*br*), the gene that defines the dorsal appendage primordia. Our results show that *pip* expression is conserved, while its upstream regulator Mirr appears to be part of the novel branch of the network in *D. melanogaster*. This observation suggests that Mirr, rather than Br, operates as the key node of the network underlying the evolution of dorsal appendages.

However, it has recently become clear that detectable *mirr* may not fully represent Mirr activity throughout the follicular epithelium. Global de-repression of *mirr* through loss-of-function of the upstream repressor Cic only results in visible expression of *mirr* in anterior follicle cells (Goff et al., 2001). Conversely, local de-repression of *mirr* through follicle cell clones in the posterior part of the epithelium is still sufficient to repress *pip* (Andreu et al., 2012). Therefore, the fact that *mirr* expression is not seen with *in situ* hybridization does not preclude its activity in the follicular epithelium at a level sufficient to repress *pip*. In other words, we cannot conclude that *mirr* expression is absent in *C. capitata* from our data alone.

Although low (undetectable) *mirr* expression may be sufficient in *D. melanogaster* for regulation of *pip*, high (detectable) expression levels are necessary for activating the late enhancer of *br* and defining the dorsal appendage fate (Goff et al., 2001; Atkey et al., 2006; Fuchs et al., 2012). These high levels of *mirr* expression are clearly absent in *C. capitata*, and constitute a novel expression pattern related to the formation of a novel trait. Understanding the regulation of *mirr* in *D. melanogaster* then is necessary to understand how Mirr could have been co-opted to regulate *br*, and possibly *pip*, in a novel manner.

The best substantiated link between EGFr activation and *mirr* expression is the HMG-box transcription factor Capicua (Cic). Cic is a repressor of *mirr* in ventral and lateral follicle cells, and is downregulated in response to EGF signalling (Astigarraga et al., 2007; Ajuria et al., 2011). Cic loss-of-function clones repress *pip* in a cell-autonomous fashion, depending on a Mirr-response site in the regulatory region of *pip* (Andreu et al., 2012). Moreover, *mirr* is ectopically expressed in *cic* mutant egg chambers, though only in anterior follicle cells. This observation

suggests the involvement of Dpp activity in *mirr* regulation (Atkey et al., 2006).

Paradoxically, *pip* expression is unaffected when the Dpp pathway is disrupted (Shravage et al., 2007). Additionally, computational analyses have shown that the two-dimensional EGF signalling profile is sufficient to explain the *pip* expression pattern, without any additional requirements for Dpp or other factors (Goentoro et al., 2006). Expression of *br*, on the other hand, does depend on input from the Dpp pathway (Peri and Roth, 2000; Shravage et al., 2007).

Based on this data and our observations in *C. capitata* we propose a model that separates the contribution of *mirr* to dorsoventral polarity from its function in epithelial patterning, using two regulatory modules in *mirr* to obtain two distinct levels of expression (Fig. 2.5A, B). One of these, responding only to EGFr activation—presumably through Cic down-regulation—is sufficient to generate the low expression levels required to repress *pip* (and likely also act on the early enhancer of *br*). Conversely, the other module requires Dpp signalling in addition to Cic down-regulation, and is able to regulate *mirr* expression to the high levels observed with *in situ* hybridization in the dorsal-anterior follicle cells of wildtype *Drosophila* egg chambers.

With this model we predict that dorsal de-repression of *mirr* through Cic is sufficient for *pip* repression, and constitutes an ancestral signalling cassette linking EGFr activation to embryonic dorsoventral patterning. Due to a non-crossreactive antibody we were unable to confirm whether the localization of Cic in the *C. capitata* follicular epithelium fit our model, but we do note that *cic* mRNA is expressed in the egg chambers (supplementary material, fig. 2.6C, D).

Alternatively, *mirr* expression could be absent in *C. capitata* altogether, and *pip* could be regulated by another transcription factor. However, given the important role of Pip in embryonic dorsoventral axis determination, and the dramatic defects that are caused with minimal variation in factors along the anteroposterior axis (Roth and Schüpbach, 1994; Roth et al., 1999), we consider it likely that *pip* regulation happens through a conserved mechanism, involving *mirr*.

Presenting both modules as enhancers of *mirr* provides us with a hypothesis regarding their evolution (Fig. 2.5C). The predicted '*mirLo*' enhancer is expected to be ancestral, as we base its existence on the *mirr* and *pip* expression patterns in *C. capitata* egg chambers. MirLo would drive *mirr* expression in dorsal follicle cells in a level sufficiently high to repress *pip*, thus regulating dorsoventral polarity of the future embryo, downstream of EGF signalling and independent of Dpp. The appearance of the second enhancer '*mirHi*' would allow *mirr* to start responding to information from the Dpp pathway, and open up the evolutionary road to new patterns on the follicular epithelium.

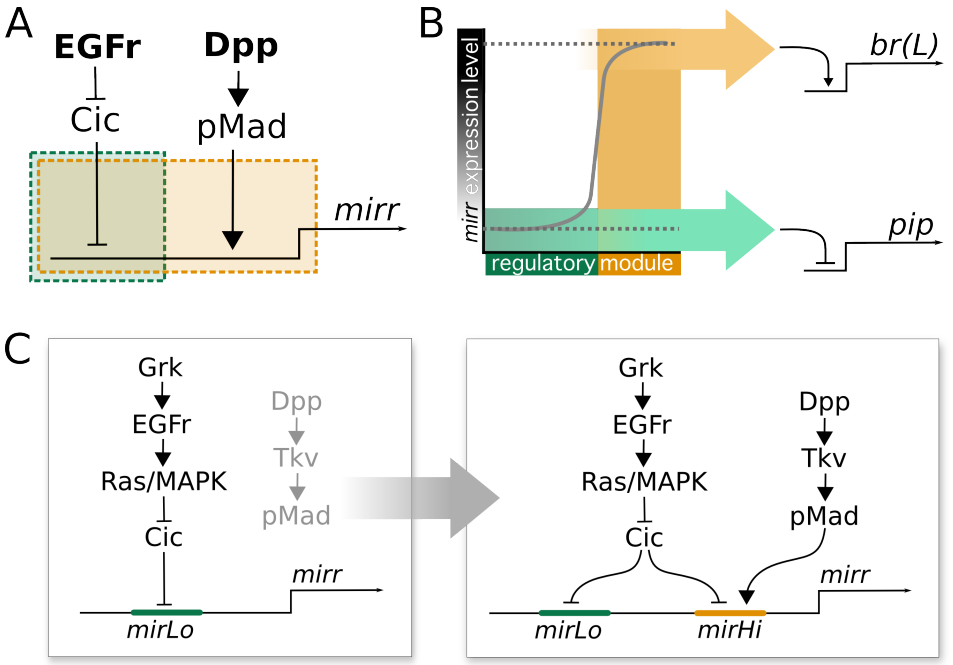

Figure 2.5: **A proposal for the evolution of *mirr* regulation in *D. melanogaster*.** (A) Two separate input modules regulate *mirr* expression: the green module uses only the input of the EGF pathway via Cic, whereas the orange module requires both EGF and Dpp input. (B) The two regulatory modules drive different expression levels of *mirr*. *pip* repression requires only low levels of *mirr*, which are provided through the green module, whereas the *br(L)* enhancer is activated only when *mirr* levels are sufficiently high, which is achieved through the orange module. (C) A proposal for the evolution of two regulatory modules, using two enhancers. A single enhancer (*mirLo*) is responsive to EGF signalling only, and sufficient to provide the low level of *mirr* expression required to repress *pip* as part of an ancestral signalling cassette. A second enhancer (*mirHi*) has evolved in *Drosophila*, which now drives *mirr* expression in response to both EGF and Dpp signalling, in high levels that are sufficient for the activation of the *br(L)* enhancer.

2.4.2 Upstream differences in Dpp signalling between *D. melanogaster* and *C. capitata*

Despite the fact that the Dpp pathway is active in *C. capitata*, in the same or largely the same cells as in *D. melanogaster*, the differences in the underlying expression of its ligand *dpp* are puzzling. Not only are there differences in the expression patterns of *Dm-dpp* and *Cc-dpp*, the transcripts are produced by a different cell type. *Cc-dpp* is likely expressed by the nurse cells and transported to the oocyte, both of which are germline, while *D. melanogaster* requires *dpp* expression in the somatic follicle cells.

Several functions have been described for Dpp signalling in *D. melanogaster* (Martinez Arias and Stewart, 2002). In the context of oogenesis, the need for Dpp signalling in the formation of anterior eggshell structures has been clearly established: *dpp* is expressed in the cells that will form the operculum, and disruptions of Dpp signalling cause misplaced and deformed appendages (Twombly et al., 1996; Deng and Bownes, 1997; Dobens et al., 2005; Chen and Schüpbach, 2006). Additionally, Dpp signalling is needed for the centripetal migration of follicle cells, to maintain structural integrity of the egg chamber, and for dumping of nurse cell content into the oocyte (Twombly et al., 1996). However, expression of the signalling molecule Dpp is only required in the somatic follicular epithelium: in *D. melanogaster* germline *dpp* is not required during oogenesis (Irish and Gelbart, 1987). In *C. capitata*, *dpp* is clearly expressed in the germline, and the signal acts through receptors in the soma. While it cannot be ruled out that the Dpp activity in the follicular epithelium is a response to early *dpp* expression in the border cell cluster, the transcript in the nurse cells as well as the ring of *dpp* in the oocyte are a likely origin for Dpp signalling in the stretched and centripetally migrating follicle cells, respectively (Fig. 2.3D′, I).

Although it is intriguing to observe such apparent dramatic changes in expression patterns, it is important to remember that the functional event, the actual Dpp signal, remains a cooperative act between the ligand and its receptors. Thus, the selective pressure for Dpp function will be on this signalling event, as opposed to the source of the ligand. Interestingly, a similar interaction between germline and soma has been described regarding Dpp signalling in the honeybee *Apis mellifera* (Wilson et al., 2011). In this system, *dpp* mRNA is localized to a dorsal stripe in the oocyte, and signalling activity is observed in the overlying follicle cells. While the absence of data on *dpp* expression in other closely related dipteran species precludes a clear evolutionary interpretation of these patterns, it does suggest that *dpp* expression in the follicle cells is a recent adaptation. A possible reason could be to prevent Dpp from remaining in the perivitelline cleft at the end of oogenesis, which could interfere with future embryonic dorsoventral

patterning in which Dpp plays a large role.

2.4.3 Pre-existing functional signals provide positional information

Our results show that activity of the EGF and Dpp pathways during oogenesis preceded the evolution of dorsal appendages and their underlying epithelial patterns. The ancestral role of EGF signalling lies in determining the dorsoventral axis of the future embryo (Lynch et al., 2010), while Dpp is involved in various cell migrations required for the developmental progression of the egg chamber (Twombly et al., 1996). As a by-product of their original function, the activity from these pathways provides the epithelium with positional information, which in the case of *Drosophila melanogaster* is interpreted by an elaborate genetic network responsible for patterning the epithelium, resulting in the formation of dorsal appendages. Our speculation is that such 'functional pre-patterns' may constitute an important facilitator for novel patterns to evolve, and thus form a crucial foundation for the evolution of novel morphologies.

2.5 Conclusion

In the evolution of dorsal appendages, several genes have been co-opted into a network that originally regulated only dorsoventral polarity, using the input from a second signalling pathway active in the tissue. This co-option event thus redefined not only their roles, but interpreted an ancestral coordinate system for a novel function. The main regulators in this novel genetic network are the transcription factors Mirror, Pointed, and Broad. The latter integrates the information from upstream Mirr and Pnt to specify the appendage primordia, and drives morphogenesis of the appendage (Deng and Bownes, 1997; Fuchs et al., 2012). Interestingly, while all three factors have gained novel expression patterns and interactions to provide the main regulatory information for the epithelial positional cues to be translated into a novel morphology, both *br*, *pnt*, and very likely *mirr* were already expressed in the ancestral non-appendage forming epithelium. In this case it is notable that evolution may have taught "old genes new tricks" (Carroll et al., 2005), within the same broad spatial and developmental context.

Publication

An earlier version of this chapter was accepted for publication in BMC EvoDevo as "Co-option of a coordinate system defined by the EGFr and Dpp pathways in the

evolution of a morphological novelty", authored by Barbara M.I. Vreede, Jeremy A. Lynch, Siegfried Roth, and Élio Sucena.

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Supplementary material

Supplementary material for this chapter consists of (I) Supplemental expression patterns: *in situ* hybridization of of *brinker*, *bunched*, *capicua*, *CF2*, and immunohistochemistry of Notch in *Ceratitis capitata* egg chambers; and (II) analysis and comparison of the *pipe* locus between *C. capitata* and *D. melanogaster*.

Supplementary material to chapter 2

I—Supplementary expression data in *Ceratitis capitata*

The following expression patterns are additional to the dataset presented in chapter 2. As in our current dataset the corresponding images in *Drosophila melanogaster* are missing, these are presented as supplementary data. Nevertheless, these patterns are of interest in the context of the work described. Expression of both *capicua* and *cf2* in the *C. capitata* follicular epithelium may indicate the involvement of the corresponding proteins in the EGF signalling pathway. Furthermore, while *brinker* and *bunched* are required in patterning the dorsal anterior follicular epithelium in *D. melanogaster* (Dobens et al., 2005; Chen and Schüpbach, 2006), their expression during *C. capitata* oogenesis is markedly different—yet it is of interest that they are expressed. Finally, we include this image from a small sample of staining for Notch. Future research should explore this pattern further, but it appears that even in *C. capitata*, Notch may be involved in defining a boundary between CMFC and main body follicle cells. While this is an intriguing result, because of a small sample size it is also very preliminary, and this result deserves proper further investigation before any speculation about its relevance.

II—Map of the *pipe* locus in *Drosophila* and *Ceratitis*

Identifying putative regulatory sequences in the *Cc-pipe* locus

A test for our model presented in chapter 2 (Fig. 2.5) would be to identify Mirr-responsive elements in the *Cc-pip* regulatory region. Unfortunately, the *Cc-pip* regulatory region has not yet been identified, and the genomic sequence of *C. capitata* is incomplete. However, we were able from the available data (Handler et al., 2012) to reconstruct a significant part of the *Cc-pip* locus (Fig. 2.7A). The known *Dm-pip* regulatory element is around 1.5kb upstream of the first exon (Technau et al., 2011; Andreu et al., 2012; Fuchs et al., 2012), and on contig #265915 of the *C. capitata* genome, a stretch of 4kb upstream of the first exon is avaible.

Bioinformatic analysis: GenomeVISTA and MEME/MAST

From the genomic sequence of *D. melanogaster* and *C. capitata* we took a region of 4kb upstream and 3kb downstream of the first exon, and used the GenomeVISTA browser (Frazer et al., 2004) to look for conserved non-coding sequences. However, none were found (Fig. 2.7B). For comparison, the same regions in the genomic sequence of *D. virilis* and *D. pseudoobscura* were also plotted against *D. melanogaster*.

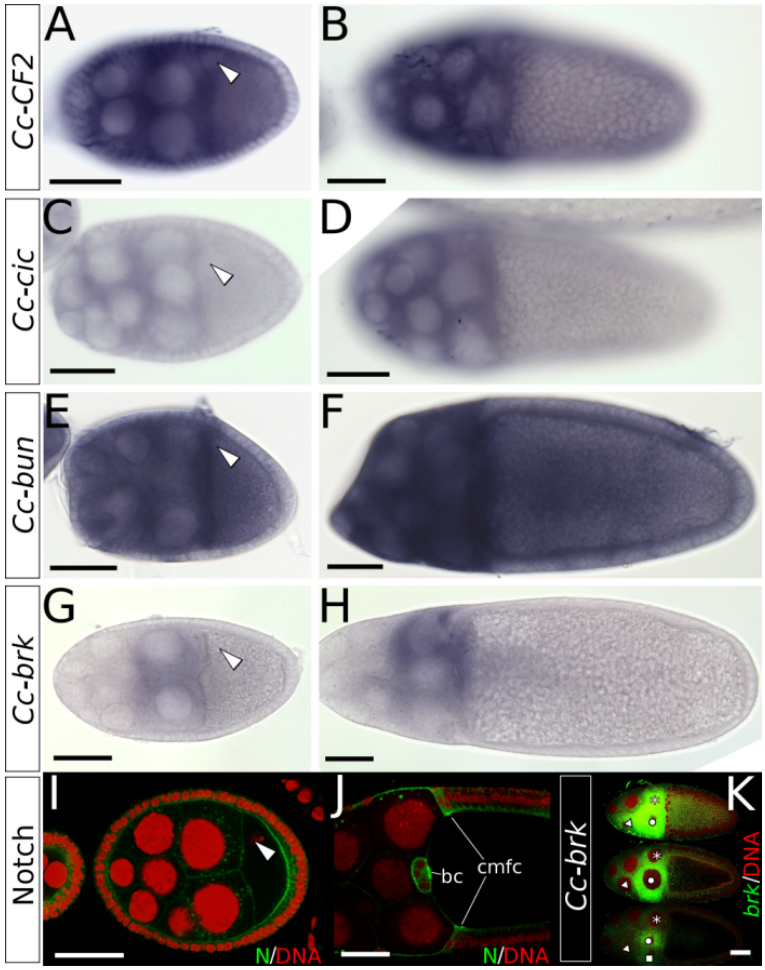


Figure 2.6: **Expression of *CF2*, *cic*, *bun*, *brk*, and Notch in *C. capitata*.** White arrowheads indicate the oocyte nucleus; in images with a marked nucleus (A, C, E, G, I) dorsal is up. Anterior is left in all images, and the scalebar is always 50μm. (A-B) Expression of *CF2* and (C-D) expression of *capicua* is ubiquitous throughout the follicular epithelium of *C. capitata*. (E-F) The *bunched* transcript localizes to the anterior cortex of the oocyte in early and late stages, and is strongly present in the nurse cells. (G-H, K) The *brinker* transcript localizes specifically to a set of nurse cells, posteriorly located in the cluster. (K) The nurse cells expressing *brk* in three optical cross sections of the same egg chamber. The cells have been marked in each section with either an asterisk, a circle, a square, or a triangle, to show which cell is which. Thus, four cells could be counted expressing *brk*. Also, the image shows differences in signal strength of the same cell between the sections, indicating nonuniform subcellular localization of the *brk* transcript. (I-J) Notch protein in *C. capitata* oogenesis. (I) The posterior follicle cells show increased levels of N in a stage 8 egg chamber. (J) In a stage 10a egg chamber, the border cells (bc) and putatively centripetally migrating follicle cells (cmfc) appear marked by increased levels of Notch.

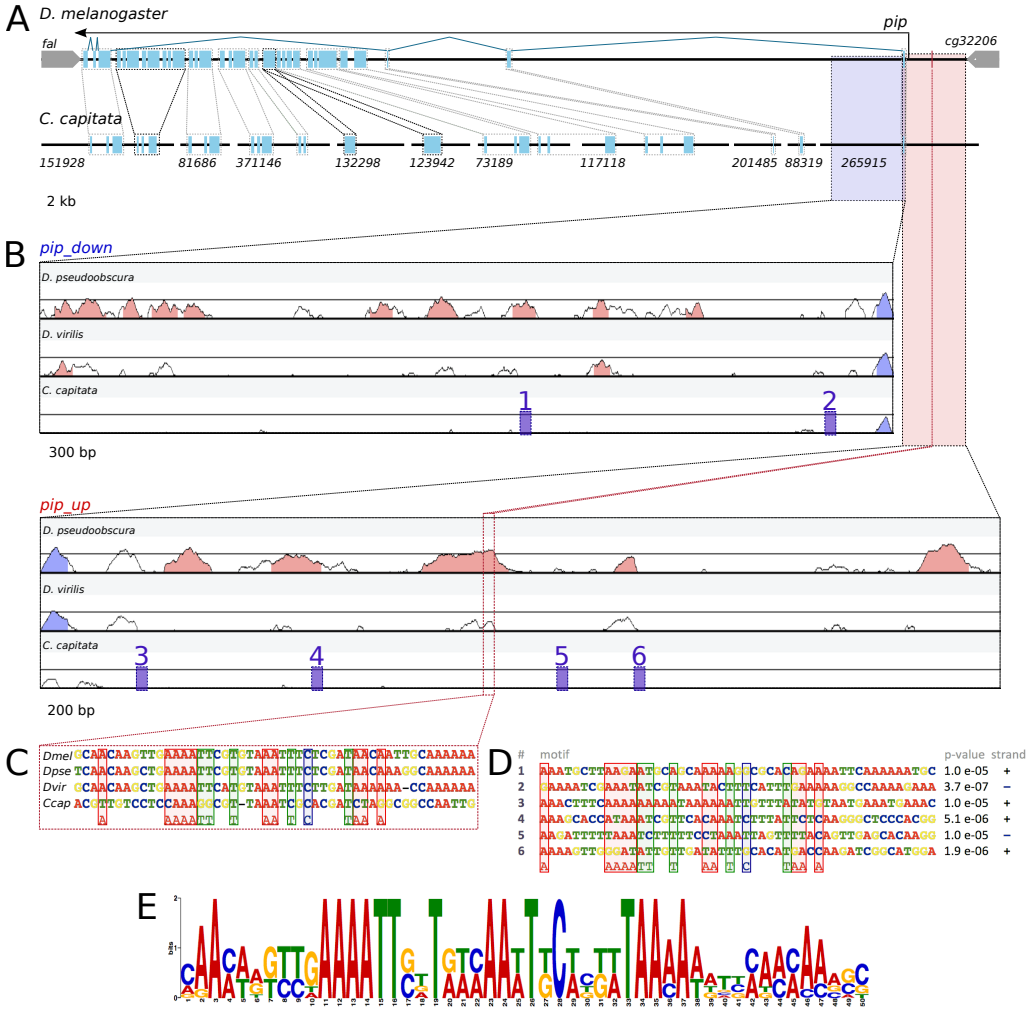


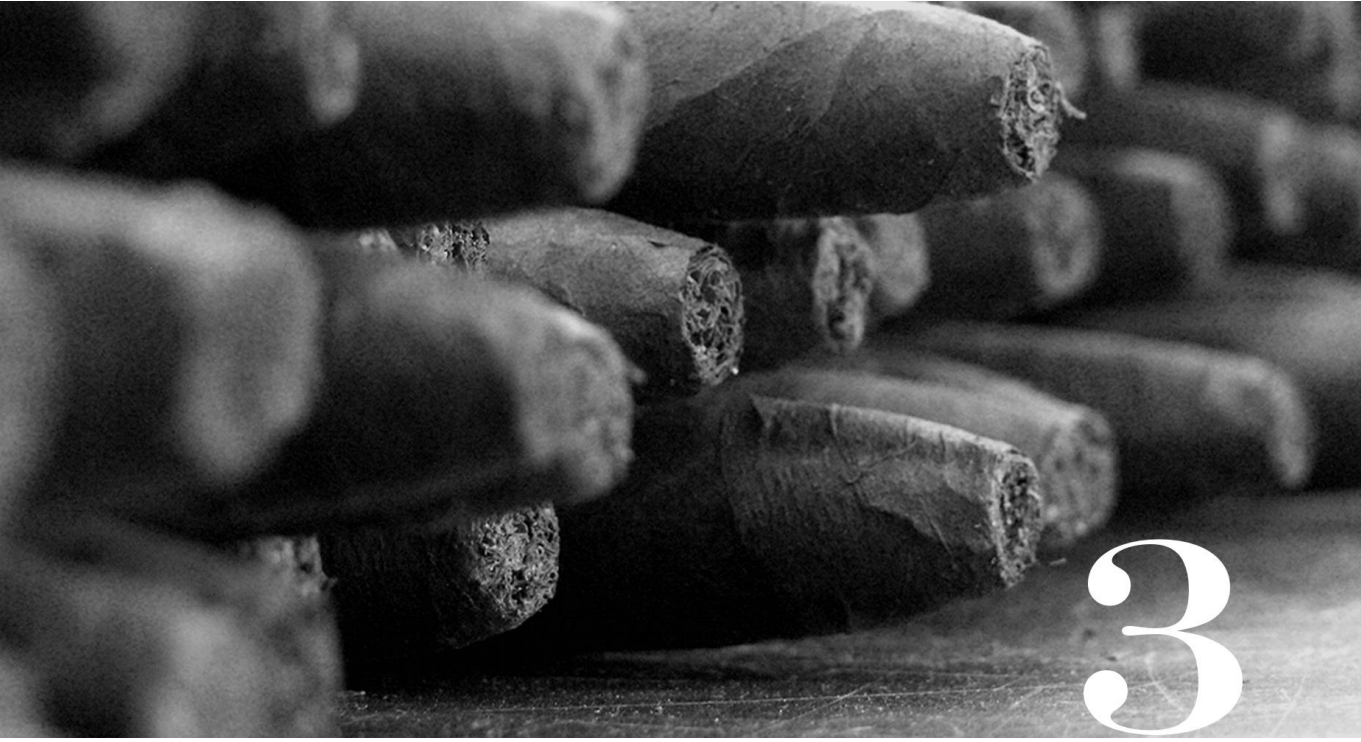
Figure 2.7: **A map of the *pipe* locus in *D. melanogaster* and *C. capitata*.** (A) A reconstruction of the *Cc-pip* locus from a set of contigs that align to *D. melanogaster pip*. Coding sequences (*pipe* exons) are shown in blue. Homologous exons are indicated with grey dotted lines; duplicated exons in one species or the other are indicated with black dotted lines. The splicing pattern indicated on the *D. melanogaster* locus follows the splicing of *pip-ST2*, and this map was used to identify the corresponding exon in *C. capitata*, which was used in the *in situ* hybridizations shown in chapter 2. (B) The GenomeVISTA plots of the non-coding region upstream (*pip_up*) and downstream (*pip_down*) of the first exon. Each track indicates conservation between the species noted, and *D. melanogaster*. Putative MREs found by the MEME/MAST search are numbered 1-6 in the track of *C. capitata*. (C) Alignment of the MRE sequences identified by GenomeVISTA. (D) Alignment of the six putative MREs in *C. capitata* to the conserved basepairs in the MRE position weight matrix. (E) The MRE position weight matrix.

The Mirror-response element (MRE) identified in *D. melanogaster* (Technau et al., 2011; Andreu et al., 2012; Fuchs et al., 2012) is conserved between the three Drosophilids, but probably not in *C. capitata* (Fig. 2.7C). However, the alignment used in Fig. 2.7C was done by GenomeVISTA on the basis of the whole sequence, and it possible that the 50bp long MRE was not identified correctly. Searching specifically for the known Mirror-binding sequence within the region could yield better results.

To do this, we generated a position weight matrix (PWM) for the MRE using the MEME/MAST package (Bailey and Elkan, 1994; Bailey and Gribskov, 1998; Bailey et al., 2009). To replicate the PWM of the MRE published by Fuchs et al. (2012) we used *br* and *pip* enhancer sequences from *D. melanogaster*, *D. pseudoobscura*, *D. virilis*, and *D. mojavensis*. The PWM thus generated (Fig. 2.7E) was used to search for similar domains within the *C. capitata pip*-locus. This way, six elements in the sequence were identified (marked 1-6 in Fig. 2.7B). However, when aligning these to the sequence of conserved nucleotides identified in the MRE PWM, none of them fits entirely (Fig. 2.7D).

Conclusions

It would be premature to conclude from this analysis that no MRE exists within the *Cc-pip* locus. Thus, we are at present generating reporter constructs for transgenesis to *D. melanogaster* with the *C. capitata* non-coding sequences, to determine (1) if they drive expression in a *D. melanogaster* backrgound, and (2) if they do so in a Mirror-responsive manner. In addition, we aim to expand the current sequence dataset, using inverse PCR and other related sequencing methods, in the search for a Mirror-responsive element further upstream.



3

Mutation of *troya* uncouples the polarity of eggshell and embryo

Abstract

Dorsal appendages on the *Drosophila* eggshell are a conspicuous manifestation of polarity on the egg. Importantly, the polarity of the eggshell and the embryo are tightly connected in *Drosophila* oogenesis; controlled to a large extent by the same underlying regulatory mechanisms. Therefore, the identification of mutants that uncouple these phenotypes is an interesting way to explore the connection between ancestral and novel traits. One such mutant was identified in a large germline screen for ventralizing mutants, and given the name *troya*. While mutation of this gene ventralizes the eggshell, some larvae still hatch, indicating that embryonic development was unaffected. Here we present preliminary data on the characterization and mapping of this mutant. We tentatively conclude that the disruption of *try* activity in the germline, not the somatic follicle cells, may be responsible for ventralizing the eggshell and uncoupling embryonic and eggshell polarity. Further characterization and sequencing of the mutation is underway, to open a new research avenue onto the mechanistic basis for eggshell/embryo patterning decoupling in this mutant.

3.1 Introduction

Breaking symmetry constitutes an important aspect of development, and is the first step in differentiation along an axis (Gilbert, 2003). The establishment of the main body axes of an embryo is a crucial manifestation of discontinuity, upon which all future development is based. In *Drosophila*, the anteroposterior and dorsoventral body axes are specified prior to fertilization, during oogenesis (Roth and Schüpbach, 1994). Importantly, not just the oocyte is polarized at this stage: the eggshell, formed around the oocyte, is asymmetrical along these axes as well (Schüpbach, 1987). A very conspicuous indication of this asymmetry is found in the appendages on the dorsal-anterior end of the eggshell (Spradling, 1993).

Eggshell and embryonic polarity share an underlying signal[1]: at stage eight of oogenesis, the TGFα-like ligand Gurken (Grk) localizes asymmetrically in the oocyte, at the future dorsal-anterior end (Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993). Grk signals to EGF receptor (EGFr) in the follicle cells of the overlying epithelium, where the EGF pathway is activated (Nilson and Schüpbach, 1999). Downstream of this signal, the dorsal follicular epithelium is patterned—ultimately generating the conspicuous eggshell structures (Deng and Bownes, 1997)—and proteins crucial for dorsoventral axis formation in the embryo are asymmetrically distributed in the perivitelline space (Stein et al., 1991).

The tight association of embryonic and eggshell polarity has been used to classify mutations that affect embryonic development (Schüpbach, 1987; Schüpbach and Wieschaus, 1989). Moreover, in the research agenda of exploring the evolutionary origin of eggshell structures—so clearly dependent on eggshell polarity—it provides an anchor in the genetic network, as the regulation of embryonic polarity is evolutionarily conserved (chapter 2 of this thesis; Lynch et al., 2010). A first step in this parallel research agenda was performed, employing a previousy performed, maternal screen by Barbosa et al. (2007). This screen specifically targeted the right arm of the *Drosophila melanogaster* chromosome 2, and identified several mutants that affect eggshell polarity.

We were primarily interested in exploring the flexibility of the relationship between the usually synonymous concepts of eggshell and embryonic polarity. Thus, we then re-screened these mutants to determine the viability of the embryos. If the embryonic axes were affected, as the eggshell phenotype from the first screen would suggest, the embryos would not be viable, and the eggs would not hatch. However, if the mutation affected eggshell polarity alone without consequences for the viability of the embryo, these two classically connected traits would be

[1] A more elaborate description of this process can be found in chapter 1 section 1.3.2, on page 14.

effectively uncoupled. From this approach resulted one candidate, a mutation that ventralized the eggshell, but where a percentage of the ventralized eggs were nonetheless viable. This viability indicated that embryonic development occurred successfully, and thus that the initial polarization of the embryo was correct. Still, the mutation had lethal effects later on, and none of the larvae reached adulthood.

As this thesis is being written, a project is underway to characterize and sequence this mutant, called *troya* (*try*), primarily for its involvement in meiotic progression (Barbosa et al., 2007). The current research aims to determine whether *try* is a component of the piRNA pathway working to silence transposable elements, thus conferring genomic stability to the oocyte. However, this is beyond the scope of this chapter, and we will here focus on the effects of *try* mutation on eggshell polarity. At this stage, only preliminary data is available, but it does provide some information regarding where in the network the gene likely acts.

3.2 Material and Methods

3.2.1 Fly stocks

The *troya* alleles try^{A28-21}, try^{A33-36}, try^{B25-61}, try^{B29-56}, try^{B35-19}, try^{B46-47}, and try^{C68-61} were identified through a mutant screen done by Vítor Barbosa et al. (2007). For genetic mapping, all deficiencies used (see Table 3.1) were from the Bloomington Stock Center. The genotypes *y w* FLP^{22} *; If / CyO hs-hid*, *w ;* ovo^{D} */ CyO ml*, and *y w* FLP^{22} *;* FRT 42B *GFP.nls / CyO hs-hid* were also from the Bloomington Stock Center.

All flies were maintained on regular fly food at room temperature. All experiments/crosses were also done at room temperature.

3.2.2 Genetic mapping

Previously, the mutation had been mapped to Df(2R)JP1, which is a deficiency spanning the cytological bands 51C3—52F9 on the right arm of chromosome 2. We used the alleles try^{A33-36} and try^{B25-61} to map the mutation in more detail to the region. For this, we used a collection of deficiencies with breakpoints between 51C3 and 52F9. By crossing them to *try* mutant alleles and examining the progeny, we assessed whether the deficiencies complemented the *try* mutation, and thus did not overlap with *try*. The deficiencies and their breakpoints are listed in Table 3.1.

Deficiency	**Breakpoints**				**Complementation**	
	cytological band		genomic location		try^{A33-36}	try^{B25-61}
Df(2R)BSC330	51D3	51F9	10818780	11237187	yes	yes
Df(2R)Exel7135	51E2	51E11	11017461	11150447	yes	yes
Df(2R)BSC346	51E7	52C2	11105513	11622946	yes	yes
Df(2R)ED2436	51F11	52D11	11260565	11887804	yes	yes
Df(2R)Exel9015	51F11	51F12	11262681	11273829	yes	yes
Df(2R)Exel6285	52A4	52B5	11371023	11563707	yes	yes
Df(2R)Exel9026	52A13	52A13	11456133	11463121	yes	yes
Df(2R)Exel7137	52A13	52C8	11463390/6117	11746753	yes	yes
Df(2R)BSC308	52B5	52D15	11567721	11918784	no	no
Df(2R)BSC482	52C8	52D5	11748787	11838157	yes	yes
Df(2R)Exel7138	52D1	52D12	11805928	11895238	no	no
Df(2R)ED2457	52D11	52E7	11887814	12017662	no	no*
Df(2R)Exel9060	52E11	52F1	12030362	12046356	yes	yes
Df(2R)BSC434	52F6	53A1	12075259	12128184	yes	yes
Df(2R)Exel6063	52F6	53C4	12075393	12274020	yes	yes

Table 3.1: Deficiencies used to map *try*, and the results of crosses with try^{A33-36} and try^{B25-61}. A 'yes' in the field for complementation crosses indicates that the deficiency complements the *try* mutant allele. In the case marked with an asterisk, Cy^+ flies ecloded from the cross, but the females were sterile.

3.2.3 Inducing homozygous germline

Homozygous try^- is lethal; for this reason, and to avoid recombination, all alleles had been balanced over CyO hs-*hid* to maintain mutant lines. To express the mutation specifically in the germline, inducing *try* mutant eggshell phenotype (Barbosa et al., 2007), we made use of a technique designed to produce female germline chimeras, employing the ovo^D mutation, which blocks oogenesis if present (Chou and Perrimon, 1992). To do this, we crossed try^- / CyO hs-*hid* females with males of the genotype *y w* FLP^{22}*;* FRT42B ovo^D */ CyO hs-hid*. Stage 3 larvae resulting from this cross were heatshocked for two hours at $37y^o$ to induce recombination.

3.2.4 Inducing homozygous follicle cell clones

For the mutant clones in the follicular epithelium we employed the FLP/FRT technique, marking homozygous mutant clones with the absence of GFP (Xu and Rubin, 1993). For this, we crossed try^- / CyO hs-*hid* females with males of the genotype *y w* FLP^{22}*;* FRT 42B *GFP.nls / CyO hs-hid*. Stage 3 larvae resulting from this cross were heatshocked for two hours at $37y^o$ to induce recombination.

3.2.5 Immunohistochemistry

Ovaries were dissected in cold PBT and fixed for 20 minutes at room temperature in 4% formaldehyde in PBTx (0.1% triton-x100 in PBS). After fixation they were washed several times in PBTx-B (1% BSA in PBTx) at room temperature during one hour. Antibody incubation was done overnight at 4^{o}C. Anti-Br-core was obtained from Developmental Studies Hybridoma Bank and was used at a concentration of 1:100. Secondary antibodies (Alexa fluor goat-anti-mouse 546) was used at a concentration of 1:2000, overnight at 4^{o}C. Imaging was done on a Leica SP5 confocal microscope. All images were processed using ImageJ (Schneider et al., 2012).

3.3 Results

The *troya* mutant eggshell phenotype was identified in a maternal screen for mutations that disrupt the polarity of the eggshell, targeted to the right arm of the second chromosome (Barbosa et al., 2007). Complementation testing grouped seven alleles together in one complementation group, and they were mapped to a 1.5 Mb region between the cytological bands 51C3—52F9 with the deficiency Df(2R)Jp1. The *try* mutation affects eggshell polarity, but—at least in some cases—not embryonic polarity, effectively uncoupling these tightly connected features in *Drosophila* oogenesis.

3.3.1 Eggshell ventralization manifests in homozygous *try* mutant ovaries to different degrees

To determine to what extent the different alleles affect eggshell polarity and embryonic viability, we assessed the degrees of ventralization in eggs with a $try^{-/-}$ germline, using all alleles available. Ventralization was not consistent: while all eggs appeared to be ventralized to some degree, there was a variety in eggshell phenotypes ranging from two appendages (weak or no ventralization), one appendage (moderate ventralization), or a complete lack of dorsal appendages (strong ventralization) (Fig. 3.1A). We scored the number of eggs in each category for each allele, and observed a variety in the degree of ventralization between the alleles (Fig. 3.1C). Further, we determined viability of the different phenotype categories (Fig. 3.1B), using try^{A33-36} and try^{B25-61}. Although viability differed per allele, the percentage of hatched eggs decreased with the degree of ventralization: the percentage of two-appendage eggs that hatched was 37% in try^{A33-36}, and and 28% in try^{B25-61}. For one-appendage eggs, these percentages were 20% and 4%, respectively. No eggs without appendages were observed to hatch. While the total

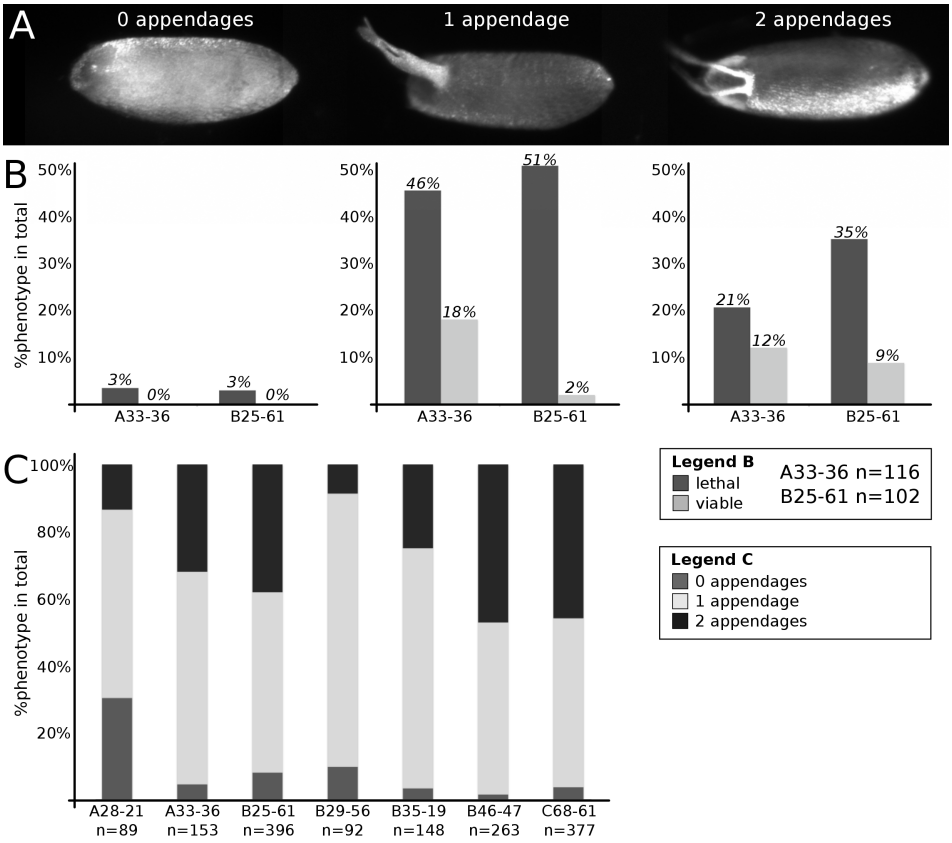


Figure 3.1: **The *troya* mutant eggshell phenotype.** Different degrees of ventralization are observed in eggs from *troya* mutant ovaries. (A) From strong to weak ventralization, the phenotype presents in zero, one, or two appendages; the latter are in the picture more closely spaced than wildtype, though while scoring phenotypes no distinction was made between wildtype-appearing and ventralized. (B) The distribution of the phenotype categories (as seen in A) in eggs of try^{A33-36} and try^{B25-61}, and the rates of hatching per phenotype category. Percentages indicated relate to the total pool of eggs per allele. The viability indicated here only concerns embryonic viability, measured in the successful hatching of the larva. Subsequent lethality was not scored, though none of the larvae survived until adulthood. (C) The distribution of the phenotype categories for all alleles, without taking into account the viability of the embryo.

amount of zero-appendage eggs used here is low, it should be noted that eggs with no appendages were never seen to have hatched: in addition to the data presented here, multiple other egglays were observed, although they were not quantified and categorized.

The data presented in Fig. 3.1C was acquired from the egglays of several independent crosses. Between those experiments, as between the alleles, variation was observed in the degree to which eggshells were ventralized. However, as all work was done at room temperature, and not within a climate chamber with controlled temperature and humidity, we cannot exclude an effect from fluctuations in climate conditions. Otherwise, it is unclear why such variety exists between the phenotype distributions in different *try* alleles.

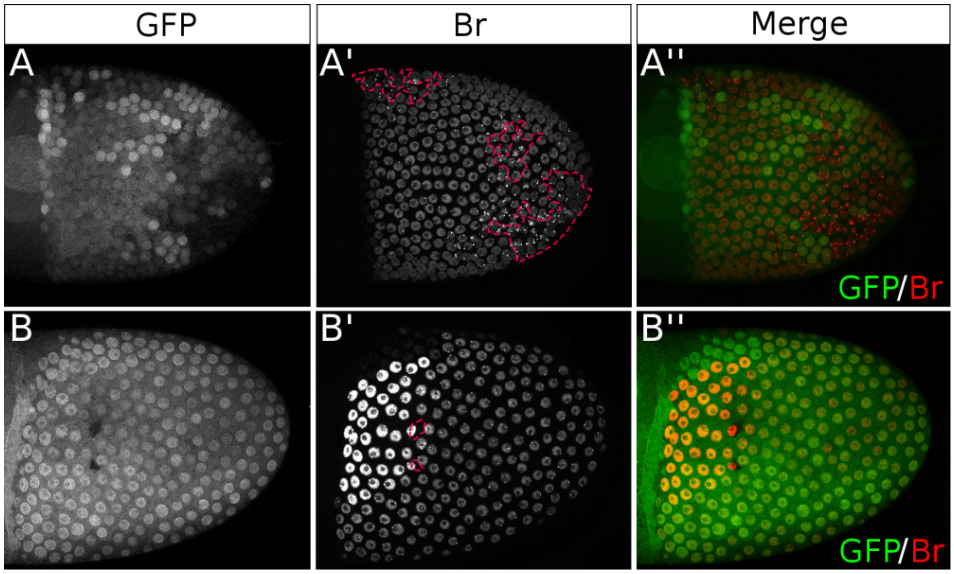


Figure 3.2: **Cell non-autonomous disruption of Br localization in try^{A28-21} follicle cell clones.** Two different egg chambers are shown carrying large (A) and small (B) follicle cell clones homozygous for try^{A28-21}. In (B), Br is visibly upregulated in the appendage primordium. (A-B) show the GFP signal, indicating the boundaries of the clone. (A′-B′) show staining for Br, with the outline of the clone marked in red. (A″-B″) show both Br (in red) and GFP (in green).

3.3.2 Subcellular localization of Br is affected in $try^{-/-}$ follicle cell clones

In contrast with the effect of $try^{-/-}$, on the germline, induced mutant clones on the follicular epithelium had no effect on eggshell phenotype, and a very minor effect on embryonic viability (eggs with induced homozygous follicle cell clones

showed 23% lethality in try^{A33-36} and 16% lethality in try^{B25-61}). However, it is possible that this lethality is a result of induced homozygosity in the germline: the heatshock-induced recombination also targets germline cells. However, when we examined the expression of Br in chambers with follicle cell clones for try^{A28-21}, we observed in some cases that the subcellular localization of Br was affected (Fig. 3.2). This occurred in a non-cell autonomous manner: in egg chambers with clones beyond a certain size, specks of increased intensity in the Br signal were observed in the cells of the clone, as well as cells surrounding them (Fig. 3.2A-A′). The same phenotype was observed in follicle cell clones of try^{B29-56}; other alleles were not yet studied.

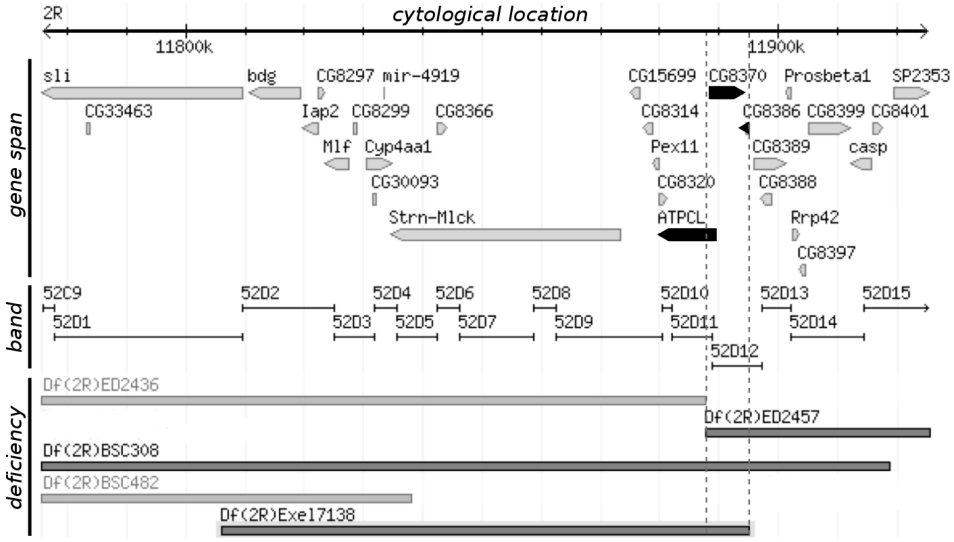


Figure 3.3: ***troya* maps to a 7 kb region containing three predicted gene coding sequences.** Deficiencies that complemented try^{A33-36} and try^{B25-61} are shown in light grey, deficiencies that did not complement the mutations are shown in dark grey. This maps *try* to a 7 kb region containing the entire coding region of CG8370 and CG8386, and part of the coding region of ATPCL (shown in black). The image is a modified and annotated screenshot from Flybase (McQuilton et al., 2012).

3.3.3 *troya* maps to a 7 kb region within 52D11 and 52D12

By fine-mapping two of the *try* alleles to deficiencies covering parts of Df(2R)Jp1, to which *try* was previously mapped, we narrowed the location of *try* down to a 7 kb region around cytogenetic locations 52D11 and 52D12 (Fig. 3.3); specifically, between the right breakpoint of ED2457, and the left breakpoint of Df(2R)Exel7138 (Table 3.1). Two complete genes are predicted within this region: *cg8370* and

cg8386. Furthermore, the region includes the 5′ end of the gene *atpcl*, encoding the enzyme ATP citrate lyase, which produces acetyl-CoA (McQuilton et al., 2012). Not much is known about the other genes: CG8386 is predicted to be a Ubiquitin-fold modifier-conjugating enzyme, and CG8370 is a PapD-like protein of unknown function (McQuilton et al., 2012). We are currently sequencing the entire 7 kb region in all alleles to determine if the coding sequence of either of these proteins is indeed affected by the various *try* mutations.

3.4 Discussion and conclusion

The project characterizing the *troya* mutant is advancing steadily. As much of the data presented is preliminary, here, we will only focus on one aspect of the results that addresses where in oogenesis *try* acts.

Given the concept of a mutant that uncouples the polarity of the eggshell and the embryo, we can speculate about the most likely location of the mutated gene in the genetic hierarchy. A logical guess would be that the mutant disrupts the eggshell polarity network beyond the point where the regulation of embryonic and eggshell polarity diverges. We know that this divergence occurs in the follicular epithelium, beyond the initial signal from Gurken in the oocyte to EGFr in the follicle cells (Nilson and Schüpbach, 1999); thus, any gene that when mutated can uncouple the two features will most likely act in the somatic follicle cells.

We created germline clones that were homozygous for try^{-}, and observed viable larvae hatching from ventralized eggshells. Conversely, we created GFP marked clones in the ovaries (both in the germline and the follicular epithelium), thus also targeting the somatic epithelium to contain cells homozygous for try^{-}. In this cross, while we did not monitor the size of the clones, none of the eggs were ventralized. These results suggest that *try* acts in the germline, rather than in the somatic follicular epithelium.

Importantly, however, these assumptions are based on *try* functioning in a cell-autonomous manner. In fact, our immunostaining for Br in egg chambers with $try^{-/-}$ follicle cell clones showed a cell non-autonomous effect of the mutation on the distribution of the Br protein. This precludes a conclusion that *try* mutation in the germline disrupts events in oogenesis during or prior to the Grk-EGFr signalling event; rather, it opens up the possibility that *try* in the germline could affect the overlying follicular epithelium, and disrupt the establishment of eggshell polarity this way.

In summary: further research is needed to explore the activity of *try*, and the way it affects the connection between embryo and eggshell. Observing Grk localization in the oocyte of germline $try^{-/-}$ clones, as well as the activation of

EGFr in the follicular epithelium, could shed light on whether *try* is involved in this crucial event. Further, the use of markers other than GFP for follicle cell clones, such as the *P[decDN]* marker (Zartman et al., 2009), can be used to explore whether *try* defects in the follicular epithelium affect eggshell patterning. This marker depends on the loss of an eggshell defective gene (Nilson and Schüpbach, 1998), and thus allows the identification of eggs deriving from mutant epithelia.

Contributions

The mutant screen that led to the identification of *troya* was done by Vítor Barbosa (Barbosa et al., 2007). The complementation tests resulting in the complementation group of seven *troya* alleles, as well as the initial genetic mapping onto Df(2R)Jp1, were also done by Vítor Barbosa. All crosses and mapping described here were done by me, with help from Triin Laos, who is currently carrying out the further characterization of this mutant.

Acknowledgements

We are indebted to Vítor Barbosa for giving us the opportunity to work on *troya*, and to Triin Laos for her unwavering assistance. Patrícia Silva helped with crosses, and Raquel Santos shared her expertise on *Drosophila* genetics and oogenesis. Raquel Santos, Triin Laos, and Élio Sucena read and commented on this chapter.

4

Explaining variation in eggshell appendages through anterior-posterior patterning

Abstract

Where the previous chapters dealt with the origin of a novelty, here we explore its variation. We aim to study the contribution of global regulators on the outcome of the eggshell patterning network. Previous work in this area has focused on the contribution of the EGF pathway to diversity in eggshell phenotypes, while the role of the Dpp pathway has remained largely unexplored. We combine a conceptual model of eggshell patterning with experimental analyses of pattern formation in different Drosophilid species, to analyse the contribution of an anterior-posterior patterning gradient on the number of eggshell appendages. Indeed, we find that changes in the patterning input along the anteroposterior axis can contribute in a meaningful way to the divergence of eggshell patterning between species with two and four appendages. To examine whether Dpp can account for variation along the anteroposterior axis, we have looked at patterns of Dpp activity in different species prior to the establishment of the appendage primordia. Indeed, we observe two qualitatively distinct gradients in early Dpp activity in species of different subgenera. However, while these patterns are possibly predictive of the spatial Br pattern in the appendage primordia, we conclude that they do not explain the diversity in appendage numbers. With these results, and a critical look at the present literature, we reassess the role of Dpp in the eggshell patterning network.

4.1 Introduction

Understanding the mechanisms through which variation is generated is an important research theme in evolutionary biology. The study of variation takes place on many levels: from exploring standing genetic variation that allows a population to adapt to a changing environment (Barrett and Schluter, 2008), to examining fixed properties that differ between species (Bateson, 1894). The emergence of a novel property can be the basis for an exploration of phenotypic space, resulting in a display of diversity within this novel property, between individuals, or between species.

The set of respiratory appendages on the eggshells of Drosophilid flies, too, come in many shapes and sizes (Fig. 4.1). Many of the Drosophilid species have four-appendage-bearing eggshells, but numbers up to 12 have been found, as well as secondary loss and single appendages. Additionally, variation exists in shape and size of the appendage, some dramatic phenotypes including a single appendage exceeding the length of the egg (Okada, 1968; Hinton, 1981).

Thus, keeping with the general theme of this thesis, we can apply the available information on *D. melanogaster* oogenesis to explore the generation of diversity in eggshell appendage phenotypes. In fact, several studies have already been done with this agenda. Most of these have focused on the diversity in the number of dorsal appendages, using the four-appendage bearing eggshell of *Drosophila virilis* as a model system (e.g. Nakamura and Matsuno, 2003; Nakamura et al., 2007). To start this chapter, we will review what is known about variation in the patterning network that defines the appendage primordia. This review will build on section 1.4 of the introduction, in which the patterning network has been elaborately discussed.

4.1.1 Variation in eggshell patterning

Grk and the EGF pathway

Much of the study of variation in eggshell patterning has focused on a member of the virilis-repleta radiation, part of the subgenus *Drosophila*, *Drosophila virilis*. Perhaps unsurprisingly, localization of the *grk* transcript does not differ between *D. virilis* and *D. melanogaster* (Peri et al., 1999). This result was confirmed experimentally using pole cell transplantation to generate chimeric egg chambers with *D. virilis* germline and *D. melanogaster* soma. No differences were detected either in the number of appendages formed this way, or the relative spacing of the two appendages, showing that the germline contribution to follicle cell patterning (i.e. Grk localization) is not sufficient to generate variation in appendage phenotype (Nakamura et al., 2007).

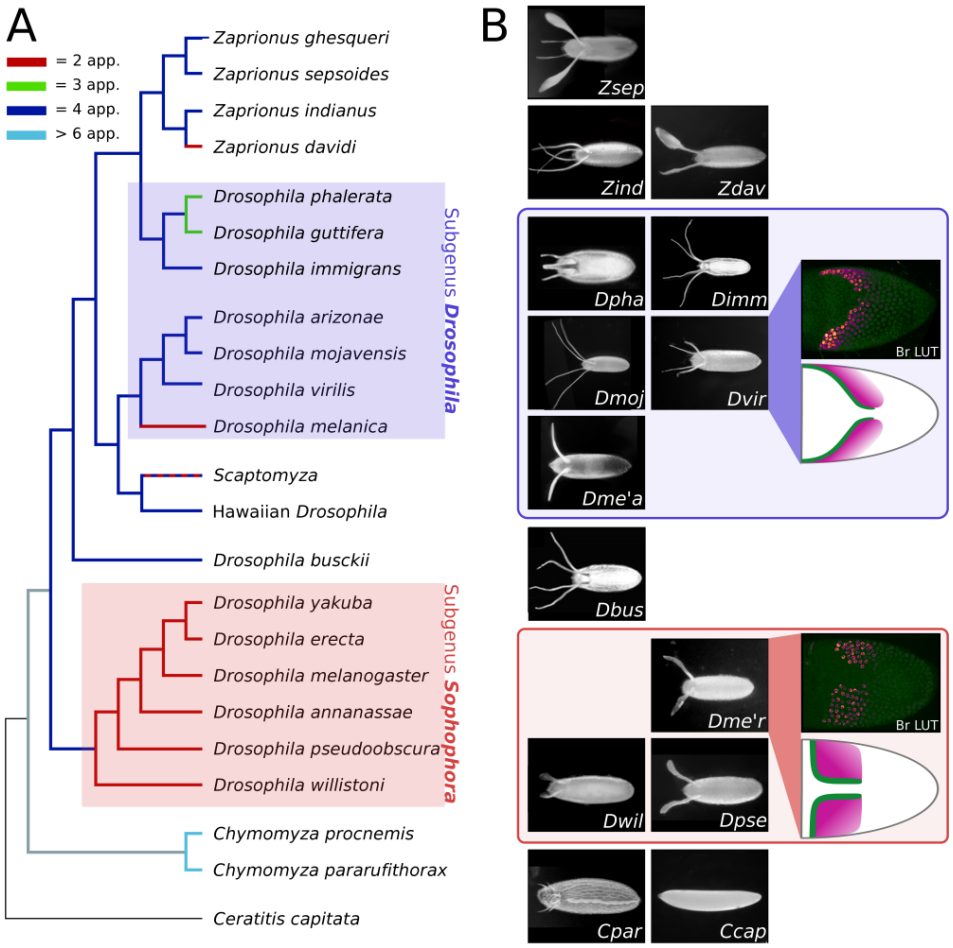


Figure 4.1: **A phylogeny of Drosophilidae demonstrating the diversity in eggshell appendage phenotypes, and their underlying patterns**. (A) A phylogenetic tree of Drosophilidae, including the outgroup species *Ceratitis capitata*. The tree was resolved as a consensus of van der Linde and Houle (2008); O'Grady and Markow (2009); van der Linde et al. (2010); Robe et al. (2010). The line colour indicates the number of dorsal appendages on eggs of the species. (B) Different egg phenotypes of species of Drosophilidae, and *C. capitata*. A diagram of the appendage primordia in members of two subgenera shows the typical V-shape of the primordia in *D. virilis*, and the L-shapes in *D. melanogaster*. The so-called 'roof' is in pink, and the 'floor' is shown in green. Above the cartoon, an immunostaining for Broad in a stage 10B egg chamber shows the roof cells. The colour is a look-up table (LUT), showing a weak signal in green, and strong signals in pink and yellow. The eggshell photos of *D. phalerata*, *D. immigrans*, and *D. busckii* were taken from Kagesawa et al. (2008), without permission.

The dynamics of EGFr activation itself and its relation to phenotypic variation have been described elaborately by Kagesawa et al. (2008). They show that the number of activated EGFr domains at the end of oogenesis (stage 12), as visualized through phosphorylated MAPK, directly correlates with the number of appendages on the future eggshell of 11 different Drosophilid species from three different subgenera (*Drosophila*, *Dorsilopha*, and *Sophophora*). Roughly, phosphorylated MAPK in members of the *Drosophila* subgenus forms a V-shape, while in *Sophophora* the pattern resembles more two L-shaped domains. Interestingly, both the L and the V-shaped domains can eventually resolve to form a two-appendage-bearing eggshell, while four appendages have only been observed in species with a V-shaped activation pattern (Fig. 4.1B).

The genetic network underlying EGFr activation dynamics has been studied in *D. virilis* by Nakamura and Matsuno (2003); Nakamura et al. (2007). Expression of *rho*, itself involved in a positive feedback loop with EGFr activation, closely resembles the pattern of phosphorylated MAPK, as is known from *D. melanogaster* (Nakamura and Matsuno, 2003; Nakamura et al., 2007; Kagesawa et al., 2008). Indeed, the cis-regulatory element driving *rho* expression seems to be largely conserved between *D. melanogaster* and *D. virilis*: reciprocal reporter assays testing enhancer activity of *Dv-rho* and *Dm-rho* have shown that the trans-landscape in both species is largely responsible for the eventual *rho* expression pattern, and diversification depends only minimally on evolution in the *rho* regulatory element (Nakamura et al., 2007).

Diversity of Br patterns and morphogenesis

James and Berg (2003) describe in detail the morphogenetic movement that translates a single patch of Br positive cells into one appendage (in *D. melanogaster*), or two appendages (in *D. virilis*). Their data clearly shows the importance of cell movement and morphogenesis in the formation of two separate appendages from a single Br patch in *D. virilis*: at the patterning stage (stage 10), the Br cells shaping the anterior and posterior appendage are indistinguishable. This change only arises around stage 12, when the anterior patch is separated from the posterior patch by a small number of cells marked by a lower Br level than cells in the two main patches.

Interestingly, in their description of the Br pattern in *D. virilis*, James and Berg (2003) point out the similarities of wildtype *D. virilis* Br and the *br* expression pattern of a *dpp* over-expression mutant published by Deng and Bownes (1997). Indeed, like the *D. virilis* pattern, both the lateral and posterior border of *br* expression have shifted with respect to the wildtype *D. melanogaster* pattern when *dpp* is overexpressed. Moreover, *dpp* over-expression flies have been known to

produce eggs with additional appendages, sometimes as much as six (Deng and Bownes, 1997; Dequier et al., 2001; James and Berg, 2003). In combination with the lateral shift, *dpp* over-expression moves the anterior border of the Br domain anteriorly (Dequier et al., 2001), which now reaches the anteriormost cell rows usually marked by the absence of Br. This, too, is a feature that is observed in the wildtype *D. virilis* Br pattern (James and Berg, 2003) (Fig. 4.1B and 4.4A′).

Dpp patterning diversifies in late stages

The dynamics of Dpp pathway activation have also been compared between Drosophilids. The spatial patterns of Dpp signalling are driven by expression of its receptor, the gene *tkv*, and can be visualized with an antibody against phosphorylated MAD (pMad) (Niepielko et al., 2011, 2012). So far, the focus of these studies has been on late stages, as the Dpp activity patterns during stage 10B and beyond show clear divergence between species. In earlier stages of oogenesis, *tkv* expression is uniform throughout the follicular epithelium (see also chapter 2 of this thesis, fig. 2.3E). However, in *D. melanogaster*, *tkv* expression is under positive regulation of Br (Yakoby et al., 2008b). This results in increased Dpp signalling in the Br positive domains, once these are specified. However, where late pMad and Br domains overlap almost entirely in *D. melanogaster* and *D. erecta* (both species with two eggshell appendages of the subgenus *Sophophora*, see fig. 4.1A), no overlap at all can be seen in the late pMad and Br domains of *D. quinaria* or *D. guttiferra* (species with three appendages of the subgenus *Drosophila*). Partial overlap, when the anterior border of the pMad pattern is anterior to the Br domain, is seen in *D. virilis* (four appendages), as well as *D. buschkii* (four appendages) and *D. tropicalis* (two appendages) (Niepielko et al., 2011, 2012). These species belong to the subgenera *Drosophila*, *Dorsilopha*, and *Sophophora*, respectively, suggesting that the partial overlap of late pMad and Br domains could be the ancestral pattern.

No in-depth studies have yet been done comparing Dpp signalling gradients in early stages, prior to the establishment of the Br domains. Early Dpp activity has only been described as consistent across all species examined (Niepielko et al., 2011), and this observation has not been further explored. Thus, a possible contribution of Dpp signalling to the observed diversity in Br patterns between species has not been investigated to date. However, given the observations of Deng and Bownes (1997) that an increase in the level of Dpp in *D. melanogaster* could produce eggshells with more than two appendages, it is worth taking another look at this data, and assess whether early Dpp activity could be a factor governing divergent eggshell patterning in Drosophilid species.

4.1.2 Modelling pattern formation on the eggshell

Models provide the opportunity to understand the generation of patterns from gene interaction networks. For a long time, EGFr activation was considered the main output of the epithelial patterning network, and consequently the focus of conceptual and computational models in this area (Wasserman and Freeman, 1998; Shvartsman et al., 2002; Goentoro et al., 2006) (Fig. 4.2A). However, the most recent models on the subject centre around the pattern of *br* expression (Yakoby et al., 2008b). This transcription factor had been established as a less dynamic output of the epithelial patterning network than EGFr activation, and is tightly connected with appendage formation (Deng and Bownes, 1997; Tzolovsky et al., 1999; Dorman et al., 2004). Meanwhile, three notable papers have been published modelling Br expression patterns, each building on the previous model: Lembong et al. (2009), Zartman et al. (2011), and Simakov et al. (2012) (Fig. 4.2B, C, D, see also Box 1).

One of these papers applies their model to explain variation between species (Zartman et al., 2011). In the model presented in this paper, EGF signalling governs both the dorsoventral and anteroposterior axis: the early posterior EGFr activity (Gonzalez-Reyes and St Johnston, 1998) is proposed to define the posterior border of an anterior competence zone that is capable of differentiating into appendage primordia. This model is supported by the experimental observation that in epithelia mutant for the EGFr inhibitor Sty the appendage primordia have not only shifted laterally, but are also shorter on the anteroposterior axis. Sty plays an important role in this model, as it is capable of modulating the levels of EGFr activity in both (i.e. posterior and dorsal) signalling events. Simply by manipulating the feedback strength of this inhibitor, it is possible to recapitulate the *br* expression patterns of *D. phalerata* (3 appendages) and *D. virilis* (4 appendages).

Furthermore, in an earlier study (not shown in the figure, but based on the concepts proposed by Wasserman and Freeman (1998)) Shvartsman et al. (2002) modelled a computational exploration of phenotypic space, where variation in the strength of an input signal (Grk), and the width of the Grk domain were related to the generation of stable peaks of activity. As the accepted model at the time equated peaks of EGFr activity with the specification of the appendage primordia, this study directly linked variation in a global input signal with phenotypic diversity.

While the model by Wasserman and Freeman (1998) has meanwhile been replaced by more accurate and detailed representations of eggshell patterning, the method used by Shvartsman et al., as well as the general concept of relating global input signals to variation in phenotypic output, is still applicable.

Box 1: The evolution of models in eggshell patterning

A—Wasserman and Freeman, 1998 Through two steps—a feedforward and a feedback loop with differential thresholds—a two-peak pattern of EGFr activation along a lateral axis is established. This model, proposed by Wasserman and Freeman (1998), is primarily one-dimensional and very elementary, but it was quantified and successfully implemented to account for variation in appendage number (Shvartsman et al., 2002). However, further research into the biological background of the model, namely the EGFr ligands Aos and Spi, and the protease Rho, revealed that these factors could not be fully responsible for defining the appendage primordia (Boisclair Lachance et al., 2009; Zartman et al., 2009).

B—Lembong *et al.*, 2009 Where prior computational attempts centred around diffusion gradients, Lembong et al. (2009) started using a distinct network-focused approach. The main players in this network, downstream of the activated EGFr and Dpp pathways, are the transcription factors Pnt and Brk, as well as the eventual output Br and its encoding gene *br*. The network is adapted from Yakoby et al. (2008b), and all connections are supported by experimental evidence. In this model, still, the calculations are made over a single dimension, but by using a diagonal (Fig. 4.2B), an anterior-posterior component was added. The model resulted in the relative quantification of expression patterns along the diagonal, calculating the dynamics of these patterns from stage 9 through 11.

C—Zartman *et al.*, 2011 As the Lembong et al. (2009) model is one-dimensional, the posterior limit may depend only on the diffusion of the Grk morphogen from a dorsoanterior source. However, a problem immediately arises when it is translated to a two-dimensional surface: the wildtype *br* pattern, with clear limits directly perpendicular to the anteroposterior axis, indicates the need for a posterior boundary other than the dorsal EGFr activation gradient. Zartman et al. (2011) propose early posterior EGF signalling as a factor responsible for the establishment of this boundary. Key in their argument is the mutant phenotype of *sty*, an EGFr inhibitor: not only are the appendage primordia shifted laterally in this mutant, but the posterior border of the primordia has moved to anterior. This indicates that the EGF signalling pathway regulates the pattern on both the dorsoventral and the anteroposterior axes.

D—Simakov *et al.*, 2012 Where the previous two models used and refined proposed patterning networks, the most recent published model has abandoned the existing networks altogether. Instead, Simakov et al. (2012) uses undefined variables in a network composed of juxtacrine and cell-autonomous interactions, responding to two input variables. Only five of the variables are identified; two remain unnamed. This model is the first to employ juxtacrine interactions, which is possible because of the context it uses: a two-dimensional hexagonal grid. The network has been stripped to the essentials, and for example no longer uses the inhibitory loop of EGFr and Sty used by Zartman et al. (2011). It does rely on a posterior signal to restrict patterning to anterior follicle cells, citing Zartman et al. (2011), but does not show the source nor the implementation of this signal.

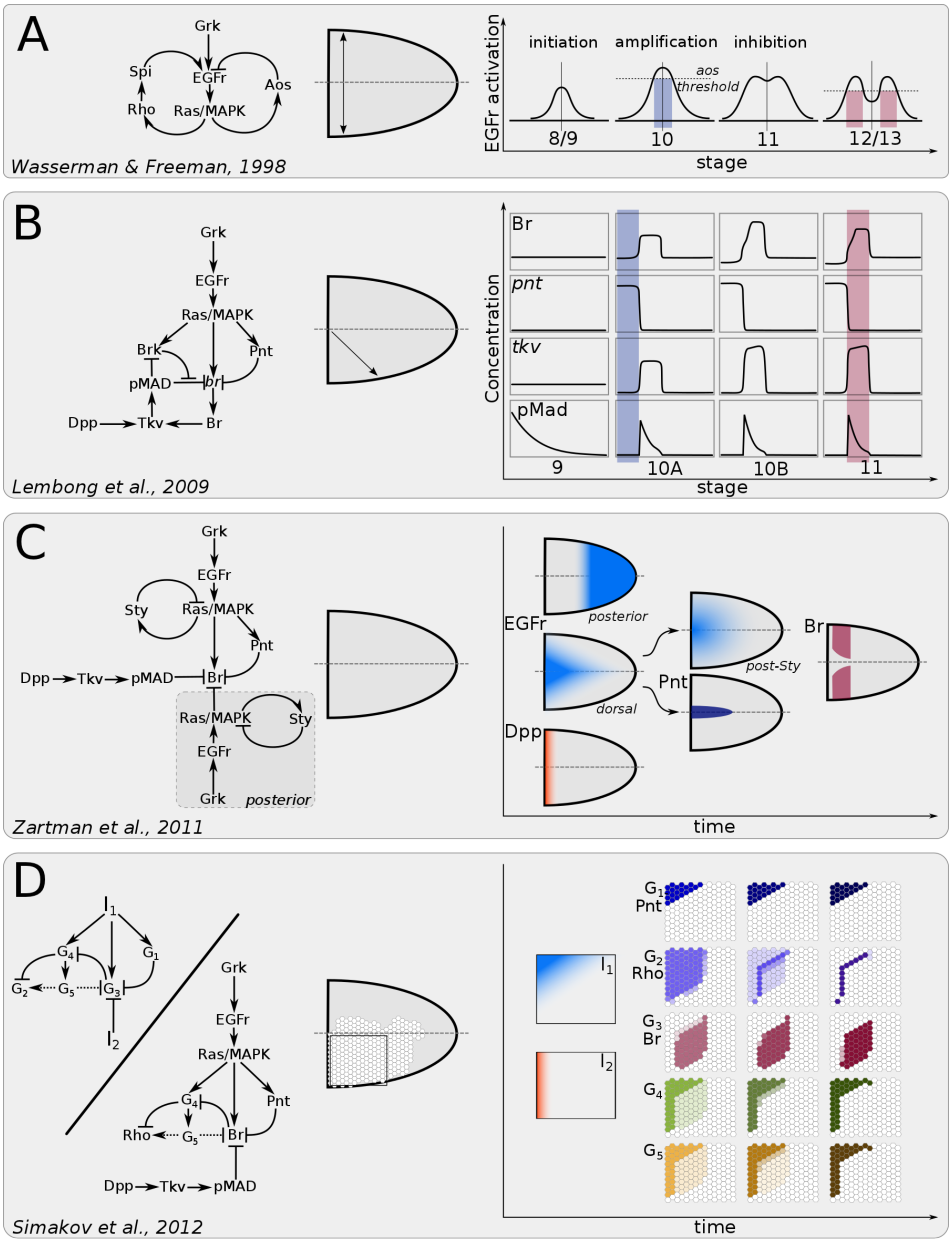


Figure 4.2: **The evolution of models in eggshell patterning**. Comparing the network (left), modelling context (centre), and main results (right) of four computational models. See box 1 for a description of each.

While both Shvartsman et al. (2002) and Zartman et al. (2011) have focused on the contribution of the EGF pathway to eggshell patterning, we will here examine how signalling along the anterior-posterior axis in general, and Dpp signalling in particular, can contribute to diversity in dorsal appendages.

4.2 Material and Methods

4.2.1 Flies

The following species were obtained from the Drosophila stock centre: *D. virilis*, *D. immigrans*, *D. erecta*, *Z. indianus*, *Z. sepsoides*, *Z. inermis*, *C. pararufithorax*, and *C. procnemis*. *Z. davidii* was a gift from Jean David. *D. pseudoobscura*, *D. mojavensis*, and *D. arizonae* were a gift from Christen Mirth. Wildtype *D. melanogaster* Oregon R was used. All flies were maintained on regular fly food at room temperature.

4.2.2 Immunohistochemistry

Ovaries were dissected in cold PBT and fixed for 20 minutes at room temperature in 4% formaldehyde in PBTx (0.1% triton-x100 in PBS). After fixation they were washed several times in PBTx-B (1% BSA in PBTx) at room temperature during one hour. Antibody incubation was done overnight at 4^oC. The rabbit anti-pMad antibody was kindly provided by Gines Morata, and was used at a concentration of 1:100 in PBTx-B. Anti-Br-core (25E9.D7) was obtained from Developmental Studies Hybridoma Bank and was used at a concentration of 1:100. Secondary antibodies (Alexa fluor goat-anti-rabbit or anti-mouse 488/546) were used at a concentration of 1:2000, overnight at 4^oC. Nuclear staining was done with DAPI and Draq5 (Biostatus). Imaging was done on a Leica SP5 confocal microscope. All images were processed using ImageJ (Schneider et al., 2012).

4.2.3 Gradient measurements

Dpp activation gradients were measured on confocal images of pMad immunostaining with a nuclear stain. Z-stack images of late stage 9 and early stage 10 egg chambers (after formation of the columnar epithelium, but prior to centripetal cell migration) were collapsed to two images covering both halves of the egg chamber. Using the nuclear staining, the ten anteriormost cell rows in the columnar follicle cells were selected for future processing. Using ImageJ, signal intensity was measured along a straight line from anterior to posterior, five times per image.

4.2.4 Bioinformatics

Gradient measurements

Gradient measurements were processed further by dividing the output into 20 brackets, and calculating the average signal of each. All measurements per species were pooled and averaged. The resulting gradient was normalized by setting the highest signal in brackets 1-4 = 1, and the average signal of brackets 13-20 = 0. Brackets 17-20 were discarded as noise, and are not shown in the results. This processing was done using python, and the script is available upon request.

Epithelial patterning model

In the patterning model, the follicular epithelium was represented as a two-dimensional hexagonal grid. The generation of the final pattern takes place in two rounds: first, for each cell, the model uses a direct logical interpretation of two input variables to generate one of two output options: Br (Broad) or Operculum (Op). The input variables used here are gradients of EGF and Dpp pathway activity; presented in three possible states for each pathway: 0 (absent), 1 (weak), or 2 (strong). The logical functions calculating the output for the first round are:

Br — Dpp [1] AND EGF [1]

Op — (Dpp [2] AND EGF[1,2]) OR (Dpp[1,2] AND EGF[2])

A second round of signalling is turned on in those cells with the identity Op: they determine whether they are adjacent to a Br-positive cell. If they are, they assume a third possible output identity: Rhomboid (Rho). Thus, in total, the logical rules for all three output options are:

Br — Dpp [1] AND EGF [1]

Rho — ((Dpp [2] AND EGF[1,2]) OR (Dpp[1,2] AND EGF[2])) AND Br_adjacent

Op — ((Dpp [2] AND EGF[1,2]) OR (Dpp[1,2] AND EGF[2])) NOT Br_adjacent

The model was developed in Python (Van Rossum et al., 1991).

4.3 Results

4.3.1 Conceptual epithelial model

In the effort to develop a comprehensive computational model of the genetic patterning network underlying dorsal appendage formation in *D. melanogaster*, our first step was a simple conceptual assessment of input and output factors in this system. We used a direct logical interpretation of the two input gradients (EGF and Dpp signalling pathways) to generate an output in three terms: operculum,

floor, and roof; the latter two represented by Rho and Br-positive cells, respectively. This model uses a hexagonal grid to represent the follicular epithelium, and is thus two-dimensional.

Key to the model's function is the multilevel input of EGFr and Dpp signalling, which allows at the same time a simple input pattern, and the generation of a relatively complex output. The transitions between the levels are comparable with activation thresholds for downstream factors used in more complicated models, but because our model directly incorporates them in the input, there is no need to quantify. The logical rules translating the two input gradients into three possible cell fates are explained in the Material and Methods section.

This method is not without precedent. The definition of a comprehensive regulatory atlas on a tissue in order to dissect observed patterns of gene expression is part of an emerging conceptual framework in evo-devo (Prud'homme et al., 2007). In fact, the method has been applied to *Drosophila* oogenesis (Yakoby et al., 2008a). Our model differs from Yakoby et al. (2008a) in that it uses only two input patterns where they use six primary components. Conversely, our input variables are not Boolean, but multilevel.

The model represents a radically simple analysis of a complicated patterning network. Yet, and perhaps surprisingly, it is able to recapitulate the output of several follicle cell mutants, which were taken from Shravage et al. (2007). (Fig. 4.3). While further versions were being developed, we have already been able to use this model to explore diversity in pattern formation, by adjusting the levels of the input gradients and observing the output.

When manipulating the quality of the Dpp gradient by extending the area with a 'weak' Dpp signal (level 1) both to anterior and posterior, the resulting pattern of Br cells closely resembled the wildtype Br pattern from *D. virilis* (Fig. 4.4; compare A′ and C). In our model, the spatial distribution of Br along the anteroposterior axis is restricted to the Dpp level 1 area (see Material and Methods, section 4.2.4.) Thus, the result of this experiment goes hand in hand with the assumption that the *D. virilis* Br pattern must contain more cell rows along the anterior-posterior axis. Indeed, when observing the wildtype Br patterns of both *D. melanogaster* and *D. virilis*, it appears that this is the case (Fig. 4.4 A-A′).

Our observation that a change in patterning along the anteroposterior axis can reproduce, to an extent, Br patterns similar to those generated in four-appendage species, is corroborated by models and experimental data alike. In order to recapitulate *D. virilis* Br patterns, Zartman et al. (2011) manipulated the strength of the EGFr inhibitor (Sty), which in their model regulates anterior-posterior as well as dorsal-ventral patterning. In addition, the inhibiting effect of Dpp on Br was removed, enabling the Br positive domain to extend anteriorly (Zartman

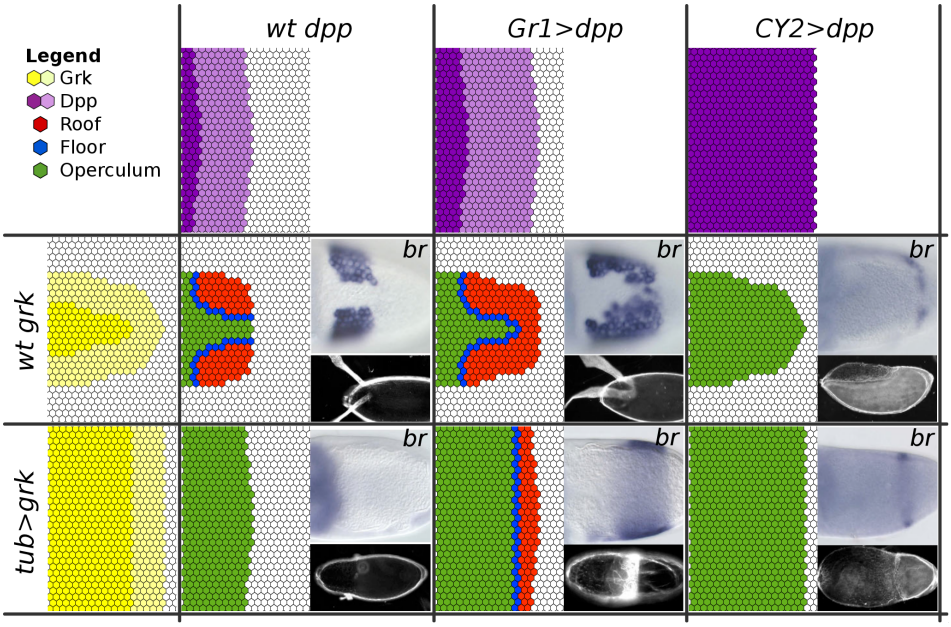


Figure 4.3: **A conceptual model of eggshell patterning**. Simple rules are able to reproduce epithelial patterning with input from the EGF (yellow) and Dpp (purple) signalling pathways. For the results of over-expression, the presumed effect of ectopic drivers Gr1 and CY2 (on *dpp* expression), and Tub (on *grk* expression) was simulated. The model is validated by comparing its output patterns with *br* expression patterns and eggshell phenotypes (showing the extent of the operculum) resulting from the use of these drivers in overexpressing *dpp* and *grk*, as performed by Shravage et al. (2007). Eggshell photos and *br* expression images were reprinted from this paper, with permission from the corresponding author.

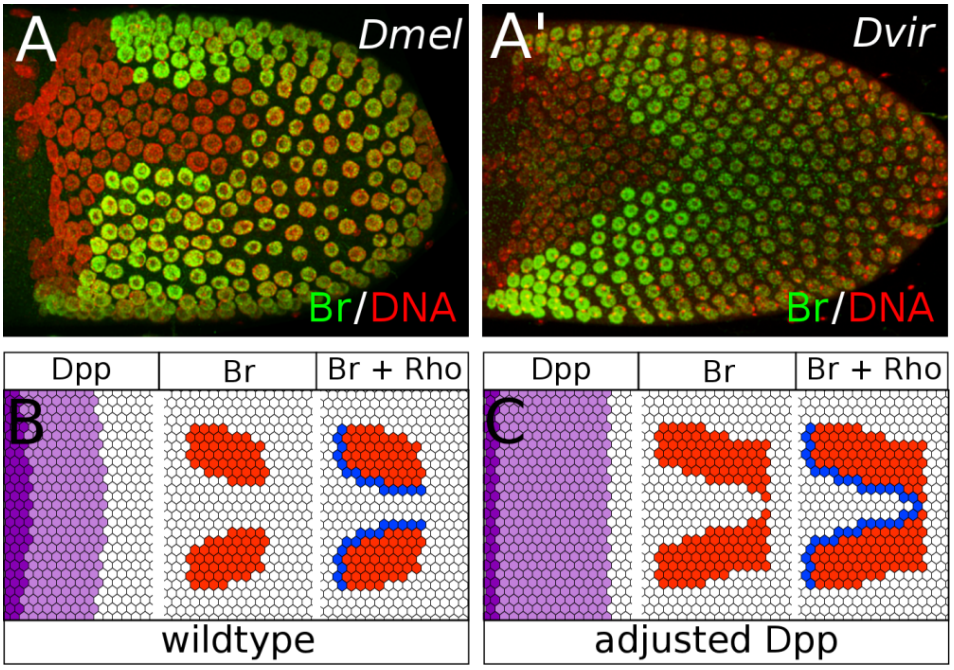


Figure 4.4: **Effects of adjusting the Dpp input in the conceptual model**. (A-A′) Spatial Br pattern in the egg chamber of wildtype *D. melanogaster* (A) and *D. virilis* (A′). (B) Dpp input, plus Br and Rho output, of the conceptual model recapitulating eggshell patterning in *D. melanogaster*. (C) By extending the region of Dpp level 1 to anterior and posterior, a Br pattern is generated that shows similarity with the Br domains of the *D. virilis* egg chamber.

et al., 2011). Deng and Bownes (1997) overexpressed Dpp in *D. melanogaster*, and observed the generation of eggs carrying four, or even six appendages.

These results are proof of principle that the pattern along the anteroposterior axis is relevant for the potential number of appendages. However, it raises two questions: (1) could the quality of the Dpp gradient indeed be responsible for the divergence of Br patterns between these two species; and (2) to what extent is this a mechanism that evolved to change appendage number? To answer these questions, we have looked in several species with different appendage numbers at the spatial pattern of pMad, which is widely used as an indication of the level of Dpp pathway activity (Niepielko et al., 2011, 2012). To ensure that our results regarding appendage numbers were not obscured by phylogenetic location of the species used, we chose species from three different subgenera: *D. melanogaster* and *D. melanica*—both species with two appendage eggshells—and *D. virilis* and *Zaprionus indianus*—species with four appendage eggshells. *D. melanogaster* is a member of the *Sophophora* subgenus, while *D. melanica* and *D. virilis* are both part of the *Drosophila* subgenus. *Z. indianus* is part of the *Zaprionus* group (Fig. 4.1).

We were interested in the Dpp activity gradient established in the columnar epithelium prior to the differentiation of the appendage primordia, thus, late stage 9 or early stage 10 egg chambers were selected. Moreover, as the anterior cell rows in the columnar epithelium move centripetally in late stage 10 and could thus obscure our results, we took care to select egg chambers prior to this stage.

Although it has been observed by Niepielko et al. (2011, 2012) that early (i.e. stage 9, 10A) Dpp signalling did not differ between species of different subgenera, we observed a stark difference between the pMad gradient in the species observed (Fig. 4.5 B-B‴). In an attempt to quantify the gradients to be able to make a more accurate comparison between species, we measured the strength of the gradient along the eight anteriormost rows (see Material and Methods, section 4.2.3 and 4.2.4). Indeed, this yielded two distinct and qualitatively different gradients (Fig. 4.5 A). As the graphs were normalized, they are not an indication of absolute signal strength, rather, they show a qualitative difference between the gradients of different species.

Importantly, the two distinct gradients correlated not with appendage number (two versus four), but with phylogenetic location (subgenus *Drosophila* or not) (Fig. 4.5A). As our earlier observation modelling the effect of different gradients used an output of Br, not appendage number (Fig 4.4), we stained for Br expression with an antibody for the Br core (BrC) in the egg chambers of several species, in order to relate the Dpp gradient to this output in a wider phylogenetic range (Fig. 4.6). Unfortunately, the Br antibody did not appear to be cross-reactive

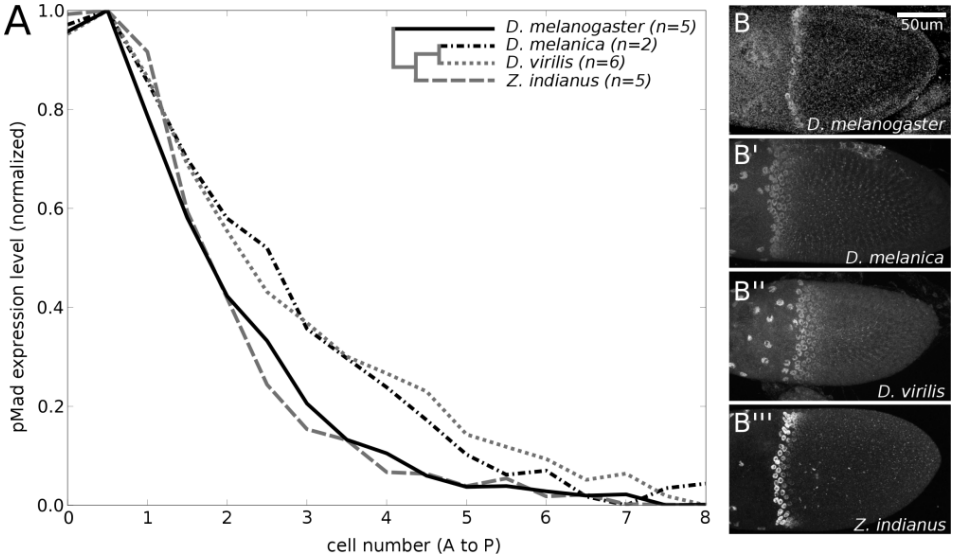


Figure 4.5: **Qualitative differences in Dpp activation gradients of early stage 10 egg chambers**. (A) Semi-quantification of the Dpp pathway activation gradient in egg chambers of *D. melanogaster* (2 appendages), *D. melanica* (2 appendages), *D. virilis* (4 appendages), and *Z. indianus* (4 appendages). (B-B‴) Examples of stage 10A egg chambers of each species used to make the measurement.

with any of the available *Zaprionus*, and we were unable to clone the *br* gene for these species. However, this experiment indeed confirms the qualitative difference between Br patterns of members of the *Drosophila* and *Sophophora* subgenera, irrespective of the number of appendages on the eggshell. Indeed, it was shown before by Kagesawa et al. (2008) that there are "two ways to skin a cat" when it comes to making two eggshell appendages, demonstrating how the typical V-shape of the EGFr activation pattern in egg chambers of the subgenus *Drosophila* can resolve into either two or four appendage primordia, depending on the species.

An unexpected observation from this dataset are the early Br patterns of *D. mojavensis* and *D. immigrans* (Fig. 4.6A-B‴): here, a distinct midline-minus is seen over the entire length of the dorsal epithelium. In late patterns this line disappears, as only the anterior cells retain the high Br expression associated with the appendage primordium fate (Fig. 4.6B‴). It is thus unlikely that this line has a significance for the phenotype, although we could not help but note that the species with this particular pattern have very long and thin appendages compared to the other species we used. Nevertheless, we favour an explanation where this midline-minus is a remnant of the EGF signalling during nuclear movement. Further exploration of this pattern could go into expression of *pointed*, which encodes a transcription factor known to be responsible for the repression of *br* in the anterior midline of *D. melanogaster* egg chambers.

4.4 Discussion

Dpp over-expression experiments done in *D. melanogater* by Deng and Bownes (1997) demonstrated how manipulations of anterior-posterior patterning could affect the total number of appendages on the eggshell. We explored this idea, and tested whether anterior-posterior patterning in general, and the Dpp activity gradient in particular, could be responsible for diversity in eggshell appendage numbers between species. Our results point to two distinct qualities in Dpp activation of early stage 10 egg chambers, but they correlate not with appendage number, rather with phylogenetic location and the shape of the Br expression pattern.

4.4.1 Limitations of the present data

Our measurements and observations on the Dpp gradient are contrary to observations published by Niepielko et al. (2011, 2012), who mentioned that no interspecific differences could be observed in the pMad patterns of early stage 10 egg chambers. Thus, it is important to present a solid body of evidence in arguing for a qualitative difference in this gradient between species. Unfortunately, our dataset is still limited. While taking extreme care that the egg chambers used

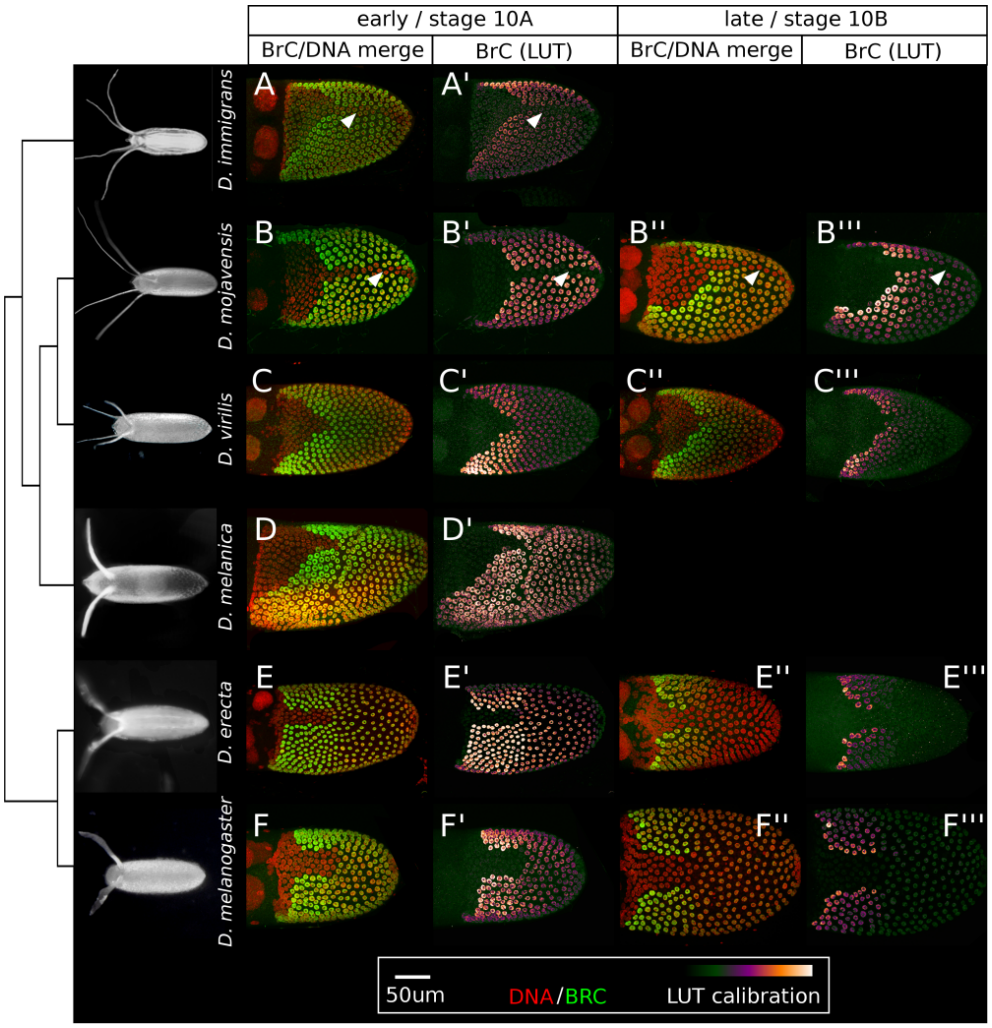


Figure 4.6: **Spatial pattern of Br in early and late stage 10 egg chambers of different Drosophilid species**. In merged images (A-F and B″-F″), Br is shown in green and a nuclear staining in red. In Br only images (A′-F′ and B‴-F‴) the signal strength shown using a look-up table (LUT). The legend below shows the calibration bar: black and dark green indicate a weak signal, while yellow and white indicate a strong signal. The midline-minus is indicated by a white arrowhead.

were correctly staged, this has reduced the total sample size used in this chapter. However, we have observed a consistent difference in quality between the pMad gradients of members of *Sophophora* and *Zaprionus*, and species belonging to the *Drosophila* subgenus, and have semi-quantified these gradients in a small subset of egg chambers (Fig. 4.5). The data presented, as well as our further observations, argue for a reassessment of the observations presented by Niepielko et al. (2011, 2012).

Nonetheless, we acknowledge the difficulty in comparing species in this regard. An important obstacle is the morphogenetic movement that takes place during stage 10 and beyond. Indeed, in addition to the centripetal migration taking place around stage 10, the columnar follicular epithelium as a whole undergoes a rigorous restructuring from this stage onwards, to shape the appendages (James and Berg, 2003). Our method of observation and measurement could be affected even prior to centripetal migration with a species (or species group) specific change in density of anterior versus posterior cells. Moreover, with a very detailed and careful analysis of movements of Br-positive cells in follicular epithelia of *D. melanogaster* versus *D. virilis*, James and Berg (2003) observe that incongruence in stages exists between these species, further complicating interspecific comparison. Thus, we advise that any future work on the subject uses double immunostaining of pMad and Br, to ensure that the Br pattern is not yet specified.

However, despite these difficulties our results are a clear argument for the need for future exploration of early Dpp signalling, and the role it may play in diversity between species. Additionally, if we accept that the patterning of eggshells along the anterior-posterior axis is instrumental in generating diversity in appendage numbers, then the question of which genetic mechanism is primarily responsible for patterning the anteroposterior axis of the eggshell becomes pertinent. There is no doubt that, in *D. melanogaster*, Dpp signalling in anterior follicle cells represses *br*, thus defining the anterior border of the appendage primordia (Yakoby et al., 2008b). However, some debate exists regarding the genetic mechanisms defining the posterior border.

4.4.2 What regulates the posterior border?

The appendage primordia are formed at the dorsal anterior end of the follicular epithelium covering the oocyte. Not only is this localization dependent on the dorsal EGFr signal, but several models and experiments have pointed out the need for an anterior 'competence field' to explain the posterior border of the primordia (Atkey et al., 2006; Zartman et al., 2011). Conflicting ideas exist regarding this competence field: one model proposes Dpp, which signals in the anteriormost columnar follicle cells, to define the anterior region in which appendages can form

(Peri and Roth, 2000). In recent years, however, a reappraisal of the role of Dpp in eggshell patterning has led to an alternative explanation, using the early EGFr signal at the posterior pole as a candidate regulator of the primordia's posterior border (Zartman et al., 2011). The models not only provide alternative explanations for the observed phenomenon of anterior competence, but some of the underlying experimental evidence regarding the role of Dpp in eggshell patterning is in direct conflict. This debate on the exact regulation of the anterior competence, as well as the role of Dpp in eggshell patterning, justifies a closer look at the evidence for each of the models.

Dpp signalling has been shown to repress appendage fate in the anteriormost follicle cells (Dobens et al., 2000; Yakoby et al., 2008b). However, as Dpp is a known morphogen that can potentially signal across large distances, its effect is not necessarily restricted to the first few cells away from the Dpp source. Low levels of signalling could be associated with a positive effect on *br* expression, and necessary to define the appendage primordia, and it is an obvious candidate for the specification of the anterior competence field.

Dpp signals through its receptor Tkv and phosphorylates the transcription factor Mad, which binds to the co-smad Med. This complex now enters the nucleus to transcribe target genes. Several experimental manipulations have been used to test the requirement of this cascade in establishing the Br positive cells of the appendage primordia. Peri and Roth (2000) showed how follicle cell clones of mutant Mad render a cell-autonomous loss of *br* expression. Shravage et al. (2007) demonstrated a similar cell-autonomous disruption of BrC expression in follicle cell clones mutant for Med. Both papers conclude that Dpp signalling is required for high Br levels associated with the appendage primordia. However, Yakoby et al. (2008b) did similar experiments, which showed that follicle cell clones negative for Mad as well as Med mutant clones have no effect on Br outside the anteriormost rows. These published results are in direct conflict, and it is unclear without a detailed examination of the raw data and the specific methods used which conclusion is correct.

Nevertheless, further empirical evidence is available to help us understand the role of the Dpp pathway in eggshell patterning. A lot of the data centres around the activity of Dpp inhibitors, on every possible level. Dad is an inhibitory Smad, blocking the intracellular association of the receptor and unphosphorylated Mad. Clones with mutant Dad have no effect on Br expression (Chen and Schüpbach, 2006), and ectopic over-expression of Dad causes an expansion to anterior, but not to posterior, of the Br positive patches (Yakoby et al., 2008b). Both these observations argue against a role for Dpp in establishing the posterior border.

However, data on other inhibitors, like the transcriptional repressor Brinker

(Brk), argues otherwise. Brk is itself a negative target of Dpp signalling (Jazwinska et al., 1999; Chen and Schüpbach, 2006), and is required for dorsal appendage formation, but not for operculum formation. Its expression is restricted to posterior follicle cells by the anterior Dpp signal. EGFr activation has a positive effect on *brk* expression, which causes dorsoventral asymmetry in the expression pattern (Chen and Schüpbach, 2006).

A third inhibitor is the oncogene dSno, which antagonizes Dpp signalling by interacting with Mad or Med (Barrio et al., 2007). *dSno* is expressed in a semicircle on the anterior dorsal follicle cells, and overlaps precisely with the posterior border of the Br domains. In eggs mutant for dSno the appendage primordia are further apart, the number of Br positive cells is decreased, and the appendages are moved slightly to posterior. (Shravage et al., 2007). More interesting, however, is the synergy between the inhibitors: for example, when *dSno* and *Dad* are disrupted simultaneously, the *br* expressing domains locate much farther away from the midline than when only *dSno* is mutated, and the posterior boundary of the patches has shifted posteriorly. Double mutants for *dSno* and *brk* have a similar phenotype (Shravage et al., 2007). Another argument for Dpp activity in the appendage primordia is the fact that dSno itself is also regulated by Dpp signalling, as cells mutant for Med do not express dSno (Shravage et al., 2007).

Further evidence for a role for Dpp in establishing, not just inhibiting, the appendage primordia, comes from combined misexpression of Grk and Dpp. Extensive over-expression of both Grk and Dpp is able to drive expression of *br* close to the posterior pole (Shravage et al., 2007) (see also Fig. 4.3). This was also shown by Peri and Roth (2000), who misexpressed *dpp* in posterior terminal cells of egg chambers treated with colchicine, which blocks nuclear movement. The resulting combined signal of Dpp and EGFr activation at the posterior pole was able to induce dorsal appendage fates in neighbouring follicle cells.

Regardless of Dpp function, an argument for the alternative explanation of posterior boundary establishment through early posterior EGF signalling is made with the phenotype of sty^{-}/sty^{-} egg chambers. When the known EGFr inhibitor Sty is mutated, not only are the primordia shifted ventrally, but the posterior boundary has moved to anterior, decreasing the size of the Br patches (Zartman et al., 2011). This leads to the conclusion that indeed it is EGF signalling from the posterior pole that establishes the posterior boundary. However, as Dpp signalling has been equally demonstrated to be involved in this, it is more likely that the two mechanisms act in concert than that they are mutually exclusive explanations.

We conclude that it is very likely indeed that it is the Dpp pathway, rather than early EGF signalling, that defines an anterior competence field for the specification of appendage primordia. This does however not preclude an additional regulatory

mechanism, such as the relay of an early posterior EGFr signal (Zartman et al., 2011).

4.4.3 Dorsal-ventral patterning and the generation of diversity

Although early Br patterns do not prescribe the appendage number (Fig. 4.6), it is possible that the specific shape of the domain facilitates multiple appendages on either side of the midline. In an attempt to generate the *D. virilis*-specific V-shape, we manipulated the input of our conceptual model along the anterior-posterior axis, which resulted in a pattern resembling the spatial distribution of Br in *D. virilis*. However, the changes between these patterns in the *Drosophila* and *Sophophora* subgenera cannot be explained along this axis alone: our model did not recapitulate the wide space between the appendage primordia in the anteriormost follicle cells. Thus, there is a need for explaining variation along the dorsoventral axis as well.

The early input of the dorsal-ventral signal (Grk and EGFr activation) is unlikely to have changed much in the evolution of Drosophilidae: this signal is instrumental for the correct specification of the dorsoventral axis of the future embryo (Roth, 2003). Conversely, the Dpp pathway's function lies mostly in directing centripetal movement of the anteriormost follicle cells, and it is very well possible that the subsequent morphogen gradient over the follicular epithelium is a by-product of this signal.

Regarding the putative contribution of the EGF pathway in phenotypic diversity: it has been shown that the germline itself, which includes the initial Grk signal, is insufficient to generate variation in eggshell phenotypes (Nakamura et al., 2007). Furthermore, ectopic activation of EGFr or over-expression of its ligands does not induce Br patterns reminiscent of *D. virilis*, or lead to the formation of multiple appendages (Deng and Bownes, 1997; Shravage et al., 2007). However, this does not preclude the involvement of network changes downstream of the EGF signalling input. Indeed, it would be interesting to assess experimentally whether strength of an EGF inhibitor like Sprouty (Sty) differs between species, and could be responsible for eggshell phenotype diversity, as proposed by Zartman et al. (2011).

4.5 Conclusion

We set out in this chapter to explore how signalling information along the antero-posterior axis of the follicular epithelium can contribute to diversity in eggshell

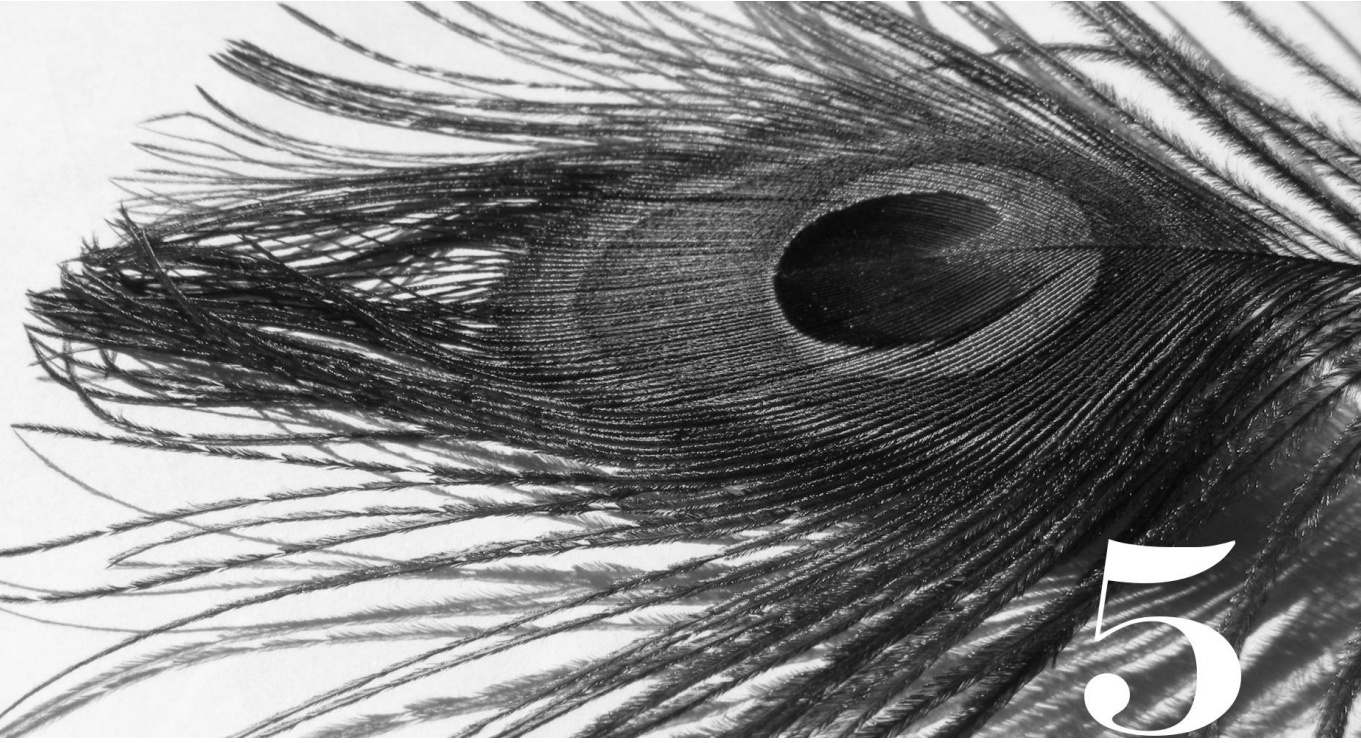
phenotypes. Importantly, we need to make a distinction between the shape of the appendage primordia, and the number of appendages on the eggshell. We conclude that anterior-posterior patterning is indeed meaningful for variation in the shape of appendage primordia. Furthermore, we propose that the quality of the Dpp activity gradient may contribute to this variation. Conversely, we are not able to connect variation in this signalling pathway to the final number of eggshell appendages, as this depends to a large extent on the morphogenetic restructuring of the follicular epithelium. Finally, if we want to fully appreciate the role of anterior-posterior patterning in generating variation in appendage primordia and phenotypes alike, we need to understand the underlying mechanisms. We have argued that for one of those mechanisms, the Dpp signalling pathway, a reappraisal is in order.

Contributions

The epithelial patterning model was developed with Adrien Fauré in the laboratory of Claudine Chaouiya. The method used to integrate and analyse gradient measurements was designed by me, and implemented in Python by Ot de Wiljes.

Acknowledgements

Thanks to Ana Marcelino for practical assistance in the lab, and many—unfortunately unsuccessful—attempts at obtaining Br patterns in *Zaprionus* species. Thanks also to Pedro Almada for help with writing an ImageJ macro to measure gradients more efficiently. Christen Mirth and Jean David provided several fly species. The pMad antibody was a generous gift from Gines Morata. The Broad-core antibody developed by G. Guild was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Finally, I thank Filipe Cadete, Marc Gouw, Adrien Fauré, and Élio Sucena for comments on the manuscript for this chapter.



5

General discussion

Abstract

In this thesis, we set out to explore what *Drosophila* oogenesis can teach us about the origin and diversification of evolutionary novelties. Although the concept of 'novelty' is difficult to define, there is little doubt that the appendages on the eggshell of Drosophilid flies can be considered a novel morphology. The work we presented shows how two major signalling pathways involved in patterning the eggshell are present and active in oogenesis of *Ceratitis*, a fly without eggshell appendages, underlining their pleiotropy in *Drosophila* oogenesis. Thus, these pathways may have defined a coordinate system on the ancestral follicular epithelium that played an important role in the evolution of dorsal appendages. This same coordination of the epithelium plays an important role in the diversity of patterns underlying the different appendage phenotypes. Furthermore, we discuss whether the gene *broad*, which fulfils a key role in the evolution and development of eggshell appendages, has also been instrumental in generating variation between species. These discussions touch on larger themes within the study of novelty. Here, we revisit the concept of co-option, and argue for a wider use for the term. We discuss deep homology in the context of the eggshell appendages, and ask whether general rules for innovations can be found.

It has been well over a century since *Drosophila melanogaster* entered the laboratory of Charles Woodworth, and subsequently the hands of Thomas Hunt Morgan. In this time, the fly has proven to be a game changer for the field of genetics as well as that of development. With the advent of evo-devo, *D. melanogaster* has become a catalog species for many comparative studies in this field. Many well-studied aspects of the fly have hence been treated as dogmas in development; the segmentation cascade of the fly, for instance, which is a textbook example in embryonic development and pattern formation (Gilbert, 2003). Yet, through comparative research it has become clear how atypical *Drosophila*'s development actually is: its 'long germ' segmentation cascade is not the prevalent mode of arthropod segmentation (Liu and Kaufman, 2005). While most of the time we may lament this property of the fly when using it in an evolutionary context, we argue it can be used to our advantage.

In addition to the segmentation, many more aspects of *Drosophila* development are derived compared to other arthropods, or even compared to Dipterans. This makes the system an ideal hunting ground for novelty. Moreover, the developmental basis of any such innovations is likely to be well-understood, which is of great benefit within a comparative framework such as the evo-devo research programme.

This thesis has centred around one such innovation: the dorsal appendages on the *Drosophila* eggshell. We have addressed not just its evolutionary origin, but also considered the genetic basis of variation observed in this phenotype. In this final synthesis of the thesis, we will very briefly review the main findings, and then focus on the question we asked in the beginning of this project: what can *Drosophila* oogenesis teach us about novelty?

5.1 This thesis: brief overview and conclusions

In this thesis, we have compared late oogenesis of *Drosophila melanogaster* with that of *Ceratitis capitata* (chapter 2), with that of a mutant affecting the eggshell phenotype (chapter 3), and with that of other Drosophilid species, such as *Drosophila virilis* (chapter 4). These comparisons served to generate hypotheses on the origin of dorsal appendages, and the diversity of this morphology, respectively.

Regarding the evolutionary origin of dorsal appendages, as well as the underlying patterning network, we asked first whether the two main eggshell patterning pathways, EGF and Dpp, were present in the oogenesis of an ancestral dipteran. This was confirmed in the formation of the appendage-less eggshell of *Ceratitis capitata*. Thus, we propose that these pathways provided positional information to an ancestral epithelium, upon which the patterning network of the dorsal appendages could evolve. This specification of patterns from a system of higher order compo-

nents is an emerging theme in regulatory evolution (Prud'homme et al., 2007). In oogenesis specifically, an elaborate analysis of expression patterns on the follicular epithelium has led to the identification of six primary building blocks from which all other patterns can be constructed (Yakoby et al., 2008a). Our proposal of a coordinate system is conceptually close to this view, with only two primary patterns defining the two axes of the follicular epithelium. From this proposal then follows that the regulatory building blocks identified by Yakoby et al. (2008a) were co-opted onto this coordinate system.

The co-option of a genetic programme for dorsal appendage formation (and the underlying patterns) should have occured in the context of an existing network structure of genetic interactions. Downstream of EGF and Dpp, we identified nodes in the known network from *Drosophila melanogaster* that may have played a key role in this co-option event: the transcription factors Mirror, Pointed, and Broad. While the latter two are likely newly co-opted in the dorsal patterning network, Mirr is known also to regulate the gene *pipe*. This gene determines embryonic dorsoventral polarity, and is expressed in both *D. melanogaster* and *C. capitata* in a similar pattern. By contrast, *mirr* itself could not be detected in *C. capitata*, but this does not preclude its presence: undetectable levels of *mirr* expression have been shown to be functional in regulating *pip* in *D. melanogaster* (Andreu et al., 2012). Thus, we propose that *mirror* in Drosophilidae acquired a regulatory module driving increased levels of expression. This second module allowed the gene to respond to a Dpp signal, and generate levels of Mirr sufficiently high to drive the *br* expression specific to the appendage primordia.

This model implies an inductive effect of Dpp on the definition of appendage primordia, through *mirr*. This effect is not without controversy in the literature, as discussed elaborately in chapter 4 (Yakoby et al., 2008b; Lembong et al., 2009). Therefore, our proposed model could kill two birds with one stone, as the identification of the predicted regulatory module responding to Dpp would make a very strong case indeed for positive Dpp involvement in defining appendage fate.

We further found that the activation of the Dpp pathway may occur in a qualitatively different way between species at early stage 10, just prior to the establishment of the appendage primordia. We propose that this qualitative difference is reflected in the subsequent shape of the appendage primordia, which roughly takes the form of two triangles in the species we examined of the subgenus *Drosophila*, while the primordia of the subgenus *Sophophora* are more square-like (see e.g. fig. 4.1B on page 72). Again, our model here supports an inductive role for Dpp in dorsal appendage fate.

In summary, our results point to a scenario where a genetic network controlled by Mirror and Pointed was co-opted into an existing coordinate system defined by

EGF and Dpp signalling. Hence, EGFr activity controlled pattern formation on the eggshell in addition to its ancestral role determining embryonic dorsoventral polarity, but even in the modern day *D. melanogaster*, these functions can still be uncoupled. Finally, following the co-option event, the graded activity of Dpp in the epithelium was used to generate variety in the outcome of the patterning network.

5.2 Limitations of the current work and some ideas for future research

Our work on *Drosophila* egg appendages as a model for evolutionary novelty is the first occasion where *Drosophila* oogenesis is used in this research theme. While we are building on a vast collection of data on the oogenesis of *D. melanogaster*, our work on gene expression in *C. capitata* oogenesis is the first such study done in this species. To our knowledge, only one *in situ* expression pattern in *C. capitata* ovarioles has been published, concerning the gene *orthodenticle* (Schetelig et al., 2008). In other words, there are still many genes left that will have to be studied in species like *C. capitata* to determine whether they are new acquisitions to, or ancestral components of, the dorsal patterning network. An indication of the number of genes active in *D. melanogaster* dorsal appendage formation comes from the work of Yakoby et al. (2008a), showing the expression data of 81 genes active in the dorsal follicular epithelium.

Specifically, we propose to focus on aspects of EGF signalling regulation. While we looked at EGF signalling targets like *rhomboid*, and *pointed*, we were so far unable to obtain expression patterns for *argos*, *kekkon*, and *sprouty*. The latter two in particular are involved in modifying EGFr activity in a manner relevant for dorsal appendage localization (Boisclair Lachance et al., 2009; Zartman et al., 2009), and examining their expression in *C. capitata* would help determine whether this modification is part of the ancestral dorsoventral polarity specifying function of EGF signalling, or not.

Most crucially, our work was unable to resolve an expression pattern for *mirr* in *C. capitata* oogenesis. This leaves two scenarios open: (1) *mirr* is not part of the ancestral network, and it was co-opted into a regulatory cascade defining embryonic dorsoventral polarity; (2) *mirr* is expressed and functional in *C. capitata* oogenesis and part of the ancestral network, but acquired a second function crucial for the formation of dorsal appendages. We explored the second option in a proposed model of *mirr* regulation and evolution, but cannot rule out the first.

This question can be approached in several manners. A very powerful method would be the identification of the *pip* regulatory region in *C. capitata*, and deter-

mining whether it responds to Mirr. We have attempted to do this by generating a transgenic construct with non-coding regions at the *Cc-pip* locus and transforming them to *D. melanogaster*. If indeed these regions contain a *Cc-pip* regulatory site, and it drives an expression pattern in *D. melanogaster* oogenesis, it confirms conservation of the regulatory cascade upstream of *pip*. Using tools available in *D. melanogaster*, we can then test which transcription factor is responsible for its regulation. Unfortunately, our results so far have been inconclusive.

A second approach is to turn the tables and transform the known Mirr-responsive *pip* regulatory domain from *D. melanogaster* to *C. capitata* (Zwiebel et al., 1995; Loukeris et al., 1995). While fewer tools are available in this system to disentangle the influence of factors, an expression of this construct in an asymmetric pattern resembling wildtype *pip* would indeed be a strong indicator of a conserved presence and function of Mirr in the specification of the embryonic dorsoventral axis, confirming at least in part the model we propose in chapter 2.

Regarding the mutant *troya*, we are looking forward to a further characterization, and the results of the sequencing project. At the moment, the project aims to determine if *try* is involved in maintaining genomic stability through the piRNA pathway silencing of transposable element mobilization. While a general effect on genomic stability is unlikely to explain the consistent uncoupling of eggshell and embryonic polarity we observed, the exploration of systems of genetic regulation beyond transcription factors and signalling activity is an exciting advance in biology that will certainly be increasingly represented in biological research, including development and evolution.

Regarding the role of Dpp in eggshell patterning, we strongly recommend revisiting the conflicting data currently available on the effect of blocking Dpp signalling on the spatial pattern of Br (Peri and Roth, 2000; Shravage et al., 2007; Yakoby et al., 2008b), to resolve the current debate. Furthermore, we would like to expand our dataset of both Br and pMad patterns in various species of Drosophilidae, to see to what extent the Dpp activity gradient is indeed involved in variation between Br patterns. While our current dataset and observations do suggest a subgenus *Drosophila*-specific Dpp pattern, at the present time it is unclear how consistent the patterns are within the subgenera, and more importantly, what their relevance is in the specification of appendage primordia. An expansion of the dataset could clarify these issues.

Importantly, we were thus far unable to obtain spatial Br patterns in *Zaprionus* species. While the Br antibody used in other Drosophilidae is not cross-reactive with *Zaprionus*, the option that Br expression is not determining the appendage primordia in this species needs to be investigated also. We have attempted to clone *br* in two *Zaprionus* species, but were unsuccessful so far. However, expanding the

information on *br* expression patterns to these species would add very valuable information to our understanding of diversity in eggshell appendage number—both in relation to a Dpp activity gradient, and to the shape of appendage primordia capable of generating four and two appendage eggshells, outside of the presently explored subgenera of *Sophophora* and *Drosophila*. Indeed, both phenotypes are represented in *Zaprionus* species, highlighting the importance of this group in addressing other fundamental concepts in the field such as convergence, and parallelism (Sucena et al., 2003; Prud'homme et al., 2006).

To close, we would like to remark on the use of *Drosophila melanogaster* as a model species in an evo-devo programme. Underlining the power of this system in an evo-devo context is unnecessary (Sucena and Stern, 2000; Skaer et al., 2002; Gompel et al., 2005). However, perhaps there is no better emphasis to this than to acknowledge the many unknowns in the genetic and developmental basis of the novel structure we studied. We have so far elaborately discussed the debate on the precise role of the Dpp pathway, but several other cases of progressive insight have radically changed the perception of function in important genes. For example, the ability of Mirr to repress *pip* was proposed to function at a distance in an important paper by Jordan et al. (2000). However, recently published evidence overwhelmingly points at a cell-autonomous repression of *pip* by Mirr (Technau et al., 2011; Andreu et al., 2012; Fuchs et al., 2012).

These genes and their mode of function are highly relevant for our interpretation of the evolution of dorsal appendages. The fact that even in *D. melanogaster* there is still uncertainty regarding their place in the regulatory network, should give us pause: what does this say about the conclusions we draw about gene function and regulatory networks in less well-known systems? Indeed, the fact that we may be wrong about aspects of development in *D. melanogaster* is a sobering reminder of the complexity of biology.

5.3 Variation: the evolution of a network

In a provocative paper, Stern and Orgogozo (2009) wonder about the predictability of genetic evolution, and introduce the concept of an 'input/output gene' to argue that in evolution not all genes are equal. An input/output gene integrates in *cis* the present information in a tissue, and turns on the appropriate genetic programmes to drive a morphogenetic output. These genes are responsible for generating variation, because they can vary in their interpretation of the *trans* genetic landscape: changes in the *cis*-regulatory region of these genes between species alters the relationship between input and output, with consequences in morphology.

In the system of eggshell patterning, a prime candidate for an input/output gene is *broad*. Not only does elevated Br expression in cells directly correlate with participation in appendage formation (Dorman et al., 2004; Boyle et al., 2010), Br is also known to drive the endoreplication of chorion genes (Tzolovsky et al., 1999), which are subsequently expressed in increased levels at the outgrowth of the dorsal appendages (Parks and Spradling, 1987). At the input level, the enhancer *brL* integrates spatial information provided by Mirr and Pnt and drives *br* expression in the appendage primordia (Fuchs et al., 2012).

The *brL* enhancer is conserved between Drosophilid species, but the expression patterns of *brL* from different species have not been tested in *D. melanogaster*. Conversely, this experiment has been done with a regulatory region for *rho*: here, it was shown that divergent *rho* expression between *D. virilis* and *D. melanogaster* depends not on the *rho cis* region, but on the respective *trans* landscapes of the two species (Nakamura et al., 2007). However, given the tight connection between the *rho* and *br* domains, it is quite possible that *rho* expression in the floor cell domains is in fact a target of Br in the adjacent cells.

In the idea presented here, the evolution of morphology hinges on one key element in an otherwise relatively invariable spatial manifestation of a regulatory network. In the model we present in chapter 4, by contrast, variation in an upstream component is implicated in driving divergent expression patterns of the downstream target *br*. A simple test should be able to resolve this issue, and determine whether *br* is indeed an input/output gene, and to what extent its *cis* regulatory region drives variation in appendage morphology between species: reporter constructs using the conserved *brL* enhancer from different Drosophilid species should be examined for the pattern they drive in *D. melanogaster*. If *br* indeed fits the label of 'input/output gene', the expression pattern of these constructs will display the variation in *br* expression patterns observed in their species of origin.

5.4 Co-option in the evolution of a novel morphology

Aside from the system-specific questions asking which genes have been co-opted and where, it is interesting to once again employ the panoramic view of pattern formation, and ask this question in a more general sense: at which stage between broad global signalling and the initiation of a morphogenetic response can we pinpoint the co-option event?

First, a note on the term co-option may be in order. Increasingly, the term 'co-option' as used in evo-devo has narrowed to mean gene co-option, specifically.

However, this is an injustice to a term that is so important in describing the mechanisms of evolution, and thus central to the field. Indeed, the reinterpretation of an existing coordinate structure defined by activity of the Dpp and EGF pathways should be considered a co-option event. This reinterpretation of the context, we argue, also falls within the boundaries of the term co-option: the verb 'to co-opt' can be defined as

> to divert to or use in a role different from the usual or original one.

This definition includes the EGF activity on the follicular epithelium, which in oogenesis of Drosophilidae has been used in the role of 'eggshell patterning' in addition to its ancestral role in 'defining dorsal-ventral polarity'.

In the following section, we will again focus on gene co-option; keeping in mind that in the redeployment of downstream factors, so changes the role of those factors upstream that control them.

5.4.1 Pinpointing gene co-option in a patterning network

In the case of *Drosophila* dorsal appendages, we see conservation of the initial global signals that coordinate the epithelium, but thus far we detected no conservation beyond this point; not in the feedback loops of both signalling pathways (insofar as they were studied), nor in other local interpretations of their signalling activity. This suggests that a large network—including the local interpretation of global signals, the demarcation of domains, and the subsequent morphogenetic response—has been acquired to generate the novel morphology.

This question gets at the heart of the novelty paradox: a complex of multiple elements needs to come together to generate a phenotype that can be maintained by natural selection. This is most obviously the case when regarding the elements of [underlying] 'pattern formation' and 'morphogenetic response': neither of these aspects makes sense without the other. A way to break the paradox is implicated by the concept of 'deep homology'. This term was coined by Shubin et al. (1997), to describe a common genetic machinery underlying different non-homologous structures. Indeed, in many novelties we can find deep homology between the novel structure and other structures in the organism or its relatives: for example, in the case of beetle horns we can find a deep homology with a regulatory programme of appendage formation that is known from legs (Shubin et al., 2009). Thus, instead of a series of genes, entire programmes are co-opted, which can subsequently be fine-tuned by natural selection.

Also in the case of dorsal appendages, this may be applicable: as has been discussed previously, Broad regulates the endoreplication of the chorion genes, increasing their expression in the dorsal appendage primordia (Parks and Spradling,

1987; Tzolovsky et al., 1999). Its ubiquitous expression in the follicular epithelium of *Ceratitis* as well as earlier stages of *Drosophila*, and the conserved regulation of chorion genes (Konsolaki et al., 1990; Tolias et al., 1990) suggest that this is an ancestral programme regulating chorion deposition. Moreover, Br has been known to regulate morphogenetic movement in many instances throughout development, such as the formation of the morphogenetic furrow in the *Drosophila* eyedisc (Brennan et al., 2001). Thus, increasing the levels of Br expression in a subset of cells, defined along the coordinate system present in the epithelium, could have been the key to a novel eggshell morphology.

5.5 Principles of innovation

So far, we have discussed how, in a coordinated epithelium, a rewiring of a genetic network could lead to the formation of novel patterns, crucially activating a programme of morphogenetic movement and chorion deposition in a subset of epithelial cells. Interestingly, the rewiring of the relationships between biological modules seems to be a frequently arising principle in innovation, regardless of the level of organization: in a classical example of novelty, the turtle shell originated in part because of a 'rewiring' of the relationships between muscles, ribs, and vertebrae (Nagashima et al., 2009). Another example includes the origin of the mammalian middle ear, in which a unique combination of skull bones created a character complex able to perform a novel function (Wang et al., 2001).

This is not to say that novelties arise in all cases where connections are rewired; rather, that the complex changes associated with a novel feature are frequently the result of a restructuring of the underlying biological modules. Importantly, the modules need not be on the same level of organization: we stress again the importance of the context in which a novel feature emerges. In the case of dorsal appendages, the co-opted gene network as well as the epithelium and its coordinates are all crucial elements in the origin of novelty.

Like every other physical structure of the organism, a novel structure finds its immediate origin in development. Here, then, is where we have to look for generative mechanisms of novel morphologies. Or, in the words of Müller and Wagner (1991),

> an empirical approach to the problem of novelty has to focus on the organizational principles of developmental systems and their ability to generate new structures.

Development provides an essential aspect to future novelties, in the intermediate stages of existing structures (Müller, 1990), offering a canvas for the creation of

novel forms. Such a canvas can be found in the early stages of bones and muscles from which the turtle shell evolved—and it can be as simple as an epithelium, in which the localized activation of a morphogenetic programme constitutes the first step in the evolution of a variety of respiratory eggshell appendages.

Defining novelty as a concept across all layers of biological organization may be a quixotic endeavour. However, naming and applying the concept spurs us to seek the most dramatic and complex changes in evolution and attempt to dissect them. The question of novelty is entangled with the history of evolutionary biology, knowing definitions central to function, and to form. In short; novelty, if anything, is an inspiration for the student of evolution—much like the diversity it has created.

Summary

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Summary

Employing development to study the mechanisms of evolutionary change has proven to be a powerful method of investigation. One central theme in evolutionary theory that has benefited extensively from this approach, is the concept of novelty. As difficult as it is to precisely define evolutionary novelty, it is far from complicated to appreciate its importance in the evolution of life. The emergence of a new trait, be it a morphological structure or a behavioural innovation, is crucial in laying the foundation for new variation, which in turn allows natural selection to operate.

A model system like the fruitfly *Drosophila melanogaster*, whose development is extraordinarily well studied, would constitute a valuable addition to the existing models of evolutionary novelty, provided *bona fide* innovations can be clearly identified. Indeed, a morphological novelty is found in the family Drosophilidae: the filaments on the dorsal-anterior end of the *Drosophila* egg, which help supply oxygen during embryonic development, first evolved in a common ancestor of the family, and have since diversified extensively.

These egg filaments, better known as 'dorsal appendages', are formed at the end of oogenesis by the appendage primordia, which are groups of specialized somatic cells of the epithelium surrounding the oocyte. These cells are specified via several signalling pathways, most important of which are the EGF and Dpp pathways. Interestingly, in addition to specifying the appendage primordia, these pathways have other functions during oogenesis: Dpp signalling, for example, is important for cell movements required for the structural integrity of the egg, while EGF signalling establishes the main body axes of the future embryo. It is thus likely that both pathways were present in oogenesis of an ancestral dipteran.

We confirm this assumption in **chapter 2** by comparing oogenesis between *Drosophila melanogaster* and *Ceratitis capitata*. *Ceratitis* is a closely related fly whose eggs do not carry appendages, thus representing the ancestral state. We demonstrate both EGF and Dpp signalling activity in the epithelium that creates the appendage-less eggshell of *Ceratitis*, and speculate that the combined activity of these pathways may have provided the ancestral epithelium with a coordinate system, which was co-opted for further patterning in the evolution of the dorsal appendages.

Moreover, our research on *Ceratitis* oogenesis revealed an interesting paradox: in *Drosophila*, one transcription factor (known as Mirror) regulates both the differentiation of the appendage primordia, and the expression of the gene *pipe*, which defines dorsoventral polarity in the embryo. In *Ceratitis*, expression of the gene encoding Mirror could not be detected. However, we found the expression of *pipe* to be identical in both species. This puzzling result prompted us to hypothesize

on Mirror as a key node in the evolution of the eggshell patterning network.

We addressed the same paradox differently by seeking mutations in *Drosophila* that uncouple embryonic polarity from dorsal eggshell patterning. In **chapter 3** we present some preliminary data on the characterization of one such eggshell mutant in *Drosophila melanogaster*, obtained in a large mutant screen by our collaborator Vítor Barbosa. In this mutant, eggs from a homozygous germline are frequently ventralized, a phenotype characterized by either a single, fused appendage or the complete lack of dorsal appendages. Despite the disrupted eggshell patterning, some ventralized eggs hatch, which demonstrates the viability and correct patterning of the embryo. Our investigation on this mutant is still at an early stage, but the existence of the mutant provides a valuable insight for discussion on developmental robustness and network evolvability, and the connection between ancestral and novel traits.

Lastly, in **chapter 4**, we explore the rich diversity of egg phenotypes within Drosophilidae, combining both *in silico* and *in situ* analyses. Using a computational model developed in collaboration with Adrien Fauré and Claudine Chaouiya, we found that by varying the input along the anterior-posterior axis we could generate patterns resembling the appendage primordia of four-appendage bearing eggshells. We then tested whether Dpp signalling could be directly responsible for the patterning differences between species.

Importantly, our computational model engages with the controversy regarding the role of Dpp in the specification of dorsal appendages. Several existing models have discarded—erroneously, we believe—a positive role for Dpp on the expression of genes in the appendage primordia. In this chapter we make a clear case for Dpp as the main pathway defining the posterior border of eggshell structures.

This thesis marks the first appearance of *Drosophila* oogenesis in the research programme of evolutionary novelty. Our results underline the importance of pre-existing patterns in the evolution of novel morphologies, and specifically identify candidate genes that are likely to have played a pivotal role in the origin of dorsal appendages. Furthermore, this work contributes to the understanding of epithelial patterning during *Drosophila* oogenesis in an evolutionary context, demonstrating that while development can teach us much about evolution, the reverse is also true.

Sumário

Utilizar o estudo do desenvolvimento para compreender os mecanismos de evolução já provou ser um poderoso método de investigação. Um dos temas centrais na teoria evolutiva que já beneficiou extensivamente desta abordagem é o conceito

de "novidade evolutiva". Apesar da dificuldade de definir precisamente o conceito de novidade evolutiva é fácil apreciar a sua importância na evolução da vida. O aparecimento de uma nova característica, seja ela estrutural ou comportamental, é crucial para estabelecer novas fontes de variação, o que, por seu turno, vai permitir à seleção natural operar.

Um organismo modelo como a mosca-do-vinagre (*Drosophila melanogaster*), cujo desenvolvimento está extraordinariamente bem estudado, seria uma adição importante aos modelos biológicos de novidades evolutivas já existentes, se novidades evolutivas puderem ser claramente identificáveis. De facto, uma novidade morfológica está presente na família Drosophilidae: os filamentos na parte dorso-anterior dos ovos, que ajudam a obter oxigénio durante o desenvolvimento embrionário. Estes filamentos primeiro evoluíram num ancestral comum da família e desde então diversificaram extensivamente.

Os filamentos do ovo, denominados 'apêndices dorsais', são formados no fim da oogénese pelos primórdios do apêndice, um grupo especializado de células somáticas do epitélio que circunda o ovo. Estas células são especificadas através de diversas vias de sinalização, sendo as mais importantes a via do Fator de Crescimento Epidérmico (EGF) e a via do Decapentaplegic (Dpp). Curiosamente, para além de especificar os primórdios do apêndice, estas vias de sinalização têm outra função durante a oogénese: a cascata do Dpp, por exemplo, é importante durante os movimentos celulares necessários para a integridade estrutural do ovo, enquanto a via do EGF estabelece os principais eixos do futuro embrião. É, portanto, previsível que estas duas vias tenham estado presentes num díptero ancestral.

Nós confirmamos esta suposição no **capítulo 2** comparando a oogénese de *Drosophila melanogaster* e de *Ceratitis capitata*. A *Ceratitis* é uma espécie de mosca filogeneticamente próxima da família Drosophilidae que não apresenta apêndices dorsais, representado assim o estado ancestral. Demonstramos a actividade de tanto a via de sinalização do EGF e do Dpp no epitélio que cria a casca do ovo sem apêndices dorsais de *Ceratitis*, e especulamos que a actividade combinada destas vias de sinalização pode ter provido o epitélio ancestral de um sistema de coordenadas, que foi utilizado posteriormente para estabelecer a padronização durante a evolução dos apêndices dorsais.

Além disso, a nossa investigação na oogénese de *Ceratitis* revelou um paradoxo interessante: em *Drosophila*, um fator de transcrição (Mirror) regula tanto a diferenciação dos primórdios dos apêndices como a expressão do gene *pipe*, que define a polaridade dorsoventral no embrião. Em *Ceratitis*, a expressão do gene *mirror* não foi detetada. No entanto, os nossos resultados mostram que a expressão do gene *pipe* é idêntica nas duas espécies. Este resultado inesperado levou-nos a propor a hipótese de que Mirror é um nó central na evolução da padronização do epitélio

que circunda o ovo.

Abordámos este paradoxo procurando mutações em *Drosophila* que desacoplam os processos que determinam a polaridade do embrião e a padronização das células epiteliais que circundam o ovo. No **capítulo 3** apresentamos alguns dados preliminares da caracterização de um desses mutantes em *Drosophila melanogaster*, obtido numa análise de uma coleção de mutantes feita pelo nosso colaborador Vítor Barbosa. Neste mutante, os ovos s são frequentemente ventralizados, um fenótipo caracterizado por possuir apenas um apêndice dorsal fundido ou a falta completa de apêndices. Apesar da padronização do epitélio que circunda o ovo estar corrompida, alguns ovos ventralizados eclodem, o que demostra a viabilidade e correta padronização do embrião. A nossa investigação neste mutante ainda está numa fase muito inicial, mas a sua existência fornece uma valiosa informação para a discussão sobre a robustez no desenvolvimento, evolvabilidade das cascatas genéticas e a conexão entre novos caracteres e caracteres ancestrais.

Por último, no **capítulo 4**, exploramos a vasta diversidade de fenótipos dentro do grupo Drosophilidae, combinando análises *in silico* e *in situ*. Usando um modelo computacional, desenvolvido em colaboração com Adien Fauré e Claudine Chaouiya, descobrimos que variando as condições ao longo do eixo ântero-posterior podem-se gerar padrões que se assemelham aos epitélios com dois ou quatro primórdios de apêndice. Seguidamente testámos se a via do Dpp poderia ser diretamente responsável pela diferente padronização entre espécies.

O nosso modelo envolve-se na discussão que diz respeito ao papel da via do Dpp na especificação dos apêndices dorsais. Vários modelos descartaram—de forma errada, no nosso entender—um papel da via do Dpp na expressão de genes nos primórdios dos apêndices. Neste capítulo propomos um cenário onde a via do Dpp funciona como a principal cascata genética a definir a parte posterior das estruturas do epitélio que circunda o ovo.

Nesta tese, pela primeira vez a oogénese de *Drosophila* é utilizada num programa de investigação de novidades evolutivas. Os nossos resultados salientam a importância de padrões pré-estabelecidos na evolução de novas morfologias e identifica especificamente genes candidatos que muito provavelmente desempilharam um papel central na origem dos apêndices dorsais. Para além disso, este trabalho contribui para compreensão da padronização do epitélio durante a oogénese de *Drosophila* num contexto evolutivo, demonstrando que enquanto o estudo do desenvolvimento pode ensinar-nos muita coisa sobre evolução, o contrário também é verdade.

Samenvatting

Het inzetten van ontwikkelingsbiologie om de mechanismen van evolutionaire verandering te onderzoeken, heeft zich bewezen als een krachtige onderzoeksmethode. Een centraal thema in de evolutiebiologie dat hier bij uitstek van profiteert, is het onderzoek naar nieuwe kenmerken, of '*evolutionary novelties*'. Hoewel het lastig is om dit concept precies te definiëren, is het verre van moeilijk om in te zien hoe belangrijk het is voor de evolutie van levensvormen. Een nieuw kenmerk, zij het een morfologische structuur of juist een nieuw soort gedrag, is cruciaal voor het leggen van een basis voor nieuwe variatie, waar natuurlijke selectie vervolgens mee werkt.

Een modelsysteem zoals de fruitvlieg *Drosophila melanogaster*, waarvan de ontwikkeling buitengewoon goed is bestudeerd, zou een waardevolle aanvulling kunnen zijn voor de bestaande modellen voor *evolutionary novelty*, indien inderdaad *bona fide* innovaties kunnen worden geïdentificeerd. Inderdaad is er een morfologisch nieuw kenmerk te vinden in de familie Drosophilidae: de filamenten op het dorsaal-anteriore uiteinde (het voorste deel van de rugzijde) van het ei van *Drosophila*, die helpen in de zuurstofvoorziening tijdens de embryonale ontwikkeling, kennen hun evolutionaire oorsprong in een gemeenschappelijke voorouder van deze familie, en hebben sindsdien een enorme diversiteit aan vormen aangenomen.

Deze eierfilamenten, beter bekend als '*dorsal appendages*', worden gevormd aan het einde van de oögenese, door de zogenaamde filamentprimordia: groepen gespecialiseerde somatische cellen die deel uitmaken van het epitheel rondom de eicel. Welke cellen dit zijn wordt bepaald door de activiteit van verscheidene signaaltransductiewegen, de belangrijkste daarvan zijn EGF en Dpp . Naast de specificatie van de filamentprimordia hebben deze signaaltransductiewegen echter ook andere functies tijdens de oögenese: Dpp, bijvoorbeeld, is belangrijk voor celbewegingen die onontbeerlijk zijn voor de structurele integriteit van het ei , terwijl EGF signalering de hoofdassen van het toekomstige embryo vastlegt . Het is dus aannemelijk dat beide signaaltransductiewegen al actief waren tijdens de oögenese van een voorouder.

Deze aanname bevestigen we in **hoofdstuk 2**, door de oögenese van *Drosophila* te vergelijken met die van *Ceratitis capitata*. *Ceratitis* is een verwante vlieg met eitjes waarop geen filamenten te vinden zijn, en vertegenwoordigt dus de oorspronkelijke staat. Wij laten zien dat zowel EGF als Dpp signalering actief zijn in het epitheel dat de filamentloze schaal van *Ceratitis* eitjes produceert, en speculeren dat de gecombineerde activiteit van deze signaaltransductiewegen in het epitheel van een voorouder een coordinatensysteem definieerde, dat gecoöpteerd is voor verdere patroonvorming in het epitheel in de evolutie van de *dorsal appendages*.

Daarnaast legde ons onderzoek aan *Ceratitis* een interessante paradox bloot:

in *Drosophila* reguleert één transcriptiefactor (Mirror genaamd) zowel de differentiatie van de filamentprimordia, en de expressie van het gen *pipe*, dat de dorsoventrale polariteit in het embryo definiëert. In *Ceratitis* konden we geen expressie vinden van het gen dat Mirror codeert, maar de expressie van *pipe* bleek identiek in beide soorten. Dit verrassende resultaat was de bron van de hypothese dat Mirror een belangrijk knooppunt is in de evolutie van het netwerk voor patroonvorming op de eierschaal.

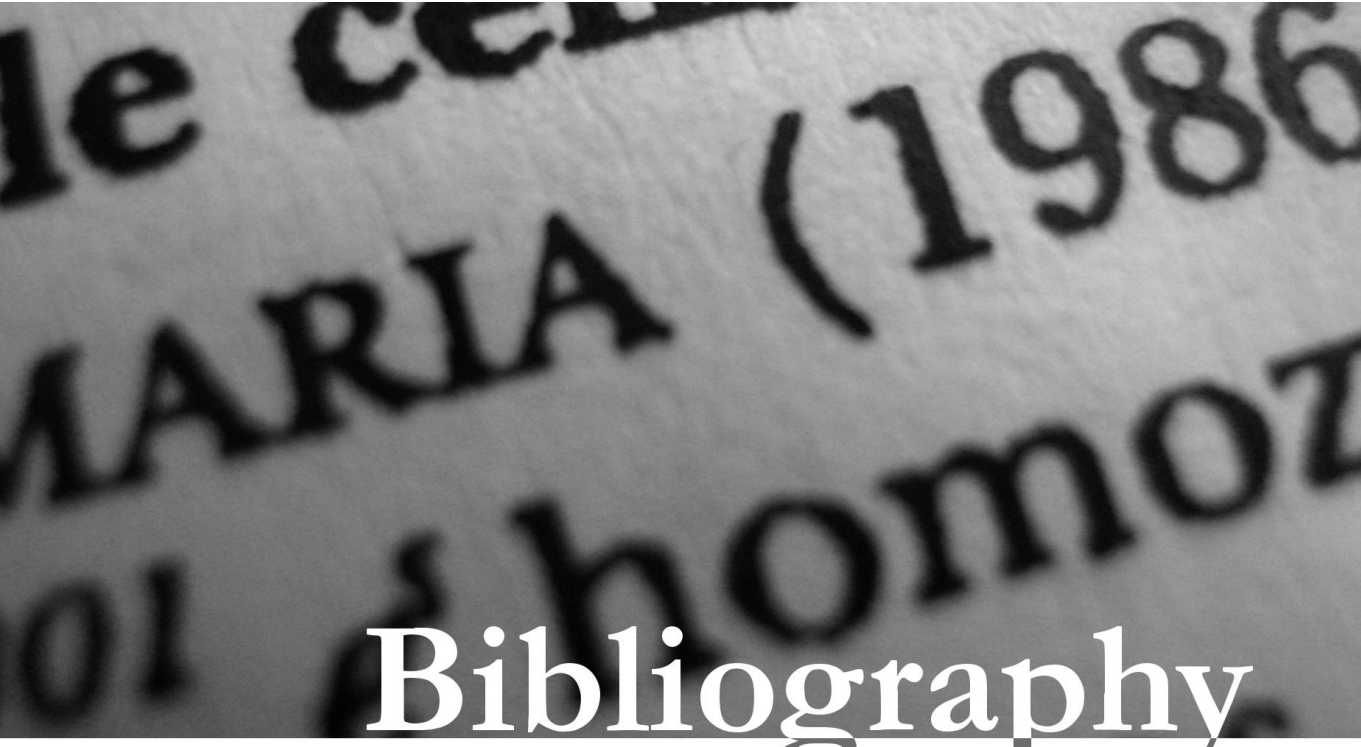
Diezelfde paradox hebben we op een andere manier benaderd door mutaties te zoeken in *Drosophila* waardoor polariteit van het embryo en patroonvorming op de eierschaal van elkaar ontkoppeld waren. In **hoofdstuk 3** presenteren we enkele voorlopige resultaten van de karakterisatie van zo een mutant in *Drosophila melanogaster*, verworven uit een uitgebreide mutantenscreen die gedaan werd door onze collega Vítor Barbosa. Eitjes die met deze mutatie worden gemaakt zijn vaak geventraliseerd, een fenotype dat gekenmerkt wordt door ofwel een enkel, gefuseerd filament, of zelfs een compleet gebrek aan *dorsal appendages*. Ondanks deze verstoorde patroonvorming van de eierschaal komen sommige eitjes toch uit, waarmee de levensvatbaarheid en de correcte ontwikkeling van het embryo worden bewezen. Ons onderzoek naar deze mutant is nog in een zeer vroeg stadium, maar het bestaan van deze mutant biedt niettemin een waardevol inzicht in de robuustheid van ontwikkeling en de evolueerbaarheid van netwerken, alsmede de verbintenis tussen oorspronkelijke en nieuwe kenmerken.

Tenslotte, in **hoofdstuk 4**, verkennen we rijke diversiteit in de verschijningsvormen van Drosophilidae eitjes, waarbij we *in silico* en *in situ* analyses combineren. Door een computermodel te gebruiken dat ontwikkeld werd in samenwerking met Adrien Fauré en Claudine Chaouiya, zagen we dat door de *input* te varieren over de as van anterior-posterior, we patronen konden genereren die leken op de filamentprimordia van de eierschalen met vier filamenten. Hierna onderzochtten we of signallering door Dpp wellicht direct verantwoordelijk zou kunnen zijn voor verschillen in de patronen tussen deze soorten.

Ons model mengt zich in de controverse over de rol die Dpp speelt in het vaststellen van de eierfilamenten. Meerdere bestaande modellen hebben—ten onrechte, zo denken wij—een positieve invloed van Dpp op de expressie van genen in de filamentprimordia verworpen. In dit hoofdstuk zetten we een duidelijk argument uiteen vóór Dpp als de belangrijkste signaaltransductieweg die de posteriore grens van de eierschaalstructuren bepaalt.

Met dit proefschrift doet de *Drosophila* oögenese zijn intrede in het onderzoeksprogramma naar *evolutionary novelties*. Onze resultaten onderstrepen het belang van reeds bestaande genexpressiepatronen voor de evolutie van nieuwe morfologische vormen, en identificeren genkandidaten die waarschijnlijk een sleutelrol hebben

gespeeld in het ontstaan van de eierfilamenten. Hiernaast draagt dit werk met een evolutionaire insteek bij aan de kennis over patroonvorming op het epitheel tijdens de oögenese van *Drosophila*, waarmee we laten zien dat het niet alleen zo is dat de ontwikkeling ons veel kan leren over evolutie, maar dat dit andersom ook werkt.



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Having all the answers
just means you've been
asking boring questions.
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