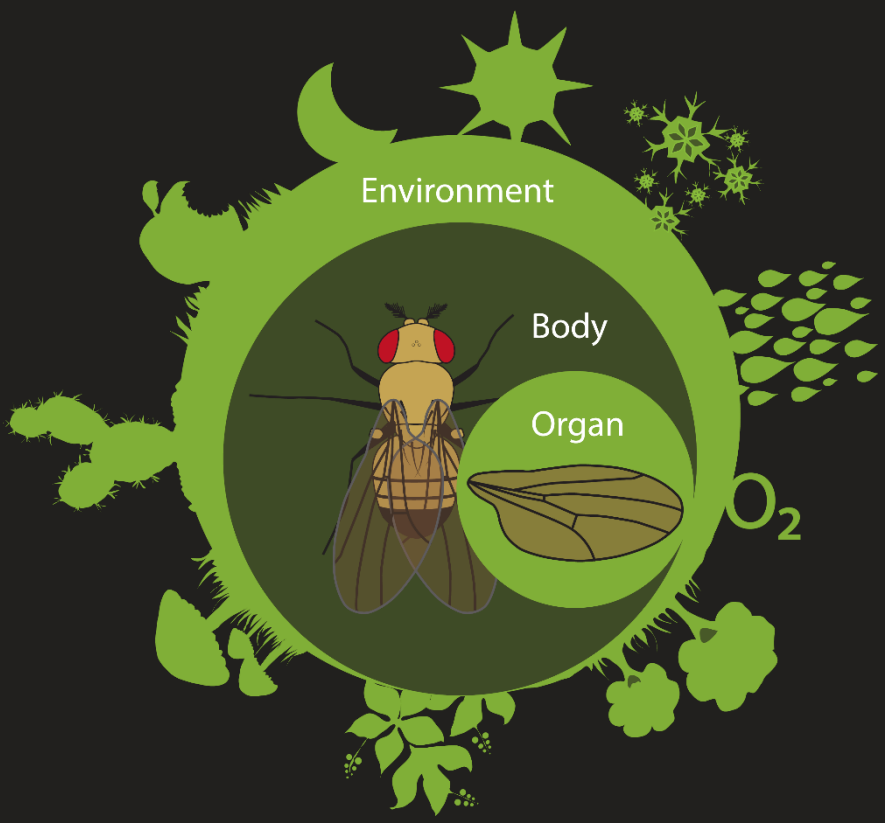
Coordinating development: uncovering the mechanisms that coordinate organ growth and patterning with the development of the whole body

Marisa Mateus de Oliveira



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
July, 2014

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Thesis successfully defended on the 1st of July, 2014 at Instituto de Tecnologia Química e Biológica António Xavier, Oeiras, Portugal, before a jury presided over by:

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Declaração/Declaration

Esta dissertação resulta do meu trabalho de investigação desenvolvido entre Janeiro de 2010 e Maio de 2014 no laboratório da Dr. Christen K. Mirth, Desenvolvimento, Evolução e o Ambiente, no Instituto Gulbenkian de Ciência (IGC) em Oeiras, Portugal, no âmbito do Programa de Doutoramento do IGC (edição 2009-2010). O capítulo 2 desta dissertação foi aceite para publicação na revista PloS Genetics, enquanto que os capítulos 3 e 4 constituem parte de dois artigos que se encontram em preparação para serem publicados.

This dissertation results from my own research, carried out between January 2010 and May 2014 in the laboratory of Dr. Christen K. Mirth, Development, Evolution and the Environment, Instituto Gulbenkian de Ciência (IGC) in Oeiras, Portugal, under the Gulbenkian Doctoral Programme (2009-2010 edition). Chapter 2 has been accepted for publication in PLoS genetics, while chapters 3 and 4 are part of two manuscripts in preparation for publication.

Apoio Financeiro/Financial Support

O apoio financeiro foi atribuido pela Fundação para a Ciência e a Tecnologia, bolsa de doutoramento SFRH/BD/51181/2010 e pela Fundação Calouste Gulbenkian de Outubro 2013 a Junho 2014.

Financial support for this thesis was provided by the Fundação para a Ciência e a Tecnologia, doctoral fellowship SFRH/BD/51181/2010 and by Calouste Gulbenkian Foundation from October 2013 until June 2014.

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Research work coordinated by:

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Tu deviens responsable pour toujours de ce que tu as apprivoisé.

You become responsible, forever, for what you have tamed.

Tu te tornas eternamente responsável por aquilo que cativas.

"Le Petit Prince" - Antoine de Saint-Exupéry

Acknowledgements

I did not feel like writing this part of the thesis, not because I am not grateful for the people that shaped my progress, but because I believe that acknowledging people is a continuous responsibility. Far more important than the experiments I have done, are the ideas that I built with the help of so many.

I start by acknowledging my family for making me believe that I can do everything that I set my mind to, for the unconditional love and continuous support, for all the challenges and for making my life so full. Most important, to my Pedro, for all the patience, love and support, and for the long hours waiting for me, especially during the long-end writing period of this thesis.

I would like to acknowledge the people with whom I started my life in science. First, Miguel Godinho for showing me what drives science: the curiosity for knowing the end of a story. Second, Patrícia Beldade, for being so honest when I asked what is like to be a scientist. “There are good and bad days” she said. She inspired me to see what the good days were like and indeed the good days are far better and important than the bad ones. Last, Filipa Alves, for the unending source of ideas and enthusiasm that inspired my way of thinking about biology.

Every student is different and certainly, every supervisor as well. I picked the best. I thank my supervisor and friend, Christen Mirth, for the kindness and generosity that always came with every smile. I cannot disregard of the brilliant and inspiring scientist she is and thank her for making me enjoying every single step of the hard path of doing a PhD. Her help and comments were instrumental for this thesis.

Thank my unofficial supervisor, Alexander Shingleton for being essential in this step of my scientific career. The beginning at MSU gave me new tools, challenges and ways of thinking and being curious about science. His enthusiasm, patience and his straightforward and truthful opinion during this four years shaped my science and made me into a better scientist.

I am very grateful for the opportunity of doing research the IGC, such a charming and friendly place to work. Thanks to Manuela who always saved us from the bureaucracy monster. Thanks to my charismatic first director of the PhD Program, Thiago Carvalho, who, during the writing of this thesis pretend to be a fish for me so I could recharge my energies laughing.

Thanks to the Evo Devo community for the continuous input on all my research projects. Thanks to the fly facility for making our lives so easy and to the UIC, specially PAlmada and Nuno Pimpão for saving me in all the times that the Confocal did not cooperate.

Thanks to everyone in my lab, past and present members for continuous support and availability to help. In special, I thank Takashi for the enthusiastic and always present friend. Thanks to Marisa for being such a young and strong inspiring scientist, although she does not know it yet. Thanks to Andreia for the precious and dedicated help during the past year, without whom my flies would die.

Friends, close and far, thanks for making my life complete. Thanks to Raquel for all the support and for the help with the abstract. Finally, I want to thank my fellow scientists and colleagues who contributed to this project.

To all, a very heartfelt obrigada.

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Summary

Organisms produce correctly patterned structures across a wide range of organ and body sizes. Despite considerable work revealing the mechanisms that regulate the growth and patterning of organs, those responsible for coordinating organ development with whole-body development are still largely unknown. In this thesis, we propose that organs coordinate their growth and patterning with the development of the whole body at discrete developmental milestones. These milestones, in turn, are regulated by hormonal and nutrition-sensitive pathways, the result of which allows animals to be plastic in terms of size but robust in terms of patterning.

In the fruit fly *Drosophila melanogaster*, the precursor of the adult wing, the wing imaginal disc, starts to grow and pattern early in development. Several patterning cascades define the axes and regions of the disc and confer cell-type identity to epithelial cells. These patterning events occur as the wing disc grows. Also, the discs grow and pattern in coordination with the whole body so that if the discs are damaged, metamorphosis is delayed.

The steroid moulting hormone ecdysone regulates the transition between life stages and the development of the body. Peaks of ecdysone orchestrate the timing of developmental events such as the larval moults and pupariation. Ecdysone production is promoted by myriad stimuli, including the insulin-like peptides, produced in response to the nutritional status of the body. Ecdysone is also necessary for the patterning of the imaginal tissues, thus is a likely candidate for the signal coordinating organ development with the development of the whole body.

There are several hypotheses explaining how organ growth and patterning are coordinated with the development of the body under varied environmental or physiological conditions. First, this coordination could result from tight, continuous communication between the mechanisms that regulate patterning, growth and developmental timing in the organs and those that regulate these processes in the body. Second, coordinated development could also arise from a checkpoint-like regulation, where patterning, growth and developmental time coordinate in a discrete manner, only at developmental milestones.

In this thesis, we examined the developmental processes and molecular mechanisms through which organs coordinate their own patterning and growth and how they coordinate their development with the development of the whole body. First, we postulated that patterning of the wing disc is robust to changes in environmental and physiological conditions by coordinating with the body development either continuously or at developmental milestones. Second, we studied the changes in growth trajectories of the wing discs in conditions known to generate variation in adult wing size. Further, we explored the nature of the relationship between growth and patterning. Last, we addressed how ecdysone and insulin/TOR (nutrition-dependent) pathways contribute to the growth and patterning of the wing disc, and consequently, coordinate wing disc with the whole-body development.

To explore how the progression of pattern changes with altered environmental/physiological conditions, we developed a staging scheme based on the change in expression of key components of the patterning cascades of the wing disc. Next, we altered developmental time either

environmentally, by altering temperature, or physiological, by changing ecdysone synthesis. Using the staging scheme, we found two milestones that coordinate patterning with whole-body development, the moult to the third instar and pupariation. These milestones align patterning when developmental time is altered, and allow for variation in patterning rate between milestones.

Using a similar approach, we examined the growth trajectories of wing discs from larvae reared in conditions known to generate large and small adult wings and asked how wing disc size is altered across developmental time. We found there are several ways to make wings of different sizes: increases in wing size tended to result from processes during pupal development whereas decreases in wing size occurred due to altered growth rates and/or altered length of the growth period. Even for discs that attained the same size at pupariation, the growth trajectories differed. Despite these differences, all trajectories showed fast initial growth rates followed by slow growth rates. We proposed that this change in growth rate could be due to active regulation of growth rates at developmental milestones. Further, we found that patterning milestones and growth milestones seem to take place at different developmental times, which suggest that are differently regulated. We then linked the size of discs to patterning stage. We observed tight correlations between size and pattern within a condition, but significant differences in the intercepts and slopes of the regression lines between conditions. Thus, discs achieved the same patterning stage over a broad range of sizes.

Finally, to gain insight into the signalling pathways involved in coordinating wing disc and whole-body development, we explored the roles

of ecdysone and nutrition-dependent signalling in regulating wing disc growth and patterning. We found that ecdysone is necessary and sufficient to promote both wing disc growth and patterning. Nutrition-dependent signalling also induced wing disc growth, although it appeared unimportant for patterning. Previous work had identified two phases of growth regulation in the disc, an early nutrition-dependent phase and a later phase characterized by morphogenetic growth that is less sensitive to nutrition. Our work provides evidence that ecdysone signalling underlies morphogenetic growth of the disc.

Furthermore, our data suggest that the growth milestones that we characterized for the disc are ecdysone and nutrition dependent, whereas the patterning milestones rely on ecdysone alone. The contribution of multiple pathways to the growth of the wing disc potentiates the range of possible sizes produced. In turn, because patterning depends only ecdysone signalling, it remains robust across environmental or physiological conditions.

Keywords:

Developmental timing
Patterning
Developmental milestones
Coordination of development
Developmental robustness
Coordinated growth
Growth trajectories
Size regulation
Ecdysone

Sumário

Os organismos têm a capacidade de produzir estruturas com padrões de expressão genética corretos, numa grande diversidade de tamanhos dos órgãos e do corpo. Apesar da extensa investigação sobre os mecanismos que regulam o crescimento e o estabelecimento de padrões de expressão dos orgãos, os mecanismos responsáveis pela coordenação do desenvolvimento dos órgãos com o desenvolvimento do corpo são pouco conhecidos. Nesta tese, propomos que os orgãos coordenam o seu crescimento e o estabelecimento de padrões de expressão com o crescimento do corpo por etapas, *milestones*, ao longo do desenvolvimento do organismo. Propomos também que estas *milestones* estejam sob regulação de vias de sinalização que respondem a alterações no estado nutricional e hormonal do organismo, permitindo aos animais plasticidade em termos de tamanho, mas robustez no estabelecimento de padrões de expressão.

Na mosca da fruta, *Drosophila melanogaster*, o crescimento e estabelecimento dos padrões de expressão do disco imaginal da asa, o percursor da asa adulta, são iniciados no início do desenvolvimento. Várias vias de sinalização definem os eixos e as regiões do disco, os tipos celulares existentes e conferem identidade a células epiteliais. Estes eventos de estabelecimento de padrões de expressão ocorrem ao longo do crescimento do disco da asa. O desenvolvimento dos discos é coordenado com o do organismo de tal modo que, se os discos forem danificados, a metamorfose é atrasada.

A ecdisona, a hormona esteróide responsável pela muda e metamorfose, regula a transição entre estádios de desenvolvimento e o desenvolvimento

do organismo. Picos desta hormona determinam a ocorrência de determinados eventos de desenvolvimento, tal como os diferentes estádios larvais e pupariação. Vários estímulos promovem a produção de ecdisona, incluindo péptidos semelhantes a insulina que são produzidos em resposta ao estado nutricional do organismo. A ecdisona é também necessária para o estabelecimento dos padrões de expressão dos tecidos imaginais constituindo, consequentemente, um bom candidato para sinal mediador de coordenação do desenvolvimento dos órgãos com o desenvolvimento do corpo.

Existem várias hipóteses para explicar a coordenação entre o desenvolvimento do corpo e o crescimento e estebelecimento de padrões de expressão dos orgãos sob diferentes condições ambientais e fisiológicas. Primeiro, esta coordenação pode resultar de uma comunicação contínua e estritamente regulada entre os mecanismos que regulam o estabelecimento de padrões de expressão, o crescimento e o tempo de desenvolvimentos dos órgãos e do corpo. Segundo, o desenvolvimento coordenado pode resultar de uma regulação em pontos-de-controlo, em que o estabelecimento de padrões de expressão, o crescimento e o tempo de desenvolvimento são coordenados de uma maneira discreta apenas em determinadas *milestones* de desenvolvimento.

Nesta tese, investigámos os processos de desenvolvimento e os mecanismos moleculares através dos quais o tamanho dos órgãos é coordenado com estabelecimento de padrões de expressão do orgão e com o desenvolvimento do corpo. Primeiro, propomos que o estabelecimento de padrões de expressão no disco da asa é robusto a alterações ambientais e fisiológicas, através da sua coordenação com o desenvolvimento do corpo,

de um modo contínuo ou discreto através de *milestones*. Segundo, estudámos as trajectórias de crescimento dos discos da asa sob condições que geram variação no tamanho da asa adulta. Além disso, explorámos a natureza da relação entre o crescimento e o estabelecimento de padrões de expressão. Por fim, investigámos como as vias de sinalização da ecdisona e da insulina/TOR contribuem para o crescimento e o estabelecimento de padrões de expressão do disco da asa e, consequentemente, coordenam o desenvolvimento do disco da asa com o do corpo.

Inicialmente, para explorar a variação no estabelecimento de padrões de expressão com diferentes condições ambientais e fisiológicas, desenvolvemos uma ferramenta para atribuição de estádios baseado nos padrões de expressão de componentes chave das vias de sinalização de estabelecimento de padrões do disco da asa. Seguidamente, alterámos o tempo de desenvolvimento manipulando condições ambientais, alterando a temperatura, ou alterando as condições fisiológicas do organismo, alterando a via de sinalização de síntese de ecdysona. Ao usar a ferramenta desenvolvida para atribuição de estádios, revelámos a existência de duas *milestones* que coordenam o estabelecimento de padrões de expressão do disco com o desenvolvimento do corpo: a muda de segundo estádio larvar para terceiro e pupariação. Estas *milestones* alinham os padrões de expressão quando o tempo de desenvolvimento é alterado.

Usando uma abordagem semelhante, analisámos as trajetórias de crescimento de discos da asa de larvas mantidas em condições em que se é sabido gerarem adultos com asas maiores e mais pequenas, e explorámos como o tamanho das asas varia com o tempo de desenvolvimento. Revelámos que existem várias formas de produzir asas de tamanhos

diferente: o aumento do tamanho das asas resultou, em geral, de processos que acontecem durante o desenvolvimento da pupa enquanto que a diminuição do tamanho das asas ocorreram por alteração das taxas de crescimento e/ou alteração da duração do período de crescimento. Discos que atingiram o mesmo tamanho quando no momento de pupariação, mostraram trajetórias de crescimento diferentes. Apesar das diferenças, todas as trajectórias exibiram taxas de crescimento iniciais elevadas que mais tarde são reduzidas. Propomos que esta alteração nas taxas de crescimento se deve à regulação que ocorre nas *milestones* de crescimento. Revelámos também que as *milestones* de estabelecimento de padrões de expressão e as de crescimento ocorrem em tempos de desenvolvimento distintos, o que sugere que são reguladas de modo diferente. Seguidamente, relacionámos o tamanho dos discos da asa com os estádios de padrões de expressão. Observámos uma estreita correlação entre o tamanho e os padrões de expressão dentro de cada condição, mas diferenças significativas nas intersecções e declives das linhas de regressão linear entre condições. Portanto, os discos atingem o mesmo estádio de padrão de expressão mesmo exibindo diferentes tamanhos.

Em conclusão, para melhorar o nosso conhecimento sobre as vias de sinalização envolvidas na coordenação do tamanho dos discos com o tamanho do corpo, explorámos as contribuições da via da ecdisona e das vias dependentes da nutrição (insulin/TOR) para regular o tamanho dos discos e os padrões de expressão. Revelámos que a ecdisona é necessária e suficiente para promover tanto o crescimento como os padrões de expressão nos discos das asas. As vias de sinalização dependentes da nutrição induziram também crescimento dos discos, apesar da ausente contribuição para os padrões de expressão. Trabalhos publicados identificaram duas fases de

regulação de crescimento de tamanho no disco, uma fase inicial que é dependente da nutrição a uma fase mais tardia caracterizada por crescimento morfogenético que é menos sensível à nutrição. O nosso trabalho mostra que a via de ecdisona é essencial ao crescimento morfogenético do disco.

Os nossos resultados sugerem que as *milestones* de crescimento que caracterizámos para o disco são dependentes tanto de ecdisona como da nutrição, enquanto que as *milestones* de padrões de expressão dependem apenas de ecdisona. A contribuição de múltiplas vias de sinalização para o crescimento do tamanho do disco potencia a gama de possíveis tamanhos produzidos. Por sua vez, visto que o estabelecimento de padrões de expressão depende apenas da via de ecdisona, este mantem-se robusto em diferentes condições ambientais e fisiológicas.

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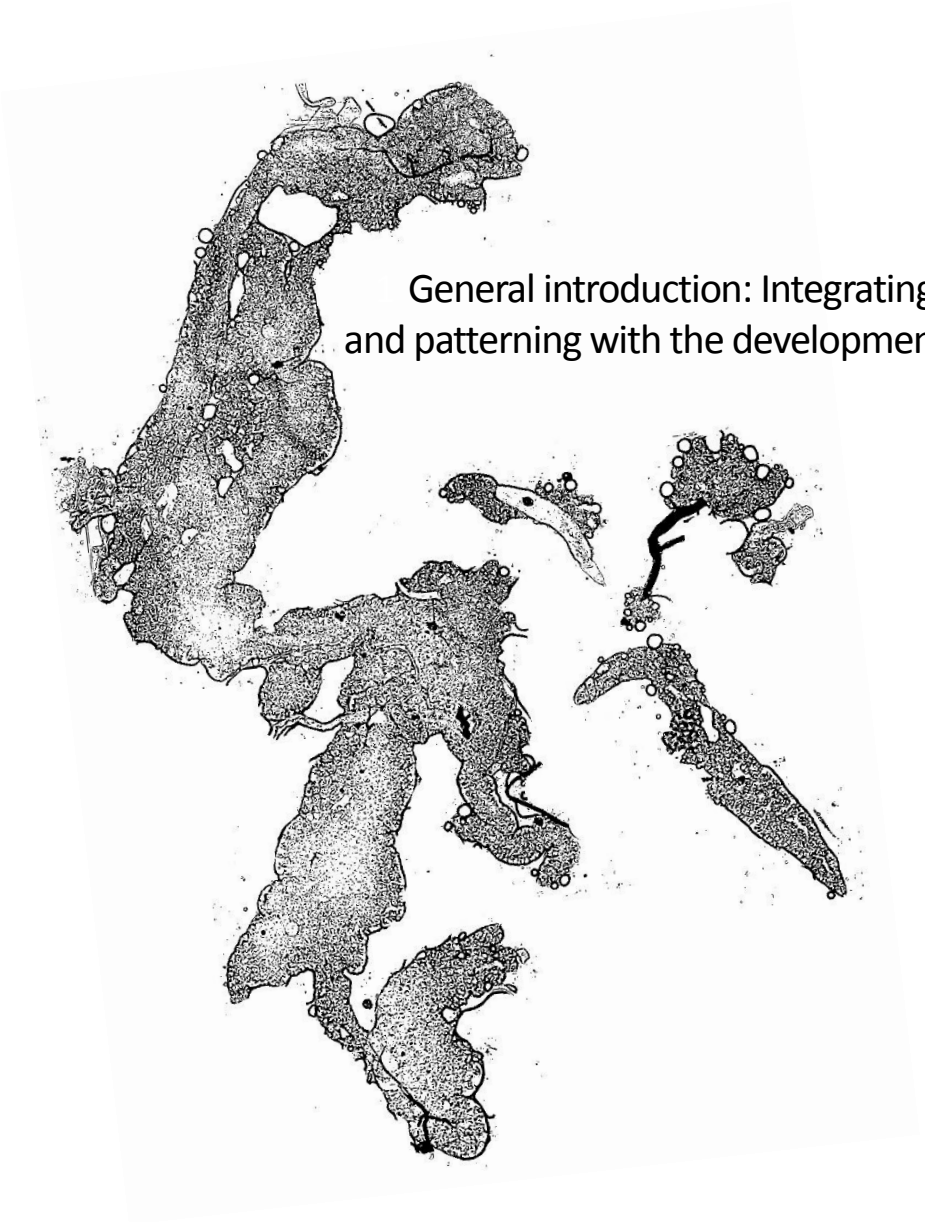


General introduction: Integrating organ growth and patterning with the development of the whole body

The diversity of sizes that organisms exhibit in nature is astonishing. Even within a species, organisms show considerable variation in size. Despite this large variation in body size, organs are robust in their pattern and proportions across a wide range of conditions, environmental, genetic, and physiological (Mirth and Shingleton, 2012; Nijhout et al., 2013). A developmental process can be considered robust if variation in this process is uncorrelated with variation in genetic, environmental or physiological conditions (Nijhout, 2002). To achieve robustness, the developmental processes that generate individual organs must, at some level, be integrated across the whole body to ensure that a correctly patterned and proportioned adult is produced at the end of development. Despite considerable knowledge of the molecular mechanisms controlling patterning and growth of developing organs, we know surprisingly little about 1) how organs coordinate their growth with their patterning and 2) how organ development is integrated with whole-body development when animals are faced with changes in environmental and physiological conditions. In this work, we aim to address both questions by studying the patterning and growth of the developing wing in relation to itself and to the development of the whole body in the fruit fly *Drosophila melanogaster.*

Drosophila has been used extensively to study the developmental mechanisms that allow organs to pattern and grow. In particular, work on the precursors of adult tissues, the imaginal discs, has provided invaluable insight into these processes (for reviews see (Andersen et al., 2013; Restrepo et al., 2014; Shingleton, 2010; Tennessen and Thummel, 2011)). Imaginal discs are pouches of tissue that grow and differentiate during larval development, which will later metamorphose to form the adult structures. The wing imaginal disc is been a favourite system to investigate the links

between patterning and growth (Whittle, 1990) due to the availability of sophisticated genetic tools, detailed knowledge of the patterning mechanisms (Cohen, 1993) and their exponential growth trajectories (Shingleton et al., 2008).

Coordination between wing imaginal disc growth and patterning

Wing disc both grows and patterns simultaneously. The wing disc forms during embryogenesis, grows and patterns as an approximately two dimensional structure during the three stages of larval development (Cohen, 1993). The progression of gene expression that occurs in discs as they pattern is somehow integrated with the systemic hormone levels that trigger transitions between developmental stages (hereafter termed developmental events) across the whole body (Tennessen and Thummel, 2011; Yamanaka et al., 2013). The timing of these developmental events changes with environmental and physiological conditions, but how these changes in timing affect wing development is not fully understood. After finishing its growth and patterning during pupal development, the wing disc differentiates and finally stretches into its final shape at eclosion (Figure 1.1).

Imaginal tissues grow by coordinating increases in cell size, known as cell growth, and rates of cell proliferation, which alters cell number. The wing disc is a highly proliferative tissue; it has roughly 30 cells at hatch (Madhavan and Schneiderman, 1977), starts to grow during the first larval instar and grows up to about 50,000 cells by the end of larval development (Bryant and Levinson, 1985; Milan et al., 1996). Until recently, more emphasis has been placed on understanding the control of cell cycle progression than on the mechanisms regulating cell growth. This has often led to the use of

proliferation and growth as synonymous terms. However, altering proliferation either in the entire wing disc or in specific disc compartments produces normally sized organs with either exceptionally small or large cells (Neufeld et al., 1998; Weigmann et al., 1997). For instance, constitutive activation of the *Myc* oncogene in the posterior compartment of the wing disc increases the size of cells in this compartment, but it has little effect on the size of imaginal discs (Johnston et al., 1999).

These results reveal that individually, the mechanisms that regulate cell growth or proliferation are not essential by themselves for the final size of

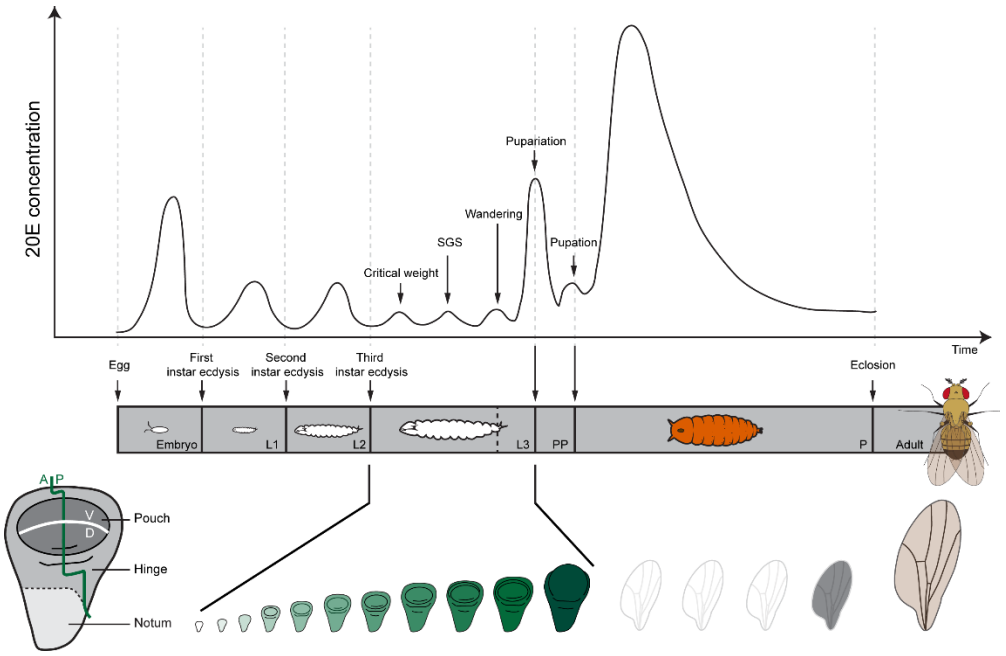


Figure 1.1. Ecdysone is produced in the prothoracic glands and released in pulses to the rest of the body to control the timing of developmental transitions like the larval moults (first larval instar (L1), second larval instar (L2) and third larval instar (L3)), critical weight (CW), salivary gland secretion (SGS), wandering (W) and metamorphosis (pre-pupal (PP) and pupal (P) stages). Peripheral tissues, most notably the larval fat body, modify ecdysone into its biologically active form, 20-hydroxyecdysone (20E). The bottom panel is a schematic drawing of wing disc development through time, of the anterior-posterior (AP), dorsal-ventral (DV) axes of the disc and of the pouch, hinge and notum regions of the disc.

the disc, but that the coordination between both is. In fact, the wings of wild-type flies show a negative correlation between cell size and cell number (Debat et al., 2011). Levels of the cell cycle regulator Cyclin G (CycG) appear to coordinate the balance between cell growth and proliferation (Debat et al., 2011). Overexpressing CycG does not affect wing patterning, but does alter wing shape and results in flies with dramatic differences in size between the left and right wing that are random in side and amplitude (Debat et al., 2011). The same phenotype is not observed by overexpressing other genes involved in cell growth or cell cycle regulation such as *dS6K*, *Myc*, *CycD* or *CdK4,* suggesting that CycG plays a distinct role in regulating the cell size/cell number balance (Debat et al., 2011).

Wing disc patterning throughout the larval period generates three distinct regions in the disc: pouch, hinge and notum (Figure 1.1). The wing pouch gives rise to the adult wing blade and is specified by the expression of the transcription factor Vestigial (Williams et al., 1993; Williams et al., 1991). In turn, the hinge region of the disc will form the wing hinge (Casares and Mann, 2000), while the notum will form the dorsal part of the thorax (mesonotum) (Grieder et al., 2009). The anterior-posterior (AP) boundary of the wing disc is inherited from the embryonic ectoderm by the expression of Engrailed (Morata and Lawrence, 1975; Vincent and O'Farrell, 1992), which subdivides the wing in two compartments. The AP boundary becomes later a source of the morphogen Decapentaplegic (Dpp) (Zecca et al., 1995). The wing pouch is further subdivided in dorsal and ventral (DV) compartments in the second larval instar through the localized expression of the Apterous protein in dorsal cells (Diaz-Benjumea and Cohen, 1993). Interactions between dorsal and ventral wing cells lead to the production of the morphogen Wingless (Wg), produced along the DV boundary (Couso et al.,

1994; Diaz-Benjumea and Cohen, 1993; Ng et al., 1996). The two signalling ligands, Dpp and Wg, each form a gradient (Entchev et al., 2000; Nellen et al., 1996; Strigini and Cohen, 2000; Zecca et al., 1996) that together provide many of the essential cues required for pattern formation.

Several lines of evidence suggest that, in addition to tissue patterning, these two morphogens regulate disc growth. For example, wings fail to grow in the absence of Dpp (Nellen et al., 1996; Spencer et al., 1982) and ectopic activation of Dpp triggers excess growth, at least in some regions of the wing disc (Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002; Nellen et al., 1996; Restrepo et al., 2014; Rogulja and Irvine, 2005). Likewise, wings fail to grow in the absence of Wg (Couso et al., 1994). Although Wg was initially thought to be a survival factor for the wing disc (Giraldez and Cohen, 2003; Johnston and Sanders, 2003), recent results suggest that Wg induces growth (Baena-López et al., 2009) and controls proliferation in the wing hinge (Dichtel-Danjoy et al., 2009). In cases where cell death is induced by injury or x-rays, compensatory proliferation is activated by Wg or Dpp, further underscoring the roles of these morphogens as growth organizers in the wing disc (for a review see (Martín et al., 2009)). Thus, the same processes that pattern the wing disc also regulate its growth, suggesting that these molecules may be necessary for coordinating both processes.

We have long known about the importance of morphogens in inducing the patterning and growth of imaginal tissues, however, recent data supports the notion that morphogens may also regulate whole-body development. Larvae with membrane-tethered Wg expressed in all tissues except the imaginal discs delay their development (Alexandre et al., 2014). This suggests that the diffusion of Wg in tissues other than imaginal discs is necessary for

the correct timing of larval development. Moreover, this systemic inhibition of whole-body development also inhibited the overgrowth of the wing discs that could have resulted from extended larval development, and these animals have normal-sized wings. These data illustrate that to understand how discs grow and pattern in coordination, we have to consider how organ development is coordinated with that of the body as a whole.

Organ/whole-body interactions during development

The scaling relationship between the size of an organ and the size of other organs or body is called allometry and is central to the evolution of morphology and shape. Most traits scale proportionally with one another and with body size such that larger adults have, for example, proportionally larger wings (for a review see (Shingleton and Frankino, 2013)). Organs that scale proportionally with one another are referred to as isometric. When the relationship in size between two organs is plotted on a log-log scale, the slope of the scaling relationship between these organs would be approximately 1 for isometric traits (Huxley and Teissier, 1936). Organs that show disproportionate increases in size with respect to other organs or the whole body have hyperallometric scaling relationships with slopes >1; those that show little increase in size relative to other organs or the whole body have hypoallometric scaling relationships with slopes <1. Finally, although most scaling relationships are linear on a log-log scale, some organs have sigmoidal or discontinuous scaling relationships (Emlen and Nijhout, 2000). Importantly, to achieve any of these types of scaling relationships organs coordinate their development with other tissues and with the whole body.

Drosophila adult body size is limited by the growth induced by feeding during the larval stages. The increase in body weight depends on nutrition from hatch until the onset of wandering (referred as the feeding period). At wandering, animals stop eating to search for a pupariation site and during this pre-pupal period they lose weight (Testa et al., 2013). Because adults do not grow, once larvae initiate metamorphosis their final size is fixed.

The timing of developmental transitions like the larval moults and the onset of metamorphosis are regulated by the steroid moulting hormone ecdysone. Ecdysone is produced in the prothoracic glands (PG) and released in pulses to the rest of the body (Figure 1.1). Peripheral tissues, especially the larval fat body, modify ecdysone into its biologically active form, 20-hydroxyecdysone (20E)[1]. Interestingly, pulses of ecdysone also regulate the development of the imaginal discs (Riddiford, 1993). In *Drosophila*, a peak of ecdysone at the beginning of the third instar regulates a developmental event called critical weight (CW). If larvae are starved before reaching critical weight, they either delay the timing of the CW ecdysone pulse, the onset of metamorphosis (Beadle et al., 1938; Mirth et al., 2005; Shingleton et al., 2005; Stieper et al., 2008) and the patterning of their imaginal discs (Mirth et al., 2009) or die. In contrast, starving larvae after they have reached critical weight accelerates the time to metamorphosis and permits continued patterning of the wing imaginal discs (Mirth et al., 2005; Mirth et al., 2009; Shingleton et al., 2005; Stieper et al., 2008).

Similarly, at larval wandering, ecdysone controls both the patterning and differentiation of sensory neurons in the wing discs and eye (Brennan et al.,

[1] For simplicity, in this thesis we use the term ecdysone to refer to both ecdysone and 20-hydroxyecdysone.

1998; Schubiger et al., 2005; Schubiger and Truman, 2000) and stimulates autophagy in larval tissues like the fat body (Rusten et al., 2004). Ecdysone pulses also control wandering and pupariation, thereby coordinating behaviour, glue production, cuticle production, disc eversion/patterning and nervous system metamorphosis. Thus, these pulses of ecdysone have been interpreted to be checkpoints that coordinate the patterning and development of organs with whole-body developmental transitions (Rewitz et al., 2013; Yamanaka et al., 2013).

Damaging discs, or slowing their growth genetically, retards the development of the non-affected discs and of the whole body (Clifton and Daniel, 1990; Halme et al., 2010; Parker and Shingleton, 2011; Pat and Howard, 1975; Russell, 1974; Simpson et al., 1980; Stieper et al., 2008). In this manner, the slow-growing discs synchronise their development with the non-affected discs, only allowing whole-body development to progress when the development of all tissues is complete. Interestingly, larvae that lack imaginal discs do not delay their metamorphosis (Simpson et al., 1980) revealing that the coordination signal is released from discs to inhibit whole-body development.

The nature of the signal that assesses/coordinates the development of tissues with the whole body was unknown for many decades. Recently, two studies identified *Drosophila* Insulin-like peptide 8 (DILP8) as a signal that acts to coordinate organ-organ and organ-body development (Colombani et al., 2012; Garelli et al., 2012). DILP8 is released from slow-growing discs and acts to reduce ecdysone biosynthesis in the PG, thereby delaying metamorphosis. Interestingly, this signalling molecule may also be a coordinator of growth between organs. *Dilp8*-mutant animals develop

normally, however, show increased size asymmetry between bilateral organs. This means that even in normal development, DILP8 may be acting to continuously coordinate the size of discs, resulting in correctly proportioned adult organs. Thus DILP8 both regulates the duration of development in response to perturbed organ growth and coordinates organ sizes.

It is obvious the role of ecdysone in regulating whole-body transitions during normal development, as well as the development of imaginal tissues. In turn, DILP8 regulates ecdysone synthesis to ensure that discs are proportional. At the level of the tissue, the coordination between growth and pattern could be achieved by morphogens. Could DILP8 function to maintain coordinated tissue patterning as well? DILP8 provides an interesting candidate for systemic coordination of the development of tissues and with the whole body. Moreover, DILP8 could coordinate development when the environment is altered.

Organs and whole-body physiology in a changing environment

Organ sizes are variable but their patterning is robust to the changes in whole-body physiology and alterations in the surrounding environment. We have a good understanding of the hormonal pathways through which whole-body development is regulated. However, we are only beginning to understand how the environmental stimuli that modify body and organ size alter these pathways to effect changes in growth and developmental timing. A wide range of environmental cues, including nutrition, temperature, humidity, infection/wounding, gut flora, crowding and oxygen levels modify the development of organisms (for reviews see (Mirth and Shingleton, 2012; Nijhout et al., 2013)). Of these factors, the effects of both temperature and

nutrition on body size and developmental time have been studied in the greatest detail.

In *Drosophila*, developmental time and body size decline with increasing temperatures (Alpatov and Pearl, 1929; Bubliy and Loeschcke, 2001; Frazier et al., 2008; Ghosh et al., 2013; Imasheva et al., 1998; Noach et al., 1996). Larvae reared at higher temperatures initiate the CW ecdysone pulse at a smaller size and at a younger age than larvae reared at lower temperatures (Ghosh et al., 2013). By altering the timing of critical weight, temperature changes the length of the growth period and affects body size (Ghosh et al., 2013).

It is largely unknown how temperature affects the developmental processes of a tissue. We know that discs can reach the same final size by either changing cell number or cell size. Potentially, changes in temperature could produce wings of different sizes by affecting either one of these processes. However, several studies report that plastic responses to cooler temperatures lead to changes in cell size, without significant changes in cell number (French et al., 1998; Partridge et al., 1994; Robertson, 1959; Shingleton et al., 2009). At high temperatures, reductions in wing size are primarily due to fewer cells (Alpatov, 1930; Robertson, 1959). Whether these differences in the plastic response to temperature reflect differences in developmental mechanisms is unknown.

Nutrition also affects developmental time and organ/body size in *Drosophila*. The insulin/target of rapamycin (TOR) signalling pathways coordinate the rate of body growth with nutritional signals. Poorly-fed larvae have lower levels of insulin/TOR signalling, develop slower and produce smaller flies than well-fed larvae. Despite their smaller size, poorly-fed flies

have normally patterned wings (Alpatov, 1930; Bryant and Simpson, 1984; Robertson, 1936; Robertson, 1959).

Nutrition regulates body size by controlling the rates of body growth, but also by controlling the timing of ecdysone synthesis. In fed flies, insulin/TOR signalling acts on the PG to regulate the production of ecdysone. In poorly fed flies, insulin/TOR signalling is suppressed, thereby reducing ecdysone synthesis and causing growth arrest and developmental delays. Delays induced by starvation also retard the progression of pattern in the wing imaginal discs. Activation of the insulin/TOR pathway in the PG increases ecdysone levels even in poorly fed or starved flies, inducing precocious developmental progression and imaginal disc patterning. In contrast, reducing insulin/TOR signalling in the PG of well-fed flies delays development and imaginal disc patterning (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2009). Therefore, altering insulin/TOR pathways in the PG alters the physiological state of the whole animal and the rate of development in the wing discs.

Interestingly, organs may use different mechanisms to respond to different environmental stimuli. For example, changing larval rearing temperature results in hyperallometric scaling between wing and body size, while the wing and body scale isometrically with changes in nutrition (Shingleton et al., 2009). Indeed, by inducing critical weight at smaller body sizes, temperature may affect the growth of organs differently than changes in nutrition. In addition, tissues show different degrees of sensitivity to environmental cues. The genital disc scales hyperallometrically with body size (Shingleton et al., 2009) with either changes in nutrition or temperature due to its reduced environmental sensitivity. The genital discs achieve

reduced sensitivity by downregulating insulin/TOR signalling (Tang et al., 2011). Overall, different environmental stimuli could change organ size via different mechanisms depending on the signalling pathways they affect and the sensitivity of a given organ to this pathway.

A much less studied question is how environmental stimuli affect the patterning of tissues. Recent work has tackled how patterning during *Drosophila* embryogenesis varies through time with temperature between closely related species (Kuntz and Eisen, 2014). The relative timing of patterning events scaled uniformly across species and with changes in temperature. Thus in the *Drosophila* embryo, patterning coordinates its progression continuously with developmental time to achieve robustness against changes in temperature. Whether organs also achieve developmental robustness by continuously coordinating their patterning processes over developmental time had not been explored.

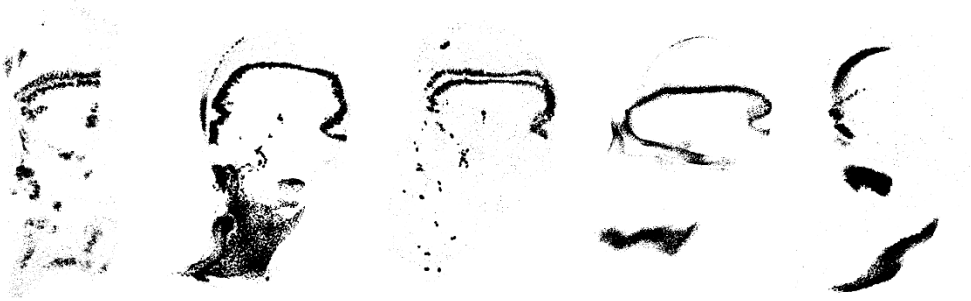
Environment-body-organ coordination: the approach

In this introduction, we first reviewed the literature describing that the development of an organ includes the coordination of growth with patterning, potentially via the action of morphogens. In turn, organ development appears to be coordinated with whole-body development by the regulation of ecdysone and insulin/TOR pathways, although we understand little about how these signalling pathways act on organs to achieve coordination. Lastly, the mechanisms ensuring robustness of organ patterning and organ/body scaling under different environmental conditions are largely unknown, but likely to be due to variation in the sensitivity of the organ to ecdysone and insulin/TOR pathways.

Although important, studying individual signalling pathways that regulate disc size and patterning provides an incomplete picture of how the growth and patterning of the wing is coordinated with itself and with the development of the whole body. To address how larvae coordinate organ and body development, we adopted an approach that integrates changes in environmental and/or physiological conditions, alterations in hormonal signalling pathways, and explorations of the changes in developmental process over time. We divided our approach into three parts. First, we postulated that patterning of the wing disc is robust to changes in environmental and physiological conditions by coordinating with whole-body development either continuously or at key developmental events, known as developmental milestones. Second, we examined the growth trajectories of wings under different physiological and environmental conditions known to generate variation in wing size and determined whether alterations in size occurred via the same or different processes. We further explored the nature of the relationship between growth and patterning. Last, we addressed how ecdysone and insulin/TOR pathways contribute to the growth and patterning of the wing disc, and consequently, coordinate wing disc with the whole-body development.

2

Coordination of wing and whole-body development at developmental milestones ensures robustness against environmental and physiological perturbations



Summary

Development produces correctly patterned tissues under a wide range of conditions that alter the rate of development in the whole body. We propose two hypotheses through which tissue patterning could be coordinated with whole-body development to generate this robustness. Our first hypothesis states that tissue patterning is tightly coordinated with whole-body development over time. The second hypothesis is that tissue patterning aligns at developmental milestones. To distinguish between our two hypotheses, we developed a staging scheme for the wing imaginal discs of Drosophila larvae using the expression of canonical patterning genes, linking our scheme to three whole-body developmental events: moulting, larval wandering and pupariation. We used our scheme to explore how the progression of pattern changes when developmental time is altered either by changing temperature or by altering the timing of hormone synthesis that drives developmental progression. We found the expression pattern in the wing disc always aligned at moulting and pupariation, indicating that these key developmental events represent milestones. Between these milestones, the progression of pattern showed greater variability in response to changes in temperature and alterations in physiology. Furthermore, our data showed that discs from wandering larvae showed greater variability in patterning stage. Thus for wing disc patterning, wandering does not appear to be a developmental milestone. Our findings reveal that tissue patterning remains robust against environmental and physiological perturbations by aligning at developmental milestones. Furthermore, our work provides an important glimpse into how the development of individual tissues is coordinated with the body as a whole.

Introduction

Organisms require robust developmental processes to guarantee that developing tissues pattern correctly in the face of a wide range of environmental and physiological perturbations (Mirth and Shingleton, 2012; Shingleton, 2010). A developmental process can be considered robust if variation in this process is uncorrelated with variation in genetic, environmental or physiological conditions (Nijhout, 2002). To achieve robustness, the developmental processes that generate individual organs must, at some level, be integrated across the whole body to ensure that a correctly patterned and proportioned adult is produced at the end of development. It is therefore thought that the progression of gene expression that occurs in tissues as they pattern needs to be somehow integrated with the systemic hormone levels that trigger transitions between developmental stages (hereafter termed developmental events) across the whole body (Tennessen and Thummel, 2011; Yamanaka et al., 2013). The timing of these developmental events changes with environmental and physiological conditions but how this affects tissue development is not fully understood.

There are several hypotheses to explain how tissue patterning is integrated with whole-body development under different environmental and physiological conditions. One hypothesis is that tissue patterning and whole-body development progress synchronously, so that the rate of the former matches the rate of the latter. If this were the case, a change in the duration of development would extend or contract the progression of patterning in a linear manner (Figure 2.1 A). Consequently, normalizing the progression of pattern to a developmental endpoint, that is using relative

rather than absolute developmental time, would produce the same progression of patterning independent of the duration of development (Figure 2.1 B).

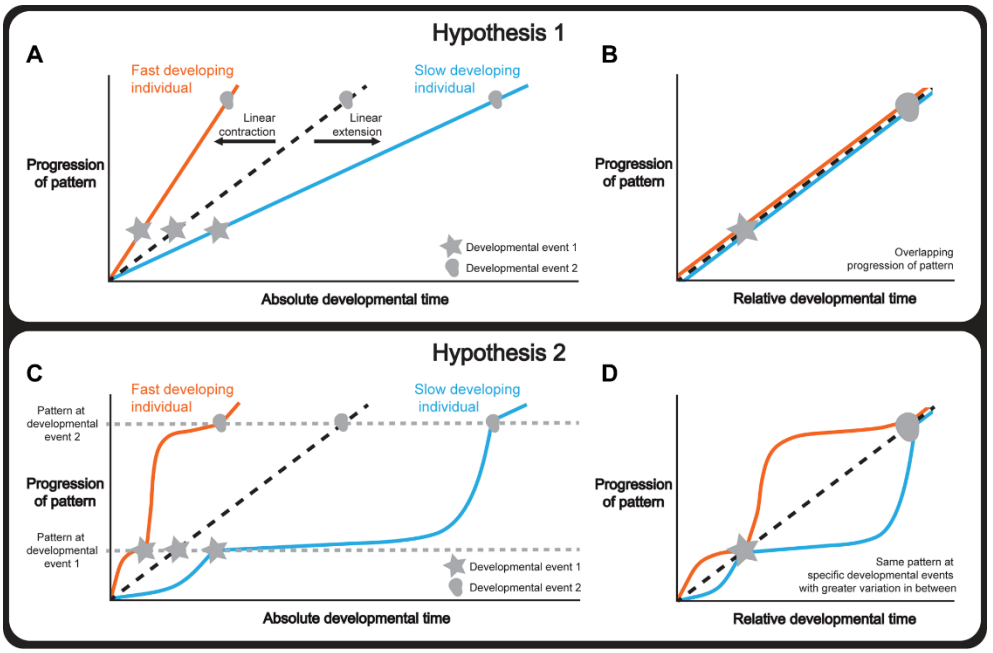


Figure 2.1. Hypotheses to explain how organ and whole-body development are coordinated. (A-B) Hypothesis 1: Whole-body development and individual tissue patterning are tightly coordinated throughout development. Changing the length of time required for development extends or contracts the progression of pattern in a linear manner (A) and, consequently, normalizing the data to a developmental endpoint, referred to as relative developmental time, produces overlapping progressions of pattern (B). (C-D) Hypothesis 2: Whole-body development and individual tissue patterning are coordinated only at key physiological transitions. Changing the length of time required for development alters the relationship between tissue patterning and developmental time non-linearly and patterning converges only at these transitions (C). Consequently, in relative developmental time, the progression of pattern overlaps at these events and shows greater variability in the intervals between them (D). Note that the curve of the two lines between the developmental events is illustrative showing two ways the curves could differ under altered developmental conditions. Tissue patterning of a reference/condition is represented in black dashed lines; fast developers are represented in orange and slow developers are shown in blue. Stars and circles symbolize developmental events 1 and 2.

Alternatively, tissue patterning may only be coordinated with whole-body development at key developmental events (Figure 2.1 C), for example moulting in holometabolous insects, or the onset of puberty in humans. Although not all developmental events act to coordinate, those that do are often referred to as developmental milestones (Levin et al., 2012). Thus if the duration of development varies, the progression of patterning would nonetheless converge at these milestones while showing greater variability between them. Consequently, normalizing the progression of pattern to relative developmental time would produce patterns that overlapped only at developmental milestones (Figure 2.1 D). This would essentially mean that if patterning were to drift in rate with respect to whole-body development, developmental milestones would ensure that the rate of patterning would decelerate or accelerate to achieve the correct stage by the onset of the milestone.

Problematically, it has been difficult to test these alternative hypotheses because, while the process of patterning has been described in exquisite detail in a variety of tissues, the dynamics of patterning is rarely tied to organismal age or whole-body physiology. Several authors have explored how genetic background contributes to the robustness of development (see examples (Matsuda et al., 2013; Nien et al., 2011)). Their approaches have focussed on the endpoints of development and on changes in the sequences of specific patterning cascades. Furthermore, studies in organisms ranging from insects to nematodes to vertebrates have explored the progression of gene expression in relation to embryonic stage to identify developmental milestones, called phylotypic stages, where gene expression converges upon an embryonic stage common across species (Domazet-Loso and Tautz, 2010; Irie and Kuratani, 2011;

Kalinka et al., 2010; Kittelmann et al., 2012; Levin et al., 2012). Such developmental milestones are thought to constrain development like an hourglass, as development across species varies more both before and after the milestones (Domazet-Loso and Tautz, 2010; Irie and Kuratani, 2011; Kalinka et al., 2010; Levin et al., 2012). However, these studies do not address how environmental/physiological conditions affect the progression and sequence of pattern, and how this is coordinated with whole-body development within a species. We therefore took advantage of the extensive knowledge of tissue patterning and whole-body physiology of the fruit fly, *Drosophila melanogaster*, to elucidate the extent to which tissue patterning is coupled with whole-body development.

In *Drosophila,* the juvenile period comprises three larval moults. This is followed by a wandering stage where larvae leave the food and search for a pupariation site. Larval development ends with pupariation, whereupon the fly metamorphoses into its adult form. These events provide useful markers of whole-body development. Each of these developmental events (moulting, wandering and pupariation) is regulated by pulses in the titre of the steroid hormone ecdysone (Riddiford, 1993), synthesized by the prothoracic gland.

Most of the adult tissues of *Drosophila* arise from pouches of cells that grow and pattern within the body of the developing larvae, the imaginal discs (Brennan et al., 1998; Mirth et al., 2009; Schubiger et al., 2005; Schubiger and Truman, 2000). Pulses of ecdysone have also been shown to regulate some stages of imaginal disc development. Early in the third larval instar a pulse of ecdysone controls the expression of three patterning gene products, Cut (Ct), Senseless (Sens) and Wingless (Wg), in response to

nutrition (Mirth et al., 2009). After pupariation, ecdysone regulates Sens expression to control the differentiation of sensory organs in the wing (Schubiger et al., 2005; Schubiger and Truman, 2000). Thus, these pulses of ecdysone have been interpreted to be checkpoints that coordinate the patterning and development of tissues with whole-body developmental events (Rewitz et al., 2013; Yamanaka et al., 2013). Nevertheless, it remains to be determined if this coordination between tissues and the whole body is necessary and happens at all developmental events, or only at specific developmental milestones.

The rate of developmental progression and the timing of these developmental events can be altered both environmentally and by genetically manipulating the timing of ecdysone synthesis. For example, *Drosophila* larvae raised at lower temperatures take longer to eclose as adults (Alpatov and Pearl, 1929; Bubliy and Loeschcke, 2001; Frazier et al., 2008; Imasheva et al., 1998; Noach et al., 1996) while larvae reared at higher temperatures eclose more quickly (Bubliy and Loeschcke, 2001; Imasheva et al., 1998). Similarly, altering the timing of ecdysone synthesis, by suppressing or activating insulin signalling in the prothoracic gland, also changes developmental timing and retards or accelerates eclosion (Colombani et al., 2005; Mirth et al., 2005; Mirth et al., 2009).

To test the extent to which whole-body development and the progression of pattern in individual tissues are coordinated, we first generated a staging scheme to describe how patterning progresses over time in the wing imaginal discs of third instar larvae. This staging scheme was based on the changes in expression pattern of key patterning genes. We then altered developmental rate either environmentally, by using

temperature manipulations, or physiologically, by altering the timing of ecdysone synthesis. We compared the progression of patterning, as determined by our staging scheme, in larvae that differ in their developmental rates. Our results indicate that the progression of patterning is coordinated with some, but not all, developmental events and varies between events.

Materials and methods

Fly stocks and rearing conditions

We used an isogenic wild-type strain, Samarkand (SAM), reared at 25˚C to develop the staging scheme, representing the baseline for all comparisons (referred to as wild type at 25˚C). To manipulate developmental time environmentally, we reared wild-type SAM flies at 18˚C and 29˚C (wild type at 18˚C and wild type at 29˚C). To alter the timing of ecdysone synthesis and manipulate developmental time physiologically, we used the progeny from *phm*-GAL4 crossed with yw *flp; UAS InR29.4* (*phm>InR*) and from *P0206-GAL4* crossed with *yw; UAS PTEN* (*P0206>PTEN*) to up- or down-regulate insulin signalling in the prothoracic gland, respectively. Even though *P0206*-GAL4 is a weaker GAL4 driver for the prothoracic gland and also drives expression in the corpora allata, we chose to use it to drive *UAS PTEN* because *phm>PTEN* larvae die as first instar larvae (Mirth et al., 2005). We used the parental lines *yw; UAS PTEN* (*>PTEN*) or *yw flp; UAS InR29.4* (*>InR*) as additional controls for genetic background effects.

Flies were raised from timed egg collections (2-6 hours) on standard cornmeal/molasses medium at low density (200 eggs per 60 x 15 mm Petri

dish) in a 12 h light-dark cycle with 70% humidity, and maintained at 25°C unless stated otherwise. Larvae that were reared at 18˚C or 29˚C were maintained in incubators without lights due to equipment constraints.

Animal staging and developmental time

Larvae were staged into 1-hour cohorts at ecdysis to the third larval instar and wing-imaginal discs were dissected at the following times (in h AL3E): wild type at 25˚C: 0, 5, 10, 15, 20, 25, 30, 35, 40, 46 (wandering) and 49 (pupariation) h AL3E; wild type at 18˚C: 0, 10, 20, 30, 50, 70, 96 (wandering) and 101 (pupariation) h AL3E; wild type at 29˚C: 0, 10, 20, 30, 35, 40 h, 48 (wandering) and 52 (pupariation) h AL3E; *P0206>PTEN*: 0, 10, 20, 30, 40, 60, 73 (wandering) and 80 (pupariation) h AL3E; *phm>InR*: at 0, 10, 20 and 30 h AL3E, 32 (wandering) and 36 (pupariation) h AL3E; *>PTEN* control: 0, 10, 20, 30, 48 (wandering) and 53 (pupariation) h AL3E; *>InR* control: 0, 10, 20, 30, 48 (wandering) and 51 (pupariation) h AL3E.

We measured the average time to wandering and pupariation by counting the number of larvae wandering/pupariating within a cohort every two hours. To measure the average eclosion time, we allowed flies to oviposit for 2-6 hours in food bottles. Larvae were maintained at low densities, and we checked for adult eclosion every 12 h.

Dissections and immunocytochemistry

To develop our staging scheme, we examined the expression of eleven patterning gene products in the wing discs of wild-type larvae at 25˚C by immunocytochemistry: Achaete (Ac), Cut (Ct), Delta (Dl), Hindsight (Hnt), Notch (N), Scabrous (Sca), Senseless (Sens), Dachshund (Dac), Engrailed (En), Patched (Ptc) and Wingless (Wg). These patterning gene products

represent the main cascades involved in wing disc patterning: the Notch signalling pathway (represented by Ac, Ct, Dl, Hnt, N, Sca and Sens), the Hedgehog signalling pathway (represented by Dac, En and Ptc) and the Wnt/Wg signalling pathway (represented by Wg). In the wing discs of larvae with altered developmental time (wild type at 18˚C, wild type at 29˚C, *P0206>PTEN* and *phm>InR*) as well as the genetic controls (*>PTEN* and *>InR*), we examined the expression of four gene products: Wg for the 0 h AL3E time point, and Ac, Sens and Dac for all time points. Although it was impossible to simultaneously stain for all gene products at all time points for all genotypes under all conditions, we minimized the effects of variation between experimental blocks by conducting experiments between at least two genotypes/conditions in parallel. Further, for any given time point for each of the genotypes/conditions, we stained for different patterning gene products on different days.

For each time point, wing imaginal discs from 10 larvae were dissected in cold phosphate buffered saline (PBS) and fixed for 30 min in 4% paraformaldehyde in PBS. Number of dissected discs varies from 5-16 depending on the treatment/genotype (Supplementary Tables S2.1 and S2.2). The tissue was washed in PBT (PBS + 1% Triton X-100) at room temperature, blocked in PBT-NDS (2% Normal Donkey Serum in PBT) for 30 min and then incubated in a primary antibody solution (Supplementary Table S2.3) overnight at 4°C. After washing with PBT, tissue was incubated with fluorescently-conjugated secondary antibody overnight at 4°C. Tissue was rinsed with PBT and wing discs were mounted on a poly-L-lysine-coated coverslip using Fluoromount-G (SouthernBiotech). Samples were imaged using a Zeiss LSM 510 confocal microscope and images were processed using ImageJ.

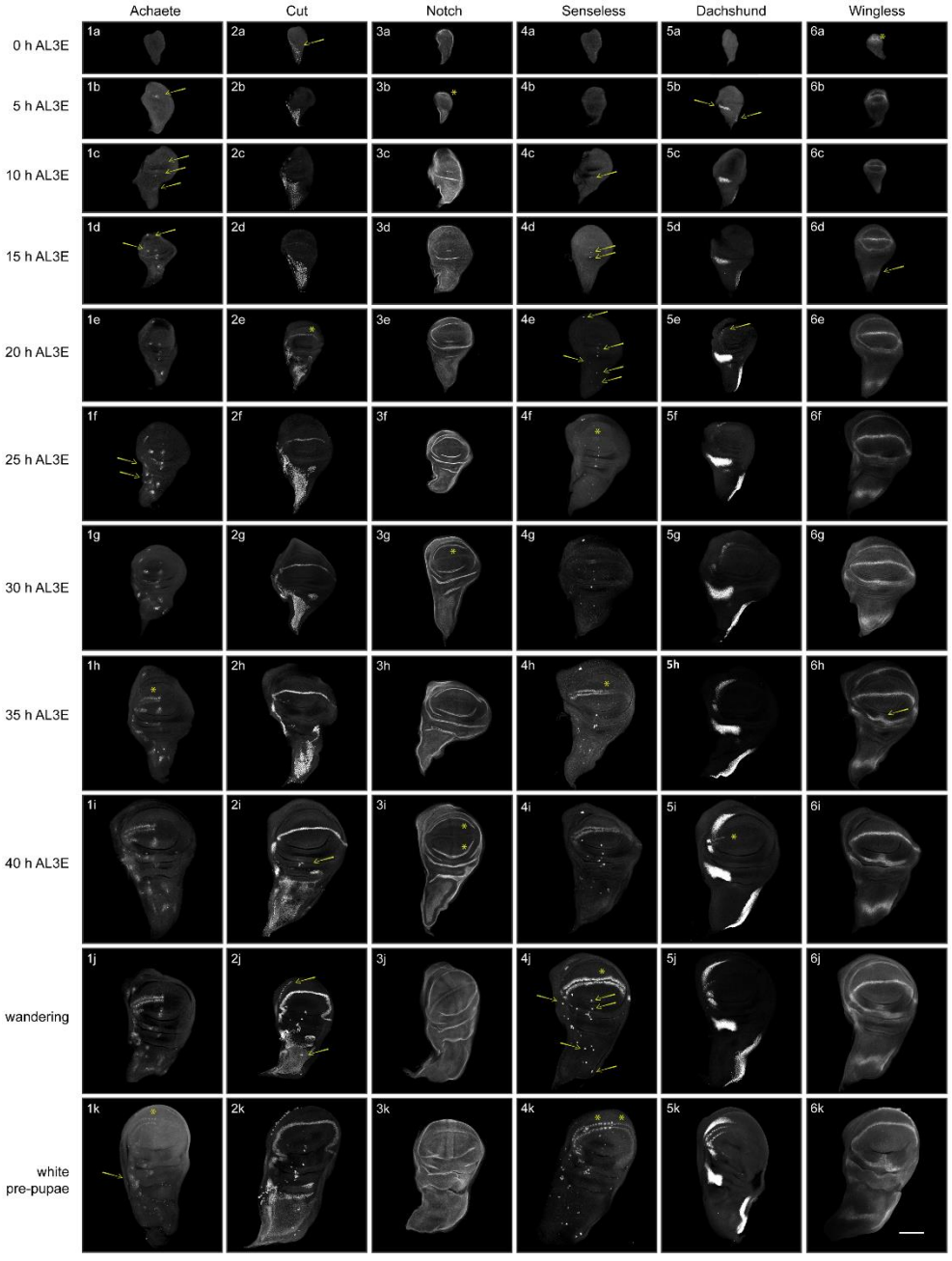
Qualifications and quantifications used to characterize gene product patterns

The expression patterns of each of the gene products examined had previously been characterised in the literature: individual cells, patches of cells, or stripes (Brower, 1986; Fehon et al., 1991; Phillips and Whittle, 1993; Romani et al., 1989) (Supplementary Figure S2.1). To compose the staging scheme, we initially conducted a qualitative analysis of the patterns observed for each gene product at each time point (Figure 2.2 and Supplementary Figure S2.2) and described their progression. We then quantified these expression patterns in two ways (Supplementary Figs S2.2, S2.3). First, we divided the area of gene product expression by the total area of the disc, to generate a measure of pattern area. Second, we quantified the number of specific elements (cells, patches of cells or stripes) that each expression pattern exhibited. By both quantifying gene product expression and characterising the addition of new pattern elements through time, we were able to identify the gene products that varied the most during the third instar as well as those patterning elements that changed through a stepwise progression. We then used the change in patterns of these gene products to generate a staging scheme.

Statistical analysis

We used a Naïve Bayes Classifier (NBC) to test the power of our staging scheme to classify dissected discs from each time point into their correct stage. We first tabulated the observed gene-specific stages for all the patterning-gene products in the dissected discs from each time point. We then permuted the data from each time point 1000 times to simulate a population of 1000 discs with the range and frequency of gene-specific

stages that was characteristic of wing discs from that time point. We then trained an NBC using our staging scheme and applied it to the permuted data set to determine what proportion of the 1000 simulated discs from each time point would be classified into the 'correct' stage. We repeated



this analysis to assign stages to discs dissected from larvae reared under all experimental conditions.

All data analyses and statistics were conducted using R (www.r-project.org) (Ihaka and Gentleman, 1996). The R scripts used to analyse the data, as well as the complete data, are available for download from Dryad (doi:10.5061/dryad.fq134).

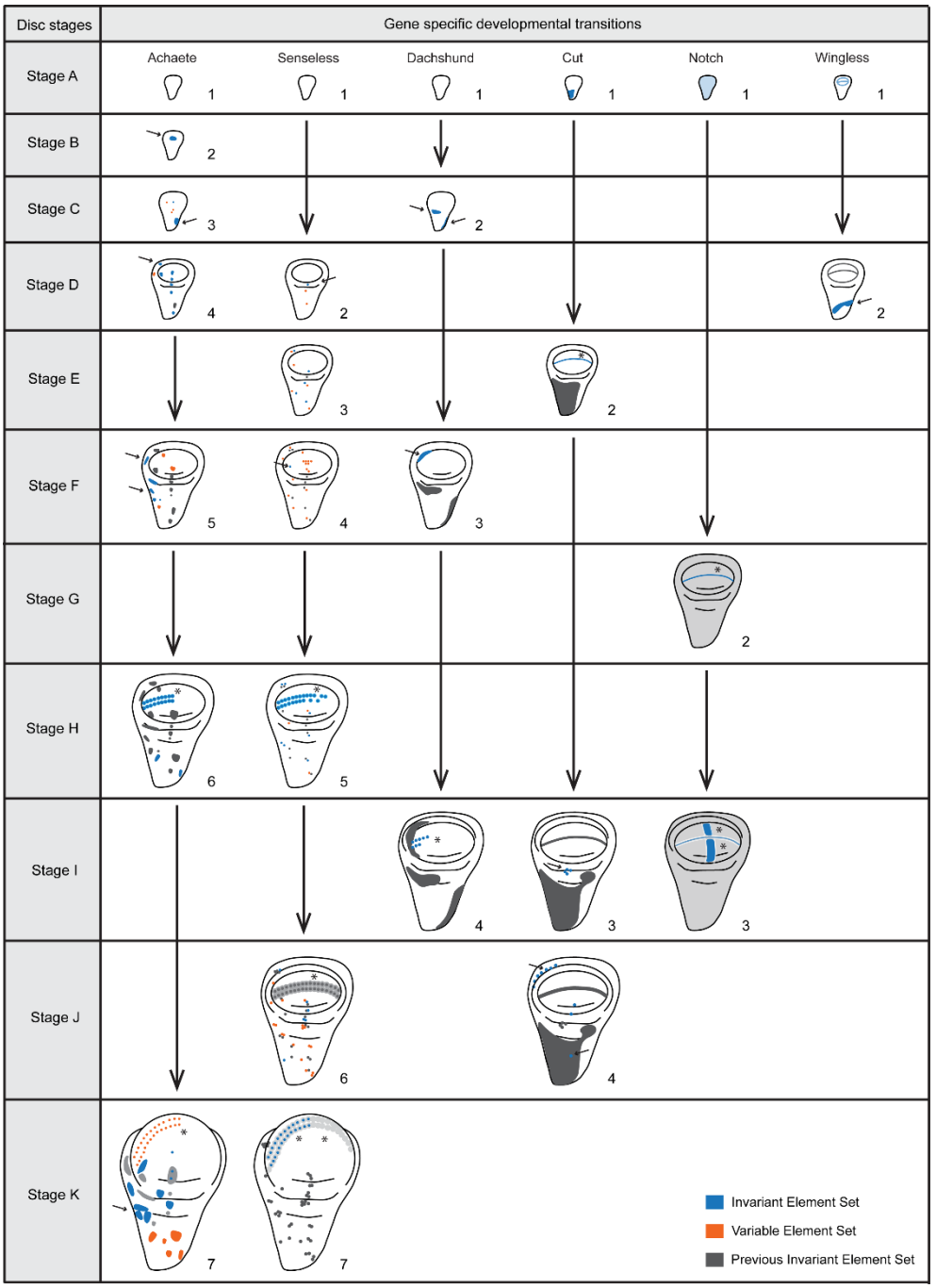
Results

Developmental staging scheme for wing discs

To compose our developmental staging scheme for wing discs, we used immunocytochemistry to identify changes in the expression of eleven patterning gene products at five hour intervals from 0-40 h after third instar ecdysis (AL3E), at wandering, and at pupariation for a total of eleven time points (Figure 2.2 and Supplementary Figs S2.1, S2.2, S2.3). We used wing discs from larvae of an isogenic wild-type strain Samarkand (SAM) reared at 25˚C (wild type at 25˚C). Three of these time points coincided with three developmental events – the moult to the third instar, wandering and pupariation. We have a strong understanding of the physiology underlying these developmental events, and so assaying patterning at

Figure 2.2. *(preceding page)* Patterning progression of six of the eleven gene products used to construct the staging scheme. The expression of Achaete (1a-1k), Cut (2a-2k), Notch (3a-3k), Senseless (4a-4k), Dachshund (5a-5k) and Wingless (6a-6k) at 0 (1a-6a), 5 (1b-6b), 10 (1c-6c), 15 (1d-6d), 20 (1e-6e), 25 (1f-6f), 30 (1g-6g), 35 (1h-6h) and 40 (1i-6i) hours after third instar ecdysis (h AL3E), wandering (at the average time of 46 h AL3E, 1j-6j) and white pre- pupae (at the average time of 49 h AL3E, 1k-6k). Arrows show addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Scale bar is 100 µm.

these time points allowed us to test for coordination between tissue patterning and whole-body development.



Collectively, we used the progression of patterning in wild type at 25˚C as a baseline for all comparisons in this work. We identified elements of pattern that we could reliably distinguish across discs of a given time point (Figs 2.2, 2.3). New elements of pattern included the addition of a new region of expression, for instance the appearance of expression in a cell or in cells that previously had not expressed a particular gene product; the refinement of an expression field from diffuse expression in a group of cells to more focussed expression in a reduced subset of cells;[3]or the disappearance of expression in a region that had previously expressed that gene product. For each patterning gene product, we discerned the time each patterning element arose, thereby characterizing the transitions in pattern for each gene. From this, we defined stages for each gene product (referred to as gene-specific stages) (Figure 2.3).

Figure 2.3. *(preceding page)* Staging scheme – the developmental transitions for each gene product arranged according to disc stage. Each column represents a gene product (Achaete, Senseless, Dachshund, Cut, Notch and Wingless) and each row a disc stage (A-K). We characterized disc stages by the combination of gene-specific stages (numbers under each disc for each gene). We highlighted the key elements characterizing each gene-specific stage either with arrows (cells or patches of cells) or asterisks (stripes). For Dachshund (Dac) stage 4 where the stripe is highlighted, we did not consider the length of the stripe for this character, although it increases in length during development. Further, in each disc, in blue we represent addition or change of elements that are common to all discs sampled (Invariant Element Set), in orange we represent the addition or change of elements that are variable (Variable Element Set, ie. do not appear in all the discs sampled) and in grey the elements that do not change in comparison to the previous gene stage (Previous Invariant Element Set). We only used the invariant element set to construct the staging scheme. Black vertical arrows represent the transition between the gene-specific stages. Disc stages A-K correspond to the time points sampled (0, 5, 10, 15, 20, 25, 30, 35, 40, 46 (average time for wandering) and 49 (average time for pupariation) hours after third instar ecdysis (h AL3E).

Not all gene products displayed clear gene-specific stages. Engrailed and Patched did not undergo patterning transitions in the third larval instar, consistent with previous studies (Mirth et al., 2009). Scabrous localization within single cells appeared to be restricted to vesicles, making changes in pattern hard to identify. Hindsight expression in the wing disc was difficult to distinguish from expression in associated tracheal cells. Finally, the patterning transitions for Delta and Notch (N) occurred at the same time. For these reasons, we chose to exclude Engrailed, Patched, Scabrous, Hindsight, and Delta from our characterizations of overall disc stage.

We tabulated the gene-specific stages for each time point from the remaining six gene products, Achaete (Ac), Ct, N, Sens, Dachshund (Dac) and Wg. These combinations of gene-specific stages allowed us to define eleven disc stages (A-K), corresponding to each of the eleven time points sampled from wild-type larvae at 25˚C (Figure 2.3 and see Materials and methods). Two of the gene products, Ac and Sens were staged simultaneously in individual discs (Ac is a mouse monoclonal antibody and Sens is a guinea pig antibody). Using these two gene products alone, we can assign discs to nine of the eleven disc stages (Figure 2.4 A). The bubbles in Figure 2.4 A represent the proportion of discs at each time point that fall into a particular disc stage based on their Ac and Sens pattern combined. These data show that using Ac and Sens alone, for five time points all discs are categorized into a single stage. For the remaining six time points sampled, most discs (67-89%) can be attributed to one disc stage, with a smaller proportion of discs (<24%) falling into one or two additional stages. Thus, staging with Ac and Sens alone provides a reliable measure of disc stage across developmental time.

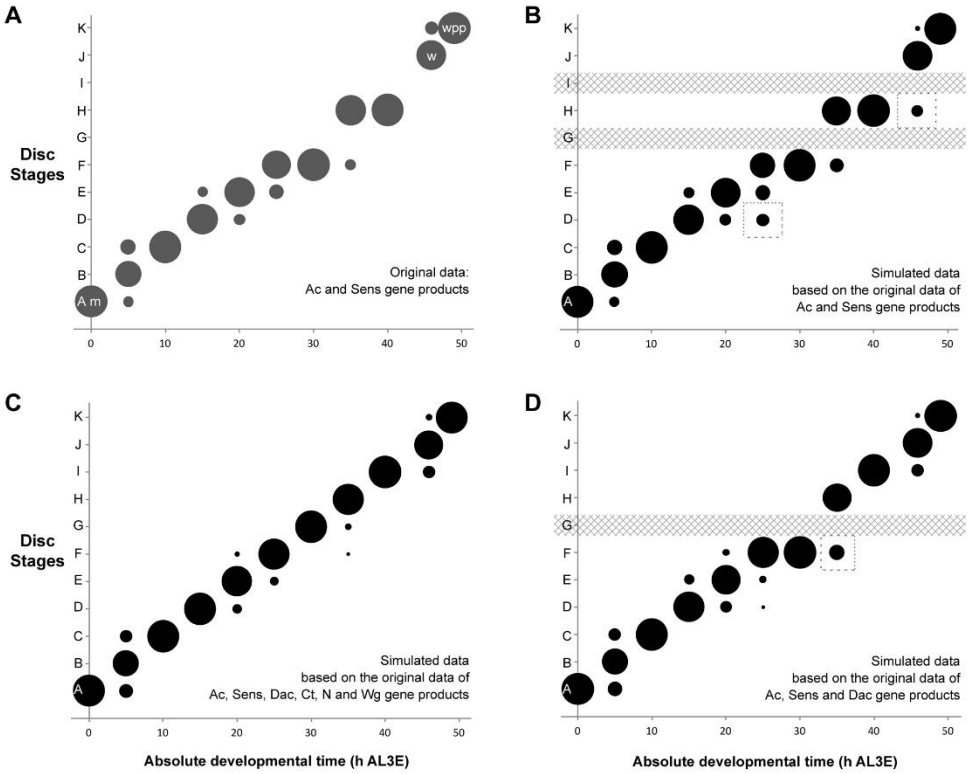


Figure 2.4. The probability of attributing a wing disc dissected at a given age to a particular disc stage. (A) Proportion of discs attributed to each disc stage based on individual discs simultaneously staged for Ac and Sens. (B) By applying the Naïve Bayes Classifier (NBC) to the permuted data set based only on Ac and Sens expression patterns, our staging scheme was able to distinguish between discs from each time point and classify them into their appropriate stages. Dashed boxes highlight regions where the simulated data set showed greater variation than the actual data from a. (C) We repeated the NBC classifier analysis using the expression patterns of Ac, Sens, Dac, Ct, N and Wg. (D) Using the expression patterns of Ac, Sens and Dac to classify the discs, the results were nearly identical, except the NBC classified all discs at 30 h AL3E (stage G) as stage F. This is because stages G and F share the same Ac, Sens and Dac expression patterns. The dashed box marks a time point where the simulated data from Ac, Sens and Dac alone showed greater variation than the simulated data generated using the complete panel of six patterning gene products. Developmental events are identified by m (moult to the third instar), w (wandering) and wpp (white pre-pupae – pupariation).

We expected that adding more markers to our staging scheme would increase its resolution. Problematically, due to the nature of antibody staining, it was not possible to stain a single disc for more than two gene products. Consequently, we cannot assign an individual disc to a particular developmental stage with the complete set of markers. To circumvent this problem, we simulated what a disc would look like if we could stain the same disc for all six gene products. We first tabulated the observed stages for each gene product at each time point. The number of discs scored for each gene product ranged from five to sixteen (Supplementary Table S1), depending on the time point and the gene product. We then randomly sampled from this table to simulate all the possible combinations of gene-specific stages for a single disc dissected at this time point. We repeated these permutations 1000 times to generate 1000 simulated discs for each time point. We then applied a Naïve Bayes Classifier (NBC) to the simulated data set to assign each simulated disc to a developmental stage, based on our staging scheme. The NBC analysis does not return a p-value, but instead provides the probability that a disc of a given time point would be assigned to a particular disc stage.

The results of this analysis are represented using a bubble plot (Figure 2.4 B-D). In this plot, the area of each bubble is the proportion of the 1000 simulated discs that were assigned to each disc stage, using the NBC. As a proof of principle, we applied our analysis to the staging scheme devised from the Ac and Sens data. The plot generated from the simulated discs looks very similar to the staging scheme derived from the sampled disc data (Figure 2.4 A, B), although the NBC appears to slightly overestimate the amount of variation in the data (dashed boxes in Figure 2.4 B). Overall,

however, our stimulated data set represents well the patterns seen from the sampled discs.

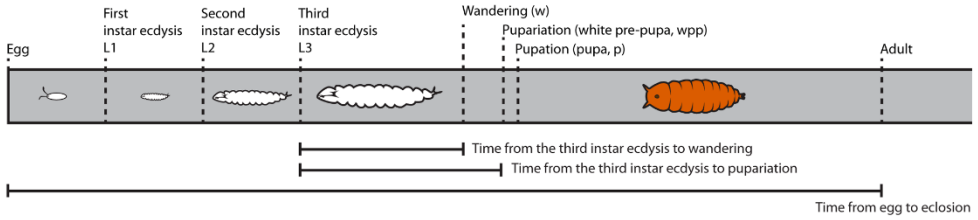
Next, we simulated discs with all six patterning gene products and applied the NBC (Figure 2.4 C). Using all six gene products, we could resolve eleven disc stages in the simulated discs. For six time points, there is a single bubble, indicating that all the simulated discs at that time point share a stage-specific combination of gene-product patterns. This suggests that the criteria for classification are unambiguous at that time point. In the remaining time points, the NBC assigned discs to two or three stages. This indicates that the discs dissected at these time points did not all share the characteristics used to define a single stage. That is, there is variability in patterning among discs dissected at the same time point. Nevertheless, even at these time points the NBC classified the majority of simulated discs (65-94%) to a single stage. Further, the amount of variation for these time points was reduced if the complete data set was used in the simulation instead of using Ac and Sens alone.

We repeated the NBC analysis using only the expression patterns of Ac, Sens and Dac to classify the discs. The results were nearly identical from the complete gene set simulations (Figure 2.4 D), except the NBC classified all discs at 30 h AL3E (stage G) as stage F. This is because stages G and F share the same Ac, Sens and Dac expression pattern, and so the NBC classified the discs into the earliest stage by default. This combination of three gene products provides greater resolution than Ac and Sens alone and was one of the combinations that identified most of the disc stages from the moult to the third instar until pupariation. Hereafter, to minimize the number of gene products necessary to stage wing discs, we established the staging scheme composed from Ac, Sens and Dac as the baseline for all

subsequent comparisons. Additionally, we choose to use Wg for the first time point because Ac, Sens and Dac were not expressed at the moult to the third instar.

Changing the rate of development by modifying environmental conditions: the effects of temperature

Once we had a method of defining the developmental stage of a disc, we then asked whether the progression of pattern through these developmental stages was tightly coordinated with whole-body development when developmental rate was altered by changes in rearing temperature. Rearing wild-type larvae at 18˚C lengthened the time to adult



	Time from the third instar ecdysis to wandering				Time from the third instar ecdysis to pupariation				Time from egg to eclosion			
	AVG (h AL3E)	95% CI	p value	N	AVG (h AL3E)	95% CI	p value	N	AVG (h AEL)	95% CI	p value	N
wild type @ 25°C	45.9	± 0.7	-	76	49.0	± 0.4	-	157	215.2	± 1.8	-	95
wild type @ 18°C	96.4	± 1.4	<2e-16	156	100.7	± 1.6	<2e-16	153	495.5	± 4.1	<2e-16	98
wild type @ 29°C	48.2	± 0.6	0.0077	122	51.7	± 0.4	0.00012	157	199.0	± 1.8	9.9e-16	123
P0206>PTEN	72.9	± 2.9	<2e-16	34	80.0	± 2.2	<2e-16	42	272.3	± 1.8	<2e-16	398
phm>InR	29.1	± 1.2	<2e-16	49	35.8	± 1.1	<2e-16	61	211.0	± 2.0	0.065	99
>PTEN	47.9	± 0.5	<1.9e-05	179	53.0	± 0.3	<2e-16	181	211.8	± 1.8	0.0148	181
>InR	47.6	± 0.4	0.00014	244	51.0	± 0.3	4.3e-15	230	219.5	± 1.3	0.0023	271

Figure 2.5. Schematic representation and table of time from the third instar ecdysis (L3) until wandering, pupariation and time from egg to eclosion. Developmental times are represented in hours after third instar ecdysis (h AL3E), hours after egg lay (h AEL) and characterized by mean (AVG) and 95% confidence intervals (CI). Data was tested for normality using Q-Q plots and analysed using one-way ANOVA (α=0.025). P values are from pairwise t-tests, and refer to differences between the temperature treatments (wild type at 18°C and 29°C compared to 25°C), differences between physiological treatments and wild type at 25°C (*P0206>PTEN* and *phm>InR* versus wild type at 25°C) and differences between parental backgrounds (*>PTEN* and *>InR*) and wild type at 25°C.

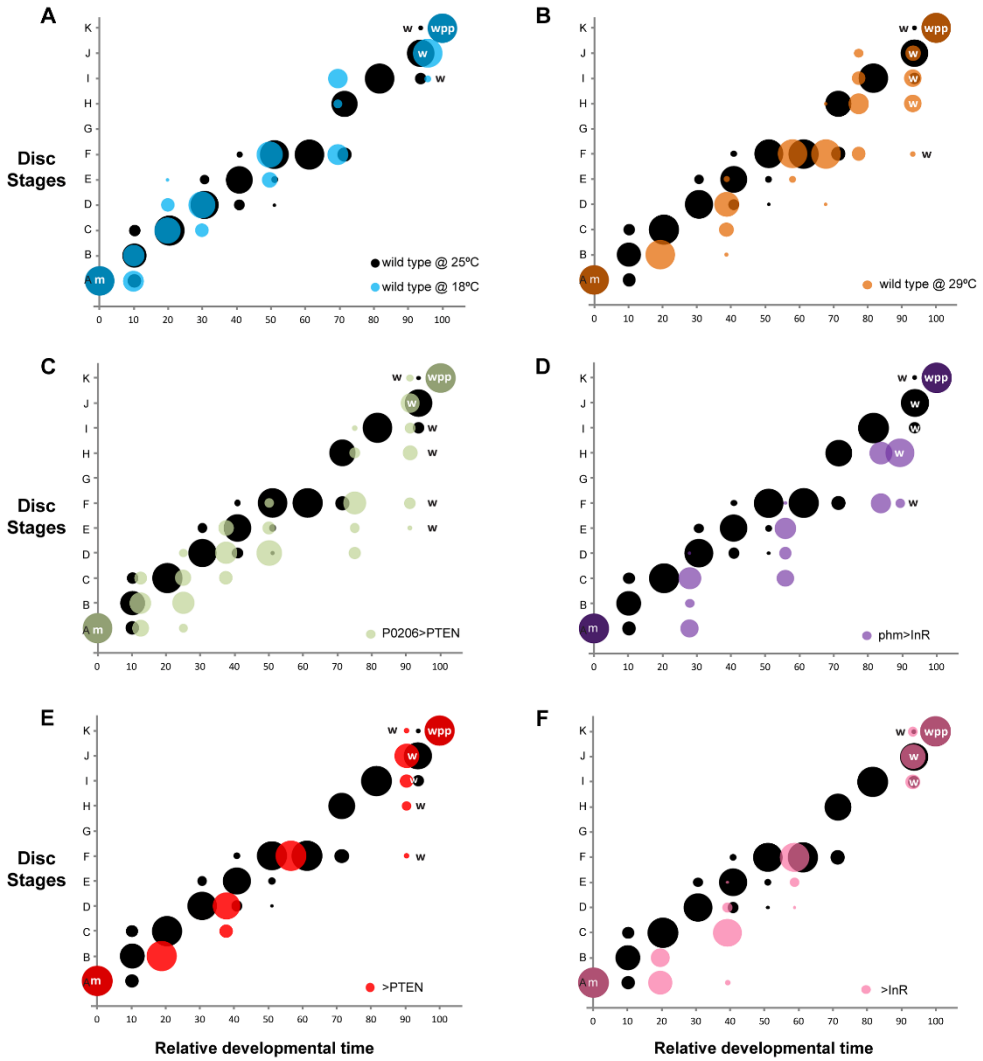
eclosion from larval hatching, while rearing larvae at 29˚C shortened the time, compared to wild-type larvae raised at 25˚C (Figure 2.5). Surprisingly, however, the duration of the third larval instar was slightly longer at 29˚C than at 25˚C (Figure 2.5), as was the time to larval wandering from the beginning of the third instar. Thus, for the purposes of our study, larvae reared at 29˚C were slow developers.

To assay whether the progression of disc patterning relative to whole-body development was affected by rearing temperature, we used a bubble plot to chart wing disc stage, as assigned by the NBC classifier applied to a permuted data set, expressed in relative developmental time (normalized to pupariation), at 18˚C, 25˚C and 29˚C. At all three temperatures, patterning in the discs was the same at the moult to the third instar and at pupariation (Figure 2.6 A, B and Supplementary Figure S2.4 a, b). At 18˚C the progression of disc patterning when normalized to pupariation time was largely the same as at 25˚C, indicated by the overlapping bubble plots at the two temperatures (Figure 2.6 A and Supplementary Figure S2.5). In contrast, at 29˚C patterning was initially delayed, evident from discs dissected at the same relative developmental time showing earlier patterning stages at 29˚C than at 25˚C (Figure 2.6 B and Supplementary Figure S2.4 b). The rate of patterning progression accelerated later in the third instar, however, to achieve the final disc stage at pupariation (Figure 2.6 B and Supplementary Figure S2.4 b). Further, there was more variation in developmental stage among discs dissected at larval wandering at 29˚C, compared to disc 25˚C (Figure 2.6 B). Earlier in development, the variation and delay observed in stage at 29°C was due to Ac and Sens expression, both of which belong to the Notch signalling pathway (Supplementary Figure S2.6). In contrast, at wandering much of the delay was caused by

variation observed in Sens and Dac expression patterns (Supplementary Figs S2.6, S2.7).

Changing the rate of development by modifying larval physiology: altering the timing of ecdysone synthesis

The timing of ecdysone synthesis is thought to be key to coordinating whole-body developmental events (moulting, larval wandering and pupariation) with imaginal disc development. To test this hypothesis, we



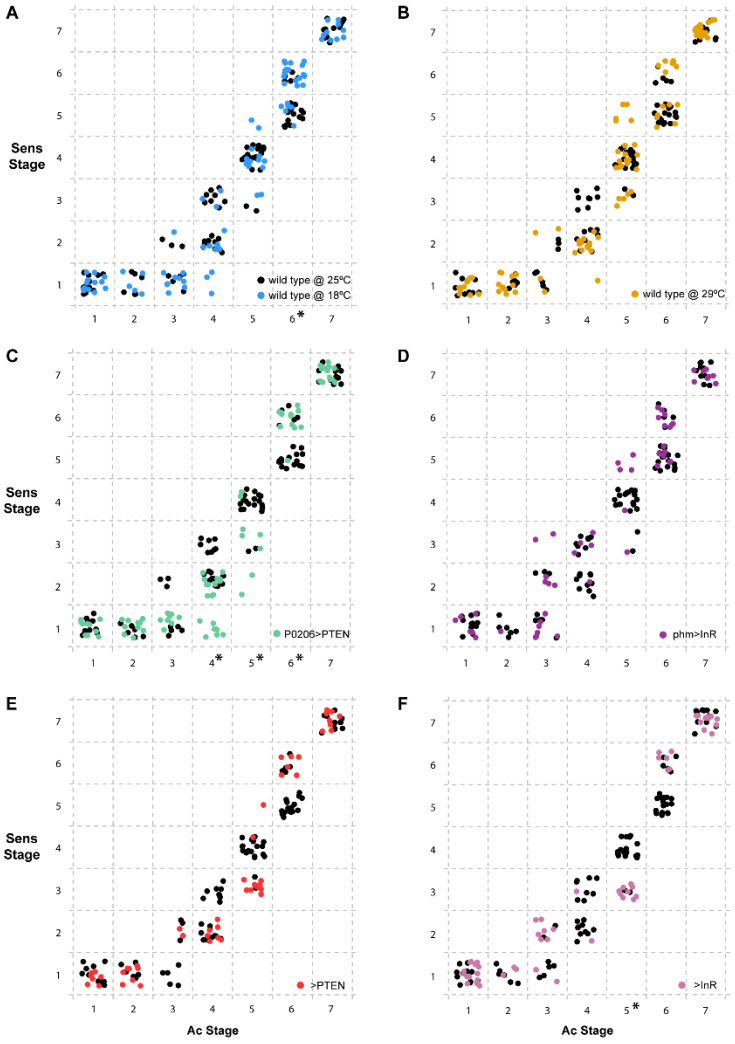
first altered the timing of ecdysone synthesis by downregulating or upregulating insulin signalling in the prothoracic gland, lengthening or shortening the duration of the third larval instar respectively (Figure 2.5) (Mirth et al., 2009). To downregulate insulin signalling in the prothoracic gland, we used the *P0206 GAL4* driver to overexpress PTEN (*P0206>PTEN)*; to upregulate insulin signalling in this tissue, we expressed InR using the *phm GAL4* driver (*phm>InR*). Together with changes in the duration of development, the rate of patterning in the wing discs was also affected. Early in the third larval instar, patterning appeared to be retarded in both *phm>InR* and *P0206>PTEN* larvae, while patterning progressed at an accelerated rate later in development (Figure 2.6 C, D and Supplementary Figure S2.4 c, d).

To explore how wing disc patterning progressed relative to whole-body development, we again used a bubble plot to chart wing disc stage in *phm>InR* and *P0206>PTEN*, as assigned by the NBC classifier applied to a permuted data set, against relative developmental time. We used wild-type SAM larvae reared at 25˚C for comparison. Under all experimental

Figure 2.6. *(preceding page)* Changing developmental time alters the progression of pattern. Probability (represented by the size of the circle) that a disc with a given set of gene-specific stages belongs to a particular disc stage, varying with relative developmental time (normalized to pupariation). In all panels (a-f), we show the wild type at 25°C in black. (a-b) Temperature manipulations: (a) disc stages attributed to discs from wild-type larvae reared at 18°C are shown in blue and (b) from wild-type larvae reared at 29°C are shown in orange. (c-d) Manipulations of the timing of ecdysis synthesis: (c) disc stages attributed to discs from *P0206>PTEN* larvae are shown in green and (d) disc stages attributed to discs from *phm>InR* larvae are in purple. (e-f) Parental lines to test for the contribution of genetic background: (e) disc stages attributed to discs from *>PTEN* larvae in red and (f) from *>InR* larvae in pink. Developmental events are identified by m (moult to the third instar), w (wandering) and wpp (white pre-pupae – pupariation).

conditions, wing discs displayed the same pattern at the beginning (moulting) and end (pupariation) of the third larval instar.

However, a bubble plot of relative developmental time (normalized to pupariation) against disc stage indicated that in both *P0206>PTEN* and *phm>InR* larvae, disc patterning is initially delayed and showed increased variability compared to 25˚C wild-type larvae at the same relative developmental time (Figure 2.6 C, D and Supplementary Figs. S2.4, S2.8,



S2.9). This delay is more evident in *phm>InR* discs (Figure 2.6 D), where it is due to changes in the relative progression of Ac, Sens and Dac expression (Supplementary Figure S2.9), than in *P0206>PTEN* discs (Figure 2.6 C), where it is primarily due to changes in the progression of Ac and Sens expression (Supplementary Figure S2.8). Furthermore, in *phm>InR* discs from wandering larvae, patterning was substantially delayed when compared to wild type at 25˚C (Figure 2.6 D).[5]

Some of the observed changes in wing disc patterning progression early in the third instar in *P0206>PTEN* and *phm>InR* larvae may result from genetic background effects. Both parental lines, *yw; UAS PTEN* (referred to as *>PTEN*) and *yw flp; UAS InR29.4* (referred to as *>InR*), showed small but significant differences in pupariation time compared to the wild type at 25˚C (Figure 2.5). Additionally, in both *>PTEN* and *>InR* larvae, we observed early delays in wing patterning relative to wild type at 25˚C, due to retardation in the progression of all three gene products – Ac, Sens and Dac (Supplementary Figs S2.4 e, f, S2.10, S2.11). However, after 50% developmental time wing disc patterning was the same in all three

Figure 2.7. (*preceding page)* Progression of Senseless (Sens) stage as a function of Achaete (Ac) stage independent of developmental time. For each individual disc sampled, we represented the combination of Ac and Sens stages observed. Inside dashed boxes, all discs were assigned the same discrete Sens and Ac stage. In all panels (A-F), we show the wild type SAM larvae at 25°C in black. (A) discs from wild type SAM larvae reared at 18°C are shown in blue and (B) from wild type SAM larvae reared at 29°C are shown in orange. (C) discs from *P0206>PTEN* larvae are shown in green and (D) from *phm>InR* larvae are in purple. (E) disc-specific stages attributed to discs from *>PTEN* larvae in red and (F) from *>InR* larvae in pink. Ac stages with asterisks (on the x-axis) are those that show significant differences between conditions/genotypes ($p < 0.01$, Wilcoxon rank test using Holm's p-value adjustment) with reference to the wild type SAM at 25°C.

lines (wild type at 25˚C, *>PTEN*, *>InR*). Further, wing disc patterning was the same in all three lines at moulting and pupariation, and largely overlapped at wandering (Figure 2.6 E, F). A comparison of wing disc patterning in *P0206>PTEN* and *phm>InR* larvae to their genetic controls suggests that the delays observed before 50% relative developmental time are due to genetic background effects while the delays after this period are due to changes in physiology (Supplementary Figure S2.12).

Examining the correlation between gene-specific stages from two genes in the same patterning cascade

Our data demonstrate that altering developmental timing of the whole body changes the progression of patterning in Ac, Sens and Dac. Next, we explored whether gene-specific stages of Sens correlated with gene-specific stages of Ac across treatments and genotypes independently of developmental time (Figure 2.7). We found that overall, Ac and Sens stages were tightly correlated and showed little significant variation with temperature, physiology or genotype. There were some exceptions; for Ac stages 4 and 5 we found that Sens stages were significantly delayed in *P0206>PTEN* larvae when compared to wild-type larvae at 25°C (Figure 2.7 C). The *>InR* larvae showed similar delays in Sens with respect to Ac at stage 5 (Figure 2.7 F). In contrast at Ac stage 6, Sens was accelerated in the wild-type larvae at 18°C and in the *P0206>PTEN* larvae (Figure 2.7 A, C). Thus, Sens stages show some degree of plasticity with respect to Ac stages, but only at Ac stages 4-6.

Discussion

In this study, we set out to examine the extent to which tissue development is coordinated with the development of the whole body. We tested two alternative hypotheses: 1) the progression of pattern is tightly coordinated with whole-body development at all times, and 2) patterning is coordinated only at developmental milestones.

Previous studies demonstrated that the development of tissues could regulate the timing of whole animal development. Specifically, larvae with slow growing discs greatly delay the development of the whole body (Halme et al., 2010; Parker and Shingleton, 2011; Simpson et al., 1980; Stieper et al., 2008). Discs induce these delays by regulating the timing of a specific developmental event that occurs early in the third instar, termed critical weight (Halme et al., 2010; Parker and Shingleton, 2011). Slowing disc growth after critical weight has no effect on developmental timing (Halme et al., 2010). Delaying patterning in the imaginal discs has also been shown to retard the development of the whole body. If the spread of Wg protein is restricted in the imaginal discs by replacing wild-type Wg with a membrane-tethered Wg allele, larvae delay the onset of pupariation (Alexandre et al., 2014). We do not yet know whether Wg signalling in the discs affects developmental timing by affecting disc growth rate nor do we know which developmental events are affected by altered Wg signalling.

Further, there is ample evidence from many insects that ecdysone controls the timing of development in the various tissues of the body (Riddiford, 1993). In third instar larvae, ecdysone signalling stimulates neurogenesis in the optic lobe via the Notch/Delta pathway (Lanet et al., 2013). The pulses of ecdysone that stimulate the onset of pupal

development are also known to initiate patterning of the sensory tissues of the wing (Schubiger et al., 2005). Thus, it seemed likely that ecdysone pulses at other stages could act as milestones to coordinate both tissue and whole-body development.

We found that patterning, as determined by disc stage, aligned at the moult to third instar and at pupariation in all conditions studied. It is important to note, however, that considerable patterning occurs in wing discs before the third instar (Ng et al., 1996). Furthermore, pupariation is not an endpoint for disc pattern, as the patterning of sensory structures and the specification of the wing veins continue on during pre-pupal and pupal development (Blair, 2007; Schubiger et al., 2005; Schubiger and Truman, 2000). Thus pupariation appears to be characterized by an alignment but not termination of patterning progression.

In contrast, disc patterning among wandering larvae showed variability, both within the wild type at 25°C and across experimental treatments. Variation in disc stage at wandering within the reference genotype at 25°C is likely to be due to the fact that the wandering stage lasts approximately 8 hours and therefore occupies a slightly longer time interval than the other intervals of the staging scheme. This, however, does not explain the difference in disc stage at wandering across experimental treatments; discs from *phm>InR* larvae were mostly at disc stage H at wandering, whereas the wild-type discs at 25°C were mostly at disc stage J. Thus, we conclude that wing patterning is not coordinated with whole-body development at wandering. This was surprising, as wandering is commonly used to stage larvae to ostensibly the same developmental point (for examples see (Mattila et al., 2004; Phillips and Whittle, 1993; Wells et al., 2006)). Overall,

our data supports hypothesis two: patterning aligns with whole-body development at specific developmental milestones, the moult and pupariation, and shows greater variation between these milestones.

Variability in pattern between the moult and pupariation showed common characteristics across treatments and genotypes. Generally, patterning showed delays relative to whole-body development early in the third instar. Disc patterning accelerated relative to whole-body development towards the end of the third instar to reach the final stage at pupariation (Figure 2.6 and Supplementary Figure S2.4). Our data highlight the possibility that because perturbations in pattern occur through delays early in the third instar, there is an intrinsic checkpoint late in the third instar that regulates pattern in the discs so that they reach a common patterning stage at pupariation.

The progression of pattern also varied with genetic background. This variation between control genotypes was most apparent early in development. In contrast to the environmental/physiological treatments, patterning was, however, aligned at wandering. This observation suggests that our staging scheme would vary somewhat with the genotype chosen as the reference background. Genetic variation in the mechanisms controlling developmental robustness has been previously described in the context of evolutionary studies. For instance, in *Caenorhabditis elegans* the types of deviations observed during the highly robust process of vulval development depend on genetic background (Braendle and Felix, 2008). We expect that genetic variation in the progression of patterning systems is common, but that it is often undetected due to alignment at developmental milestones.

Many of the delays in the progression of pattern that we observed across developmental time were due to delays in two gene products from the same pathway, Ac and Sens (Culi and Modolell, 1998; Nolo et al., 2000). This likely reflects the observation that the progression of patterning in these two genes was correlated, independent of developmental time. Consequently, when one gene was delayed, so was the other. In contrast, delays in Dac expression tended to occur at later stages of development. Taken together, this raises the question of how environmental perturbations might affect gene expression within or between signalling pathways as an interesting avenue for future study.

Collectively, our data reveal that tissue patterning is coordinated with some but not all whole-body developmental events. This raises two questions: first, across all of development which whole-body developmental events are developmental milestones for tissues? Second, do all tissues align their development to the same milestones?

Because many developmental events are regulated by ecdysone, whether or not a tissue aligns its pattern to a particular developmental event may be due to its sensitivity to ecdysone at that time. The response of a tissue to a given ecdysone pulse is likely to be tied to its function. If we had examined the development of tissues that have functions in the larvae, we might have found tighter coordination with wandering. For example, the pulse of ecdysone that initiates larval wandering also coordinates the onset of autophagy in the fat body (Rusten et al., 2004). Autophagy in this tissue is thought to sustain the growth and development of other tissues during non-feeding stages (Levine and Klionsky, 2004). In the salivary glands, a pulse of ecdysone in the mid-third instar stimulates glue

production, while the pulse at larval wandering induces movement of the glue from the cells into the lumen of the gland (Henrich et al., 1999). This glue is then expelled in response to the ecdysone pulse at pupariation to cement the animal to the substrate. Consequently, development of the fat body and salivary glands may be tightly coordinated with larval wandering. In contrast, tissues like the imaginal discs, whose differentiation into their adult form only starts after pupariation, may not need to respond to these earlier ecdysone pulses.

Despite the striking effects that environmental and physiological changes induce in developmental timing, the resulting adults bear correctly patterned structures. We originally presumed that this was because developmental time and patterning of the tissues was tightly coordinated. Using our staging scheme, however, we have shown that patterning and whole-body development are coordinated only at moulting and pupariation, suggesting these events mark milestones during development. A third event, wandering, does not appear to act as a developmental milestone, at least as far as wing disc patterning is concerned. We also found that the progression of pattern in the wing disc is far more plastic than originally supposed. Further, we found that both the duration of developmental intervals and rates of patterning can be slowed down or sped up. Thus underlying the robustness of the adult phenotype, we have revealed that developmental milestones coordinate wing disc and whole-body development to cope with environmental and physiological variation.

Acknowledgments

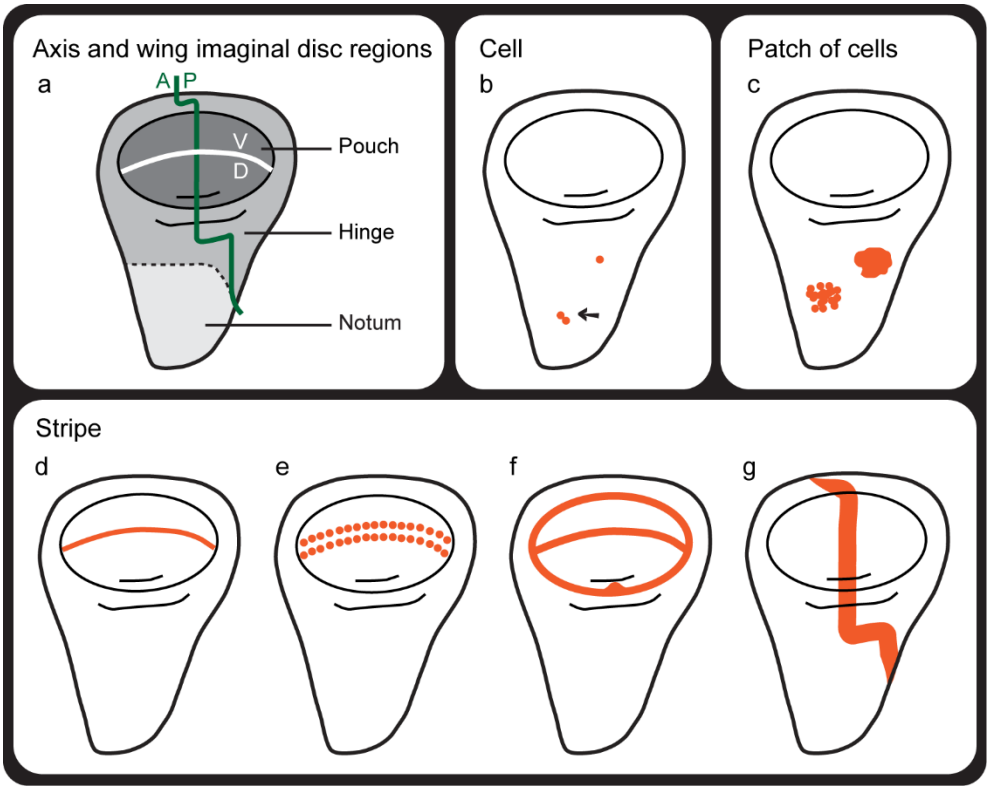
We would like to thank Dr. Florence Janody, Dr. Luís Teixeira and Dr. Pavel Tomancak for their critical reading of this chapter as a manuscript.

The following antibodies, Ac, Ct, Dac, Delta, En, Hnt, N, Ptc, Sca and Wg, were supplied by the Developmental Studies Hybridoma Bank (developed under the auspices of NICHD and maintained by the University of Iowa). Sens antibody was a gift from Dr. Hugo Bellen (Baylor College of Medicine, Houston, USA).

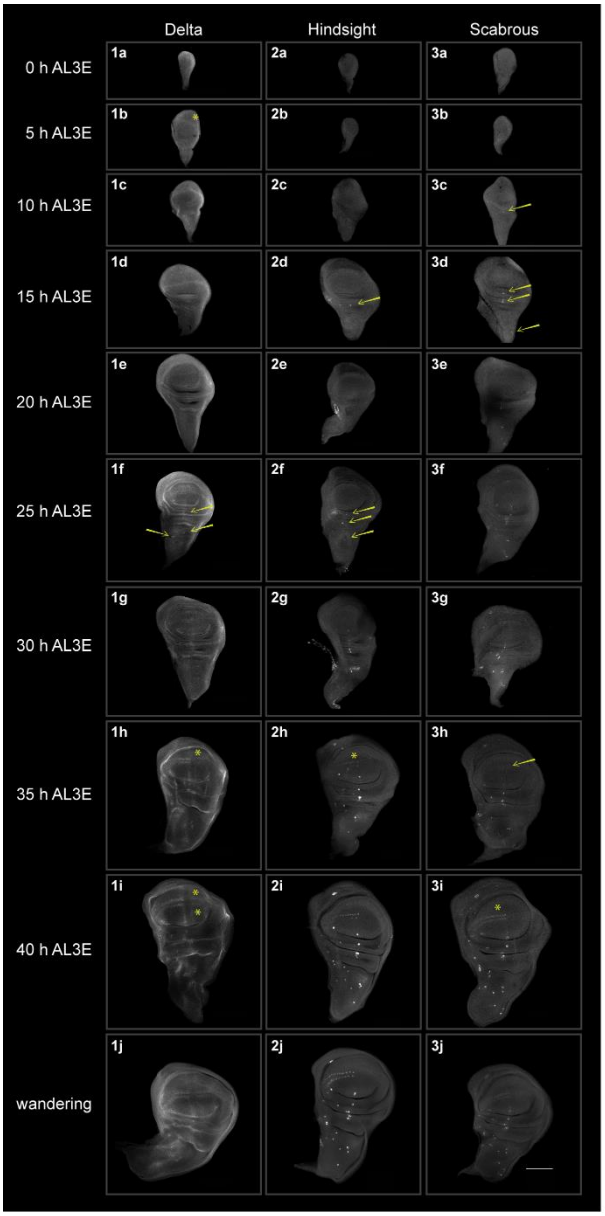
Publication

This chapter is published in PLoS Genetics as “Coordination of wing and whole-body development at developmental milestones ensures robustness against environmental and physiological perturbations”, authored by Marisa M. Oliveira, Alexander W. Shingleton and Christen K. Mirth.

Supplementary material to Chapter 2



Supplementary Figure S2.1. Definitions of patterning elements used to characterize stages in the staging scheme. (a) The third instar wing disc is already subdivided into domains that will form the wing pouch, wing hinge and notum of the adult fly. It has an anterior (A) and posterior (P) axis and dorsal (D) and ventral (V) domains. (b) The element *cell* was defined by one round dot of expression, which corresponded to the refinement of expression to a sensory organ precursor (SOP). For later time points, SOPs divide giving rise to two sister SOPs, referred as doublets (arrow). (c) *Patch of cells* refers to a region of pattern that resembles a cluster of cells, either clearly delimited or diffuse. (d-g) *Stripe* corresponds to one or more line of cells (more or less defined) (d, e), that can be parallel (double stripe) or perpendicular (forming a cross) to each other, located in the developing wing pouch along the dorsal-ventral axis. Stripes also appear as lines restricted to the anterior side of the wing pouch or along the dorsal-ventral boundary with a surrounding ring (stripe with ring, f), or along the anterior-posterior axis (g). The stripes correspond to lines of SOPs along the wing margin (e), or lines of positional information regarding wing disc boundaries (d). Different compositions of these elements describe all observed patterns for each gene product through development.


Delta
Hindsight
Scabrous
0 h AL3E
5 h AL3E
10 h AL3E
15 h AL3E
20 h AL3E
25 h AL3E
30 h AL3E
35 h AL3E
40 h AL3E
wandering
1a
2a
3a
1b
2b
3b
1c
2c
3c
1d
2d
3d
1e
2e
3e
1f
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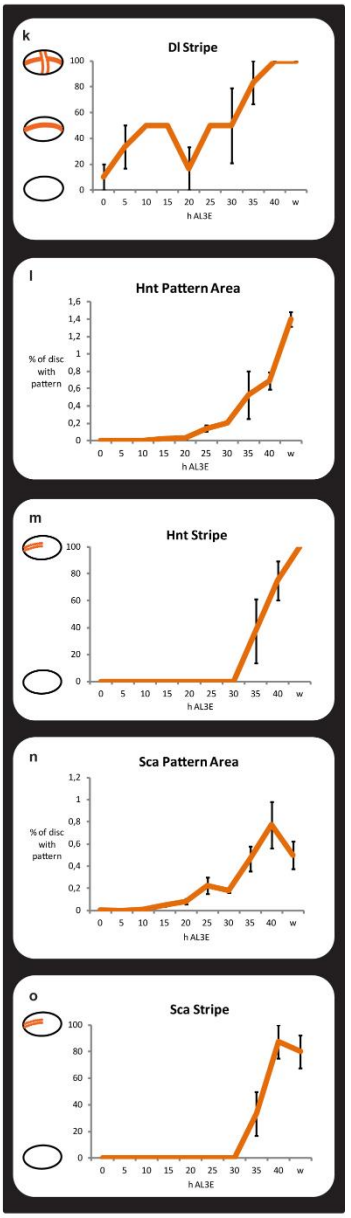
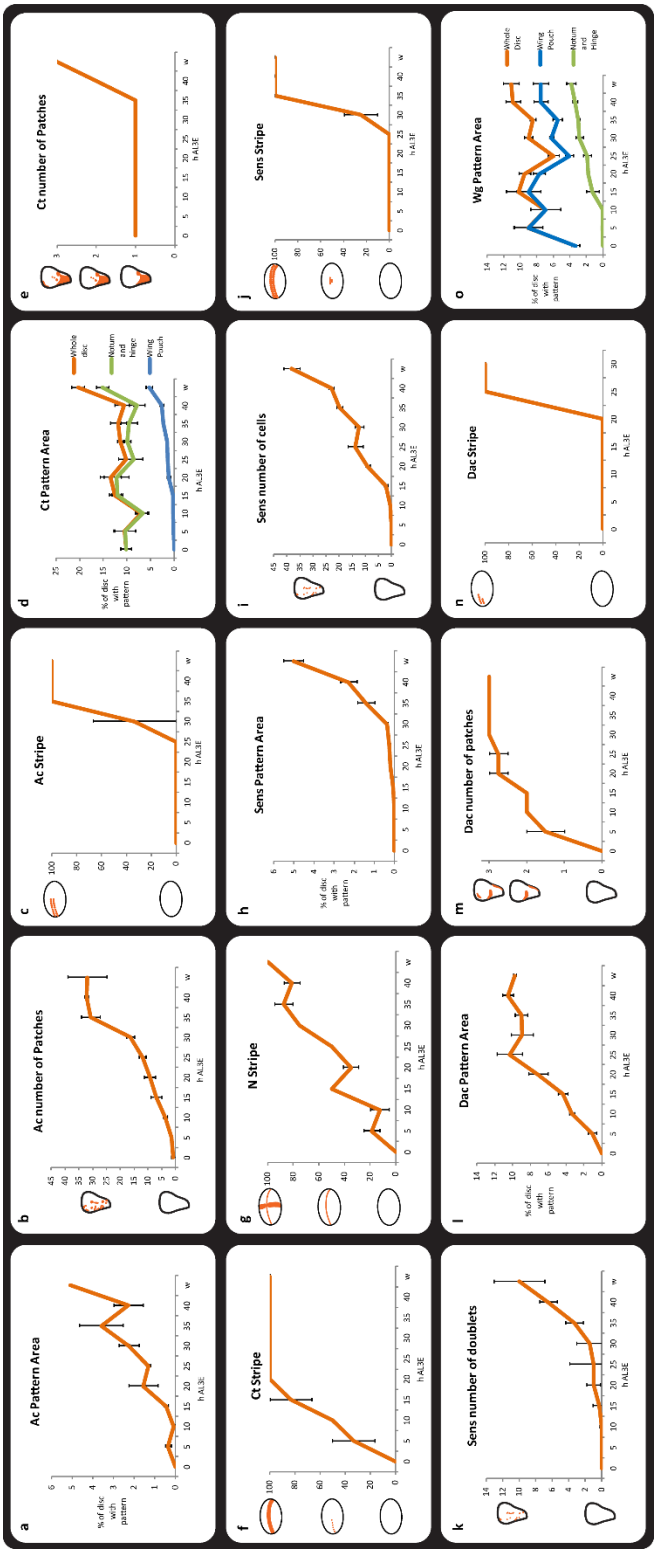
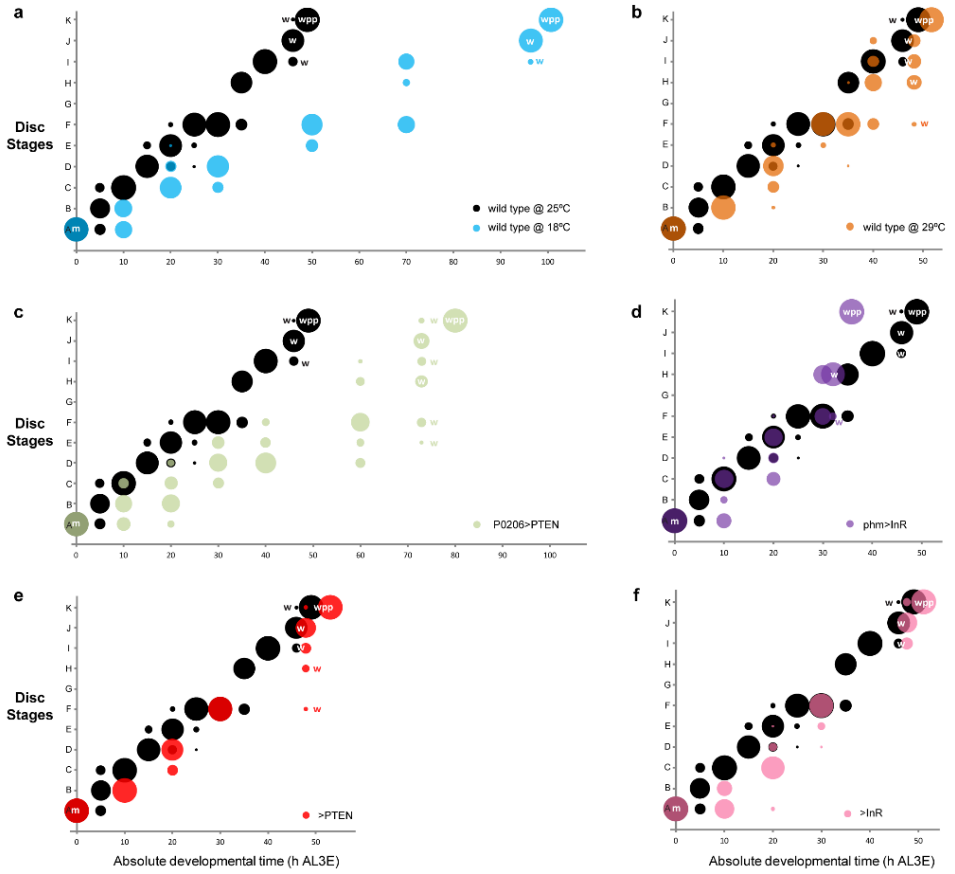
3h
1i
2i
3i
1j
2j
3j


k
Dl Stripe
l
Hnt Pattern Area
% of disc with pattern
m
Hnt Stripe
n
Sca Pattern Area
% of disc with pattern
o
Sca Stripe
h AL3E
w

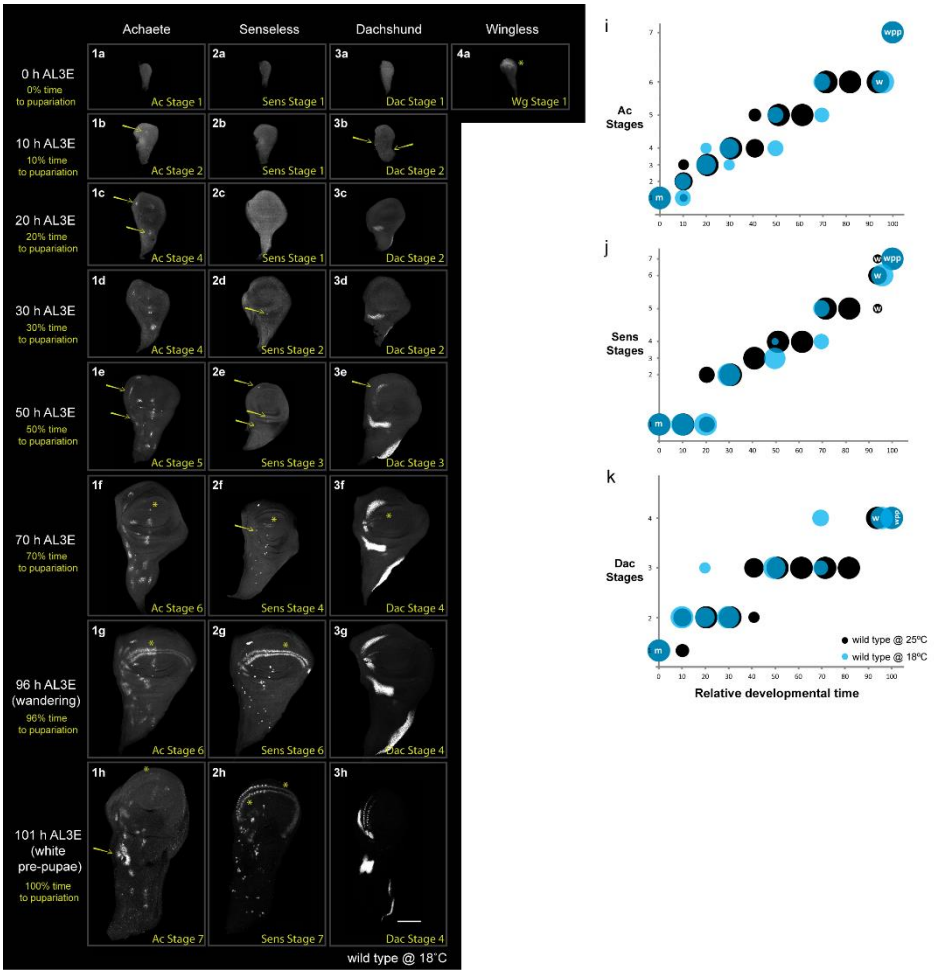
Supplementary Figure S2.2. *(preceding page)* Patterning progression of three of the eleven gene products initially assessed but not included in the staging scheme. Dynamic expression of Delta (1a-1j), Hindsight (2a-2j) and Scabrous (3a-3j) at 0 (1a-3a), 5 (1b-3b), 10 (1c-3c), 15 (1d-3d), 20 (1e-3e), 25 (1f-3f), 30 (1g-3g), 35 (1h-3h), and 40 (1i-3i) hours after third instar ecdysis (h AL3E) and wandering (1j-3j). Arrows show addition or change of cells or patches of cells, and asterisks highlight changes in stripes. (1f) Arrows highlight Delta expression mainly in the hinge and notum. Hindsight undergoes four transitions adding new elements at 15, 25 and 35 h AL3E. Lastly, Scabrous undergoes four transitions adding new elements at 10, 15 and 40 h AL3E. (3h) shows Scabrous expression in the centre of the wing pouch, before it refines to a stripe. (Scale bar 100 μm). (k-o) Quantitative measures of the relative amount of expression normalized to disc size of the different elements observed. (k) Delta expression pattern represented by the progression of the stripe. (l-m) Hindsight expression pattern decomposed into (l) pattern area and (m) progression of the stripe. (n-o) Scabrous expression pattern decomposed into (n) pattern area and (o) progression of the stripe. Delta undergoes four transitions in its pattern, adding new elements at 5, 35 and 40 h AL3E.


a
Ac Pattern Area
% of disc with pattern
h AL3E
b
Ac number of Patches
h AL3E
c
Ac Stripe
h AL3E
d
Ct Pattern Area
% of disc with pattern
Whole disc
Notum and Hinge
Wing Pouch
h AL3E
e
Ct number of Patches
h AL3E
f
Ct Stripe
h AL3E
g
N Stripe
h AL3E
h
Sens Pattern Area
% of disc with pattern
h AL3E
i
Sens number of cells
h AL3E
j
Sens Stripe
h AL3E
k
Sens number of doublets
h AL3E
l
Dac Pattern Area
% of disc with pattern
h AL3E
m
Dac number of patches
h AL3E
n
Dac Stripe
h AL3E
o
Wg Pattern Area
% of disc with pattern
Whole Disc
Wing Pouch
Notum and Hinge
h AL3E

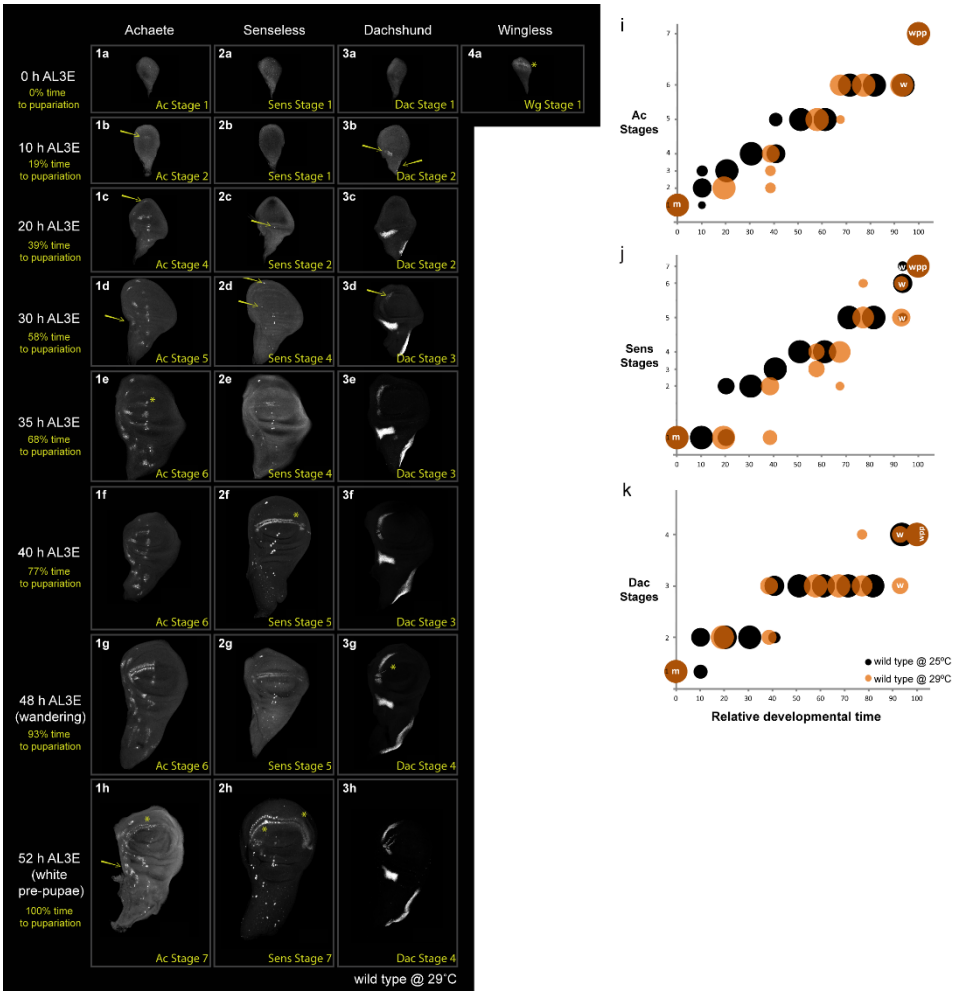
Supplementary Figure S2.3. *(preceding page)* Quantitative measures of the relative amount of expression normalized to disc size and of the different elements observed for six of the eleven gene products. (a-c) Achaete expression pattern decomposed into (a) pattern area, (b) number of patches of cells and (c) progression of the stripe. (d-f) Cut pattern broken down into (d) pattern area (whole disc, only notum and hinge, and only wing pouch), (e) number of patches of cells and (f) progression of the stripe. (g) Notch expression pattern represented by the stripe progression. (h-k) Senseless expression pattern decomposed into (h) pattern area, (i) number of SOPs, (j) progression of the stripe and (k) number of doublets. (l-n) Dachshund expression pattern in terms of (l) pattern area, (m) number of patches of cells and (n) progression of the stripe. (o) Wingless pattern area in the whole disc, only notum and hinge, and only wing pouch.



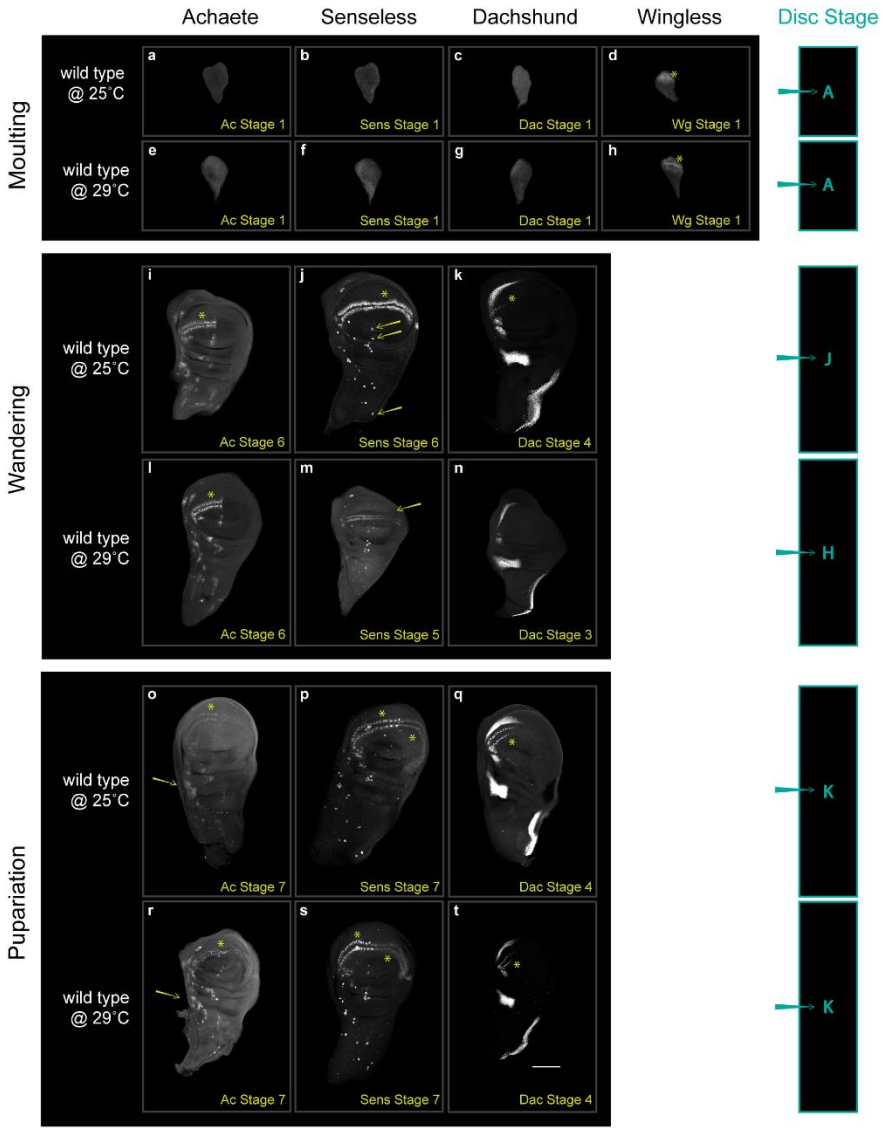
Supplementary Figure S2.4. The progression of pattern, in absolute time, in discs from larvae with altered developmental time and from two parental lines. The probability (represented by the size of the circle) that a disc with a particular set of gene-specific stages belongs to a given disc stage, varied with absolute developmental time (hours after third instar ecdysis (h AL3E)). (a-b) Temperature manipulations include (a) 18°C in blue and (b) 29°C in orange. (c-d) We manipulated the timing of ecdysone synthesis using (c) P0206>PTEN larvae (in green) and (d) phm>InR larvae (in purple). (e-f) Parental lines to test for the contribution of genetic background include (e) >PTEN in red and (e) >InR in pink. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



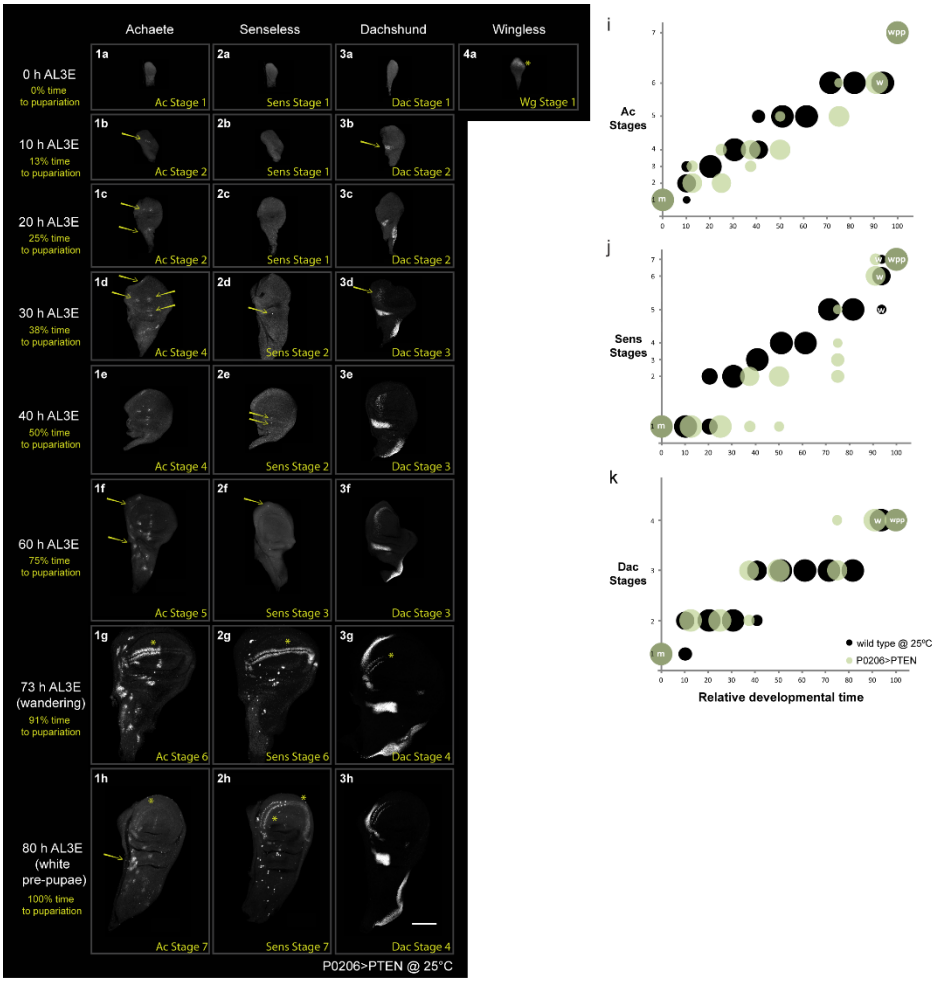
Supplementary Figure S2.5. Patterning progression of four gene products in discs from wild-type larvae reared at 18°C. The expression of Achaete (1a-1h), Senseless (2a-2h) and Dachshund (3a-3h) at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c), 30 (1d-3d), 50 (1e-3e) and 70 (1f-3f) hours after third instar ecdysis (h AL3E), wandering (1g-3g) (at the average time of 96 h AL3E) and white pre-pupae (at the average time of 101 h AL3E, 1h-3h). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows show addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (i-k) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage, represented in relative developmental time: (i) Achaete (Ac) stages, (j) Senseless (Sens) stages and (k) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. Wild type 18°C staged discs are represented in blue while the 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



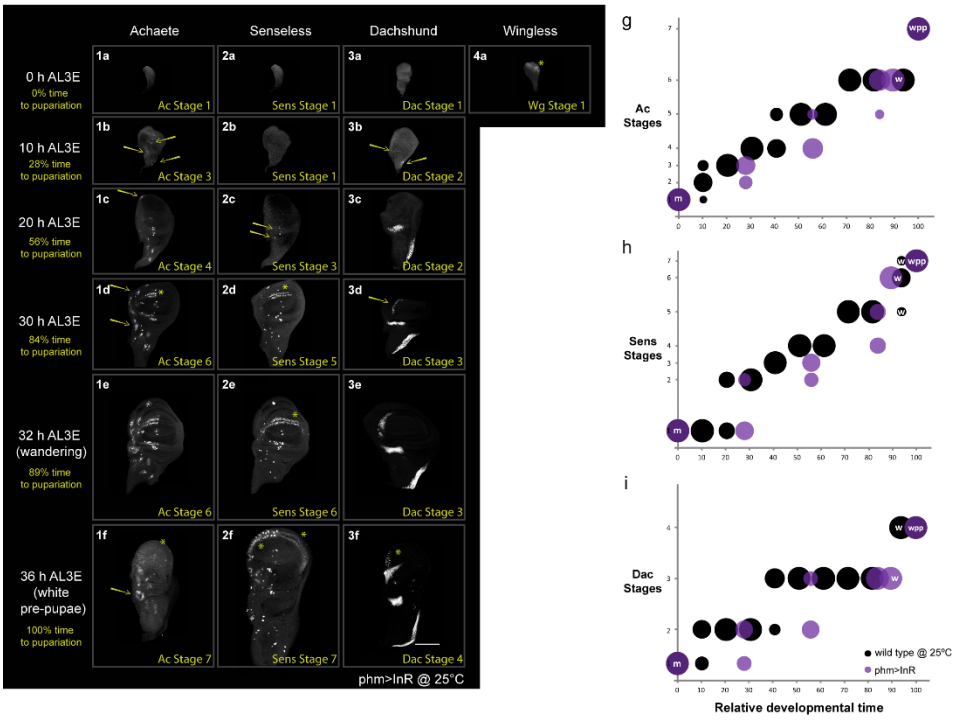
Supplementary Figure S2.6. Patterning progression of four gene products in discs from wild-type larvae reared at 29°C. The expression of Achaete (1a-1h), Senseless (2a-2h) and Dachshund (3a-3h) shown at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c), 30 (1d-3d), 35 (1e-3e) and 40 (1f-3f) hours after third instar ecdysis (h AL3E), wandering (1g-3g) (at the average time of 48 h AL3E) and white pre-pupae (at the average time of 52 h AL3E, 1h-3h). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows show addition or change in the appearance of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (i-k) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage in relative developmental time: (i) Achaete (Ac) stages, (j) Senseless (Sens) stages and (k) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. Wild type 29°C staged discs are represented in orange while the 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



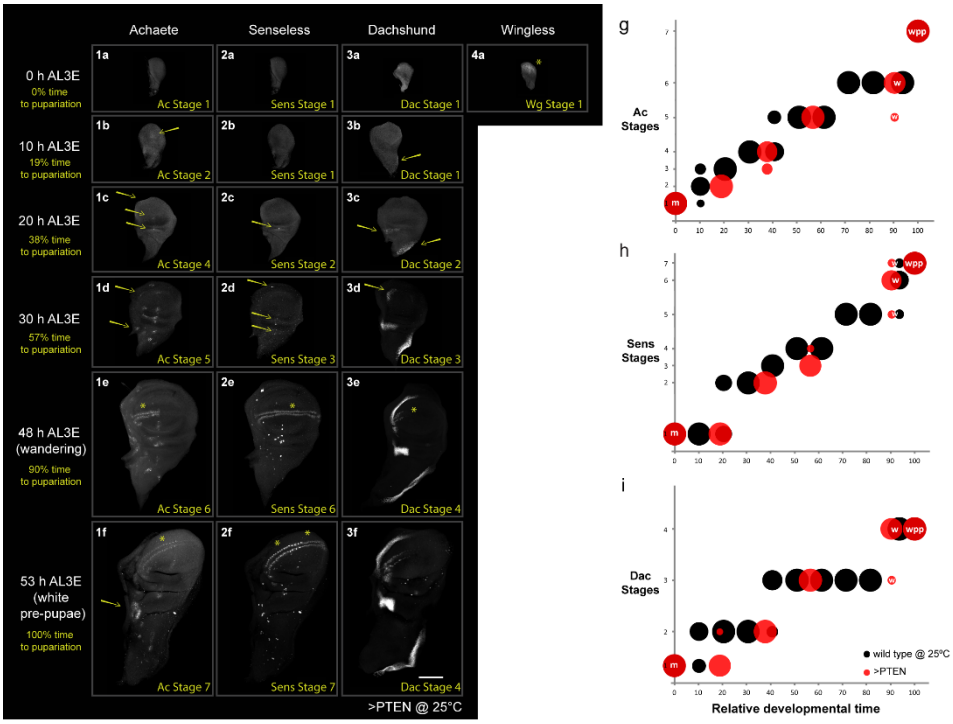
Supplementary Figure S2.7. Comparing expression patterns at moulting, wandering and pupariation in larvae reared at 25˚C and 29˚C. The expression patterns of Ac, Sens, Dac and Wg between discs from wild-type larvae reared at 25˚C and reared at 29˚C at the three developmental events of moulting, wandering and pupariation. Comparison of the expression of Achaete (a, e, i, l, o, r), Senseless (b, f, j, m, p, s), Dachshund (c, g, k, n, q, t) and Wingless (d, h) at the moult to the third instar (0h, a-h), wandering (i-n) and pupariation (o-t) between wild-type larvae reared at 25˚C (a-d, i-k, o-q) and reared at 29˚C (e-h, l-n, r-t). The corresponding disc stages are represented in the column to the right of the images. Arrows show addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Scale bar is 100 µm.



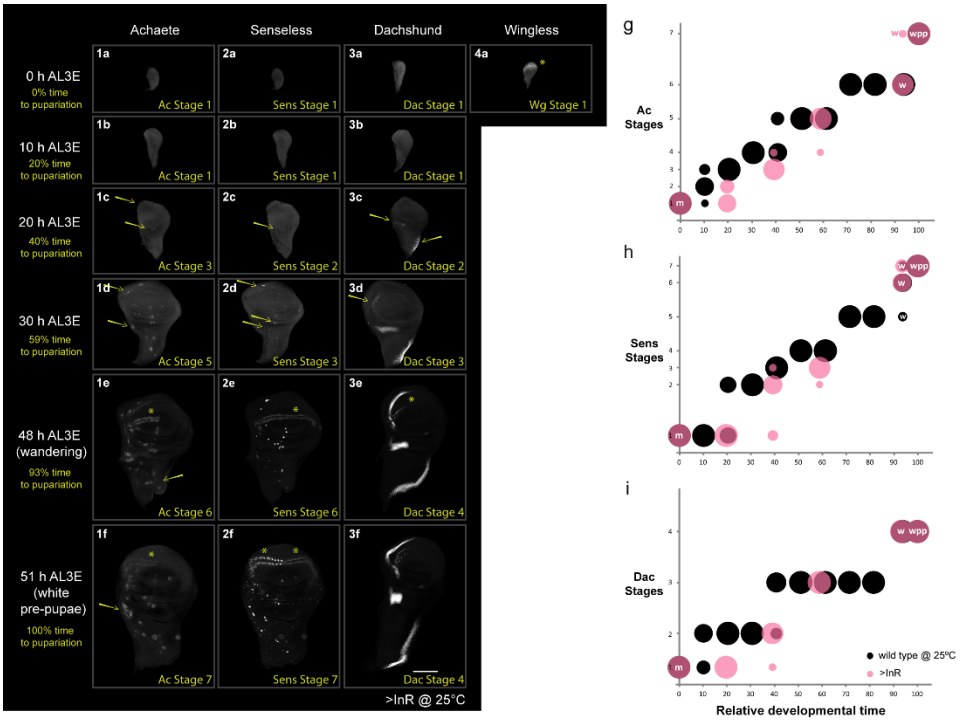
Supplementary Figure S2.8. Patterning progression of four gene products in discs from larvae reared with delayed ecdysone production (*P0206>PTEN*). The expression of Achaete (1a-1h), Senseless (2a-2h) and Dachshund (3a-3h) shown at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c), 30 (1d-3d), 40 (1e-3e) and 60 (1f-3f) hours after third instar ecdysis (h AL3E), wandering (1g-3g) (at the average time of 73 h AL3E) and white pre-pupae (at the average time of 80 h AL3E, 1h-3h). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows mark the addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (i-k) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage in relative developmental time: (i) Achaete (Ac) stages, (j) Senseless (Sens) stages and (k) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. *P0206>PTEN* staged discs are represented in green while the 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



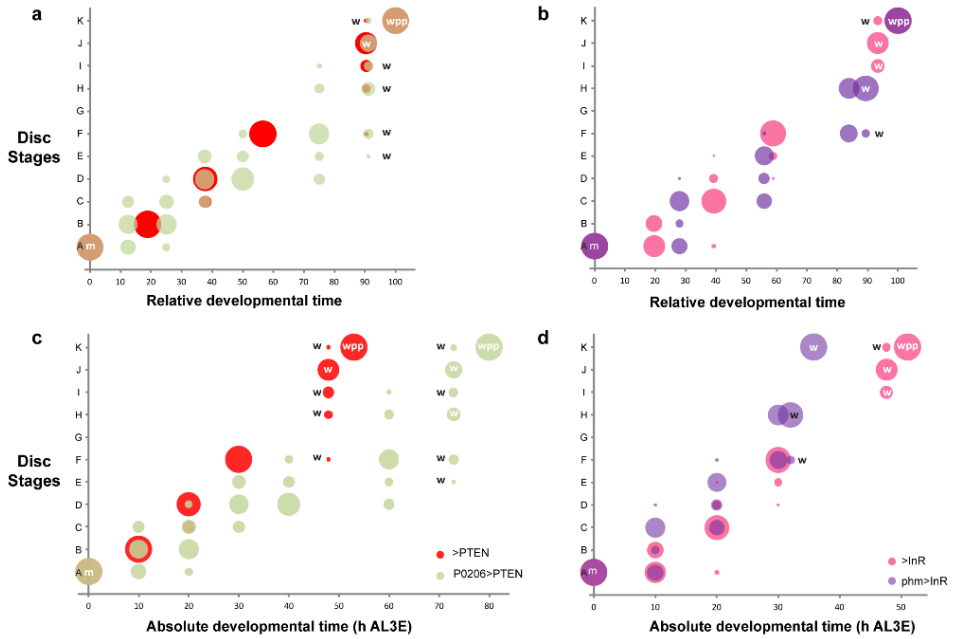
Supplementary Figure S2.9. Patterning progression of four gene products in discs from larvae with accelerated ecdysone production (*phm>InR*). The expression of Achaete (1a-1f), Senseless (2a-2f) and Dachshund (3a-3f) shown at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c) and 30 (1d-3d) hours after third instar ecdysis (h AL3E), wandering (1e-3e, samples from 30.5 h AL3E) and white pre-pupae (1f-3f). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows mark the addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (g-i) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage in relative developmental time: (g) Achaete (Ac) stages, (h) Senseless (Sens) stages and (i) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. *phm>InR* staged discs are represented in purple while the 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



Supplementary Figure S2.10. Patterning progression of four gene products in discs from the parental line *>PTEN*. The expression of Achaete (1a-1f), Senseless (2a-2f) and Dachshund (3a-3f) shown at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c) and 30 (1d-3d) hours after third instar ecdysis (h AL3E), wandering (at the average time of 48 h AL3E, 1e-3e) and white pre-pupae (at the average time of 53 h AL3E, 1f-3f). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows mark the addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (g-i) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage in relative developmental time: (g) Achaete (Ac) stages, (h) Senseless (Sens) stages and (i) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. *>PTEN* staged discs are represented in red while the wild type 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



Supplementary Figure S2.11. Patterning progression of four gene products in discs from the parental line *>InR*. The expression of Achaete (1a-1f), Senseless (2a-2f) and Dachshund (3a-3f) shown at 0 (1a-3a), 10 (1b-3b), 20 (1c-3c) and 30 (1d-3d) hours after third instar ecdysis (h AL3E), wandering (at the average time of 48 h AL3E, 1e-3e) and white pre-pupae (at the average time of 51 h AL3E, 1f-3f). Wingless expression is represented only for the moult to the third instar (0h AL3E, 4a). Arrows mark the addition or change of cells or patches of cells, and asterisks highlight changes in stripes. Under each time point is the corresponding relative developmental time (normalized to pupariation). In green under each disc is the attributed gene-specific stage. (g-i) For each time point, the size of each circle represents the proportion of discs attributed to each gene-specific stage in relative developmental time: (g) Achaete (Ac) stages, (h) Senseless (Sens) stages and (i) Dachshund (Dac) stages. The differences in axis spacing between gene-specific stages scale according to developmental time at 25°C. For example, the transition from Ac stage 1 to 2 takes 5 h while the transition from Ac stage 6 to 7 takes 15 h. *>InR* staged discs are represented in pink while the wild type 25°C staged discs from our staging scheme are in black. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).



Supplementary Figure S2.12. The progression of pattern, in relative and absolute time, in discs from larvae with altered timing of ecdysis synthesis and respective parental lines. Probability (represented by the size of the circle) that a disc with a particular set of gene-specific stages belongs to a given disc stage varied by relative (normalized to pupariation) (a, b) or absolute developmental time (hours after third instar ecdysis (h AL3E)) (c, d). Manipulations of the timing of ecdysis synthesis: (a, c) disc stages attributed to discs from *P0206>PTEN* larvae are shown in green and disc stages attributed to discs from *>PTEN* larvae are in red; (b, d) disc stages attributed to discs from *phm>InR* larvae are shown in purple and disc stages attributed to discs from *>InR* larvae are in pink. Developmental events are identified by m (moulting), w (wandering) and wpp (white pre-pupae).

Supplementary Table S2.1. Number of discs dissected for the wild type at 25°C for each gene product at each time points, used to devise the staging scheme. The asterisk represents discs that were simultaneously scored for both Achaete (Ac) and Senseless (Sens).

Condition / time point (h AL3E)		# discs for each gene product					
		Ac*	Sens*	Dac	Wg	Ct	N
wild type @ 25°C	0	10	10	10	10	10	10
	5	9	9	16	10	8	10
	10	6	6	5	10	10	10
	15	10	10	10	10	8	10
	20	8	8	10	10	5	8
	25	10	10	10	10	10	10
	30	9	9	10	10	10	10
	35	9	9	10	10	10	10
	40	8	8	10	10	6	6
	w	6	6	6	6	6	7
	wpp	10	10	8	5	10	9

Supplementary Table S2.2. Number of discs dissected for all treatments/genotypes (except for wild type at 25°C) and for each gene product at all time points. The asterisk represents discs that were simultaneously scored for both Achaete (Ac) and Senseless (Sens).

Condition / time point (h AL3E)		# discs for each gene product			
		Ac*	Sens*	Dac	Wg
wild type @ 18°C	0	9	9	9	9
	10	10	10	10	-
	20	10	10	10	-
	30	10	10	10	-
	50	8	8	10	-
	70	13	13	11	-
	w	20	20	13	-
	wpp	10	10	10	-
wild type @ 29°C	0	10	10	9	10
	10	10	10	10	-
	20	18	18	10	-
	30	9	9	10	-
	35	10	10	10	-
	40	9	9	10	-
	w	9	9	10	-
	wpp	18	18	10	-
P0206>PTEN	0	8	8	9	6
	10	8	8	8	-
	20	11	11	8	-
	30	10	10	9	-
	40	10	10	7	-
	60	7	7	8	-
	w	16	16	18	-
	wpp	10	10	10	-
phm>InR	0	7	7	8	9
	10	10	10	10	-
	20	10	10	10	-
	30	10	10	10	-
	w	10	10	10	-
	wpp	8	8	8	-
>PTEN	0	7	7	10	10
	10	8	8	12	-
	20	9	9	6	-
	30	10	10	10	-
	w	8	8	8	-
	wpp	8	8	7	-
>InR	0	9	9	10	10
	10	8	8	8	-
	20	10	10	9	-
	30	10	10	10	-
	w	9	9	8	-
	wpp	8	8	10	-

Supplementary Table S2.3. List of antibodies used in the immunocytochemistry protocol. Mouse anti-Achaete was used in combination with guinea pig anti-Senseless (Nolo et al., 2000).

Antibody Name	Antigen	Source	Host species	Dillution used
anti-achaete	Achaete protein	Developmental Studies Hybridoma Bank	mouse	1:100
anti-Senseless	Senseless protein	a gift from Dr Hugo Bellen	guinea pig	1:3000
mAbdac2-3	Dachshund protein, Drosophila	Developmental Studies Hybridoma Bank	mouse	1:100
C594.9B	Delta, extracellular domain	Developmental Studies Hybridoma Bank	mouse	1:100
C17.9C6	Notch, intracellular domain	Developmental Studies Hybridoma Bank	mouse	1:100
2B10	Cut protein	Developmental Studies Hybridoma Bank	mouse	1:100
1G9	Hindsight protein	Developmental Studies Hybridoma Bank	mouse	1:100
mAB sca1	Scabrous Drosophila gene protein	Developmental Studies Hybridoma Bank	mouse	1:100
4D9	Engrailed/Invected proteins	Developmental Studies Hybridoma Bank	mouse	1:20
Drosophila Ptc (Apa 1)	Patched	Developmental Studies Hybridoma Bank	mouse	1:40
4D4	Wingless protein	Developmental Studies Hybridoma Bank	mouse	1:100

3

Growth of wing discs from larvae with altered developmental time

Summary

Correctly patterned organs come in many sizes. Despite differences in size, organs maintain their scaling relationships with the body to ensure correct function. In the fruit fly, *Drosophila melanogaster,* adult organs come from imaginal tissues that grow and pattern during larval and pupal development. The growth trajectories of imaginal tissues under standard conditions have been well described. In addition, we know in detail how different environmental and physiological conditions change final adult wing and body size. However, we do not know how the developmental processes that regulate wing size change with alterations in environmental or physiological conditions.

To explore whether the growth trajectories of wing discs change with environmental and physiological conditions, we either reared larvae at different temperatures or genetically altered the timing of ecdysone synthesis. Our data show that growth trajectories change in different ways to generate discs of different final sizes at pupariation. Small discs either resulted from changes in growth rates or a combination of changes in growth rates and length of the growth period. On the other hand, discs reared under conditions that produce large wings were equal in size at pupariation to control wing disc. This suggests that the increase in adult wing size results from processes during pupal development. In addition, although all discs showed fast-then-slow growth rates, for four of the conditions examined, the switch from fast to slow occurred around 25-33% development and for the remaining three it occurred around 59-68% of development. To explore the relationship between growth and patterning in the wing disc, we linked disc size with the expression patterns of two canonical patterning genes, Achaete and Dachshund. Within a condition, we observed strong correlations between disc size and patterning stage. However, between conditions discs exhibited considerable variation in size with patterning stage.

Our results suggest that the developmental processes that control disc growth can be altered in a variety of ways to produce different-sized wings. In addition, we find that the growth/pattern relationship in the disc varies with condition to ensure that discs can reach their final patterning stage regardless of their size.

Introduction

Organs show characteristic scaling relationships with body size that are necessary for their function. Previous studies show that these scaling relationships can change depending on the environmental conditions (Shingleton et al., 2009) and with genetic backgrounds (Frankino et al., 2005, 2007; Tobler and Nijhout, 2010). However, the mechanisms that allow organs to scale in correct proportions with the rest of the body remain poorly understood. Furthermore, regardless of their final size organs show correct pattern. This lead us to pose two questions related to the coordination of organ/whole-body development: 1) how is organ size coordinated with whole-body development and 2) what is the nature of the organ size/pattern relationship?

In fruit flies (*Drosophila melanogaster*), the final size of the adult wing depends on many well-studied conditions. Larvae reared at cooler temperatures show larger body and wing sizes than those from larvae reared in warmer temperatures (De Moed et al., 1997; Debat et al., 2003; James et al., 1995; Partridge et al., 1994; Shingleton et al., 2009). Differences in nutrition (Bakker, 1959; Beadle et al., 1938; De Moed et al., 1997; Shingleton et al., 2009), larval density (Santos et al., 1994; Shingleton et al., 2009), and the timing of hormone synthesis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005) also alter body and wing sizes of the adults. Although many environmental conditions affect body and wing size, they do not all affect size in the same way. Changing temperature produces disproportionally larger (hyperallometric) alterations in wing size with respect to body size, whereas changes in larval density or larval nutrition result in proportional (isometric) changes between the body and

the wing (Shingleton et al., 2009). Given these differences in scaling relationships between adult wing and body sizes, we sought to understand whether discs showed alterations in their growth trajectories that could explain these differences.

The growth trajectories of wing imaginal discs under standard conditions have been well described (Bryant and Levinson, 1985; Garcia-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977; Martin, 1982; Milan et al., 1996; Parker and Shingleton, 2011). Although the imaginal discs grow throughout the three larval instars, the bulk of the increase in size occurs during the third larval instar. Many processes contribute to changes in final disc size. First, discs can start out at different sizes at the onset of the third instar. Second, discs can grow at different rates and finally they can grow for different lengths of time. Understanding how disc growth is altered with environmental, genetic or physiological conditions requires taking each of these processes into account.

In terms of the wing disc size/pattern relationship, several studies show that wings pattern correctly across a wide range of sizes caused for example, by changes in rearing temperature (De Moed et al., 1997; Debat et al., 2003; James et al., 1995; Partridge et al., 1994; Shingleton et al., 2009). This suggests that size does not limit the final pattern of a wing disc. Nevertheless, early in larval disc development morphogens like Decapentaplegic (Dpp) and Wingless (Wg) organize specific domains within the disc and regulate directions of growth. Manipulating Dpp changes disc size (Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002; Nellen et al., 1996; Rogulja and Irvine, 2005; Spencer et al., 1982), suggesting that it coordinates both patterning and growth. Potentially, we

could envision a relationship between the size and the pattern of the wing imaginal disc such that to reach a given patterning state a disc needs to attain a minimum size or vice-versa. Our previous work shows that disc patterning aligns with whole-body development at developmental milestones (Oliveira et al., 2014). Given this, we hypothesized that disc size and pattern could either be A) regulated by independent, albeit interacting, processes or B) coordinated by minimal size-dependent checkpoints.

To explore the growth trajectories and the relationship between growth and patterning in the wing disc, we altered environmental conditions, by changing temperature, or physiological conditions, by altering the timing of ecdysone synthesis. For each condition, we measured wing disc size through time to explore how growth trajectories change. Further, we compared disc size with the expression patterns of two canonical patterning genes, Achaete and Dachshund, allowing us to observe if there is a minimum disc size to progress in pattern. Our data show that discs differ in their growth trajectories depending on the condition, although all show similar fast-then-slow growth rates. Further, although within a condition disc size and pattern show high correlation, between conditions we observe significant differences in the disc size/pattern relationship. This suggests that both the coordination of disc growth with body development and the disc growth/pattern relationship are altered with changes in environmental and physiological conditions.

Materials and methods

Fly stocks and rearing conditions

See Chapter 2. Materials and methods – Fly stocks and rearing conditions.

Animal staging and developmental time

See Chapter 2. Materials and methods – Animal staging and developmental time.

Dissections and immunocytochemistry

See Chapter 2. Materials and methods – Dissections and immunocytochemistry.

Quantifications of wing imaginal disc size

We quantified wing disc size using disc area as a proxy. All quantifications were done using ImageJ. Wing discs show exponential growth in the third instar. Thus, we studied the growth trajectories of the discs by log transforming disc area or relative disc area.

Quantification of patterning stages

We quantified the patterning of the gene products Achaete (Ac) and Dachshund (Dac) in the wing imaginal discs using the gene-specific stages described in chapter 2 (Oliveira et al., 2014).

Statistical analysis

All data analyses and statistics were conducted using R (www.r-project.org) (Ihaka and Gentleman, 1996). Growth trajectories were

visualized by fitting a Loess smoothing function (Cleveland, 1979, 1981; Cleveland and Devlin, 1988) (with 95% confidence interval) to the relationship between log transformed disc area against time, log disc area over relative time (normalized to pupariation), and log relative disc area (normalized to mean size at pupariation) over relative time. We tested for changes in the slope of the curves using a Davies's test for change in slope (Davies, 1987) and estimated the breakpoints using the segmented function from the segmented package in R (Muggeo, 2008).

To explore the relationship between wing disc size and patterning stage, we fit linear regression models to log disc area against Ac stage and log disc area against Dac stage. We tested for changes in the disc size/pattern relationship with condition using an Analysis of Covariance (ANCOVA).

Results

To understand how growth trajectories changed with environmental/physiological conditions in wing discs, we altered the duration of development either environmentally or physiologically and observed the effects on wing disc size over time. To alter developmental rate environmentally, we used wing discs from larvae of an isogenic wild-type strain Samarkand (SAM) and reared larvae at 18˚C (wild type at 18˚C), 25˚C (wild type at 25˚C) and 29˚C (wild type at 29˚C). We measured wing disc size at 8-11 time points (depending on the condition) throughout the L3 that ranged from moult to pupariation (Supplementary Tables S3.1 and S3.2).

Wing disc growth trajectories change with temperature

As expected, changes in rearing temperature induced changes in developmental time (Figure 2.5). Wild type larvae reared at 29˚C showed slightly longer L3 duration when compared to those reared 25˚C. Larvae took two times longer to develop at 18˚C than at to 25˚C. Because wing

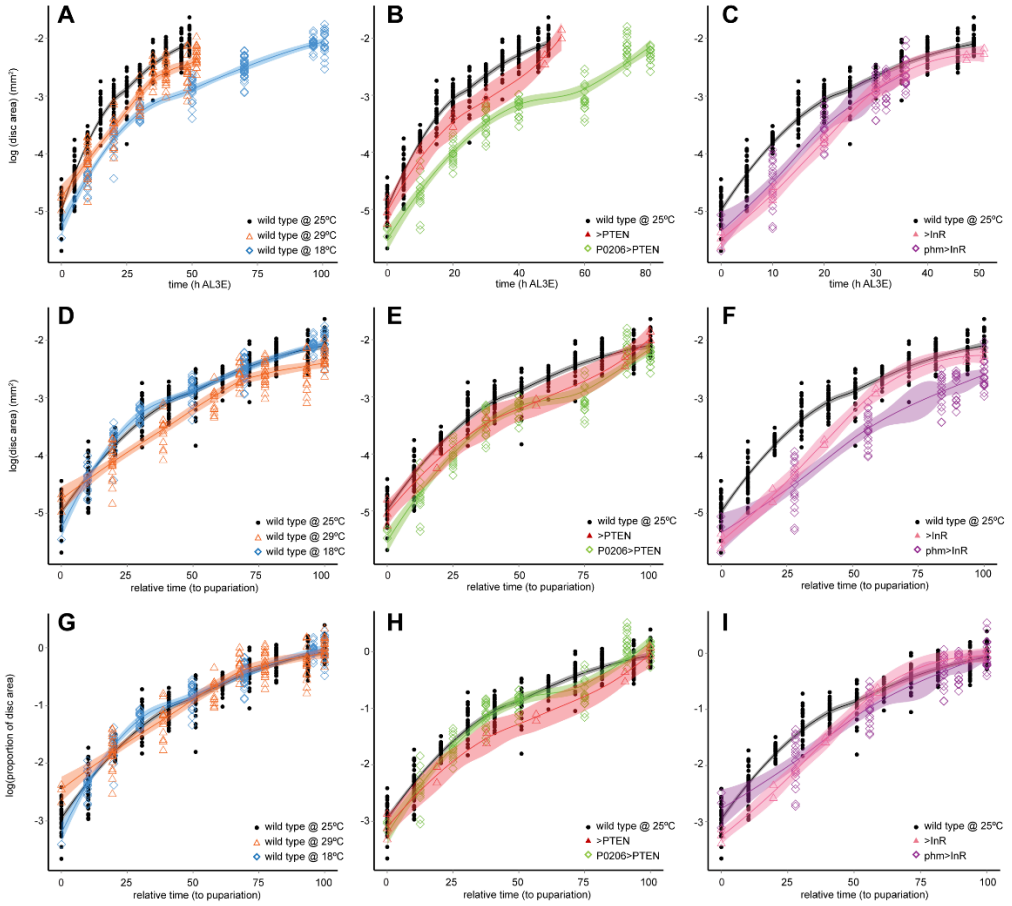


Figure 3.1. Wing disc growth trajectories depend on environmental and physiological conditions. Log-transformed wing disc area over time (A, B, C), over relative time (normalized to pupariation, D, E, F) and log-transformed proportion of wing disc area (normalized by mean size at pupariation, G, H, I) over relative time. Size is represented in mm^2 and time in hours after the third instar ecdysis (h AL3E). The data is fit using a Loess smoothing function and the shaded areas represent 95% confidence intervals.

discs grow exponentially in the L3 (Shingleton et al., 2008), we log transformed disc area to compare growth trajectories between temperatures. The log transformed data showed non-linear increases with time. Therefore we fit a Loess smoothing function, with 95% confidence intervals, to explore the changes in the growth trajectories with condition.

At the beginning of L3, wing discs from larvae reared at 18˚C are smaller than those from larvae reared at 25˚C or 29˚C (Figure 3.1 A). Surprisingly, by pupariation, discs from wild type larvae at 18˚C were not distinguishable in size from discs at 25˚C. This suggests that the differences in size observed in the adult wings from animals reared at 18˚C versus 25˚C results from processes occurring after pupariation. In contrast, discs from wild type larvae at 29˚C were smaller at pupariation than those from wild type larvae at 18˚C and 25˚C. Lastly, discs from larvae reared at 18˚C grew slower than discs from larvae at 29˚C, which in turn grew slower than discs from larvae reared at 25˚C (Figure 3.1 A).

To explore how disc growth coordinates with whole-body development independent of the changes in developmental time, we normalized developmental time to pupariation (relative time). In relative time, the growth curves of discs from larvae reared at 18˚C and 25˚C were largely overlapping (Figure 3.1 D). In contrast, the relative growth of discs from larvae reared at 29˚C was substantially slower throughout development. Furthermore, we noticed that the shape of the growth trajectories appeared different between temperatures, with a change in slope occurring early in discs from 18˚C and 25˚C and later in discs from larvae reared at 29˚C. Using Davies' test for change in slope and bi-segmented linear regression, we confirmed significant changes in slope between at

Table 3.1. Comparison of breakpoints in the growth trajectories for the different conditions.

Conditions	Breakpoint		
	Davies' test for change in slope (p-value)	Estimated breakpoint (% dev. time)	Breakpoint standard error
wild type at 25°C	8.707e-06	31.320	1.375
wild type at 18°C	2.2e-16	25.850	1.617
wild type at 29°C	2.2e-16	67.65	4.25
P0206>PTEN	2.2e-16	33.370	3.256
>PTEN	2.2e-16	25.180	7.942
phm>InR	2.2e-16	65.38	11.72
>InR	2.2e-16	59.20	19.48

26%, 31% and 68% development for discs from larvae reared at 18˚C, 25˚C and 29˚C respectively (Table 3.1).

Finally, we explored whether the differences in growth trajectories we observed were an effect of the final size of the disc. To do this, we normalized disc growth to the final size at pupariation and compared the trajectories of log relative disc size over relative time. This allows us to ask, for example, if at 50% development time discs from all conditions have reached an equal proportion of their final size. Between rearing conditions, we find greater variation in the proportion of final disc size obtained before 50% development (Figure 3.1 G). After 50% development, the trajectories from all three rearing conditions overlap.

Wing disc growth trajectories change with altered ecdysone synthesis

We also altered developmental time by manipulating the timing of ecdysone synthesis, by regulating insulin signalling in the prothoracic gland, at 25˚C. To downregulate insulin signalling in the prothoracic gland,

thereby delaying ecdysone synthesis, we used the *P0206 GAL4* driver to overexpress *PTEN* (*P0206>PTEN)*; to accelerate ecdysone synthesis, we upregulated insulin signalling in this tissue using the *phm GAL4* to drive the expression of *InR* (*phm>InR*). We used the UAS responder lines as controls *(>PTEN* and *>InR*) and compared all genotypes/conditions to the growth of wild type discs at 25˚C.

As reported, increasing ecdysone synthesis (by upregulating insulin signalling in the prothoracic gland, *phm>InR*) shortens developmental time, while delaying ecdysone synthesis (by downregulating insulin signalling in the prothoracic gland, *P0206>PTEN*) retards developmental time. The wing disc size at the moult to L3 between *P0206>PTEN* and either the control *>PTEN* discs or the wild type at 25˚C discs, and between *phm>InR* and either the control *>InR* discs or the wild type at 25˚C discs are similar. Although adult wings from *P0206>PTEN* adults are larger (Mirth et al., 2005), *P0206>PTEN* discs show similar sizes compared to *>PTEN* at pupariation and are slightly smaller than wild type at 25˚C discs (Figure 3.1 B). At pupariation, *phm>InR* discs are dramatically smaller than *>InR* or wild type at 25˚C (Figure 3.1 C). Control *>PTEN, >InR* and wild type at 25˚C are not distinguishable at pupariation.

P0206>PTEN discs show a slower growth rate compared to either *>PTEN* or wild type at 25˚C (Figure 3.1 B). Interestingly, wing discs from *phm>InR* and *>InR* larvae grow at the same rate, although both grow slower than discs from wild type larvae 25˚C (Figure 3.1 C).

In relative time, we observe that discs from *P0206>PTEN* and *>PTEN* grow at the same rates, but both grow slower in the early phases of L3 than discs from wild type larvae reared at 25˚C discs (Figure 3.1 E).

phm>InR discs grow slower than *>InR,* which grow slower than discs from wild type larvae reared at 25˚C (Figure 3.1 F). We detect significant changes in slope in the growth trajectories of all genotypes, with *P0206>PTEN* and *>PTEN* showing changes in the logarithmic disc growth rate at 33 and 25% development respectively and *phm>InR* and *>InR* changing slope at 65 and 59% development respectively (Table 3.1).

Finally, the proportional growth curves in relative time largely overlap for discs from *P0206>PTEN, >PTEN* and wild type larvae reared at 25˚C (Figure 3.1 H). In contrast, before 50% development *phm>InR* and *>InR* achieve a lesser proportion of their final disc size than wild type at 25˚C (Figure 3.1 I). After 50% development time, the proportional growth trajectories overlap.

Disc size/pattern relationship differs with conditions to produce fully patterned wings with different sizes

To address whether disc patterning is coordinated with disc growth, we compared wing disc growth to the progression of pattern for two patterning genes: Ac and Dac. We characterized the expression pattern as described in chapter 2 (Oliveira et al., 2014), attributing gene-specific stages to each disc. Because discs both grow and pattern over developmental time, we expect these two parameters to be highly correlated. However, both the intercepts and slopes from the linear regression of the log disc size over gene-specific stage provide information relating to the nature of their coordination. If disc size and patterning stage are tightly coordinated, we expect to see that the intercepts and slopes of the regression lines from the different conditions and genotypes should overlap. Significant deviations in both intercept and slope would indicate

that discs achieve the same patterning stage over a range of sizes, suggesting that the coordination between the two processes is plastic. Additionally, if growth and patterning are coordinated only at milestones, then we expect to observe discs of the same sizes showing the same gene-specific stage at these milestones.

As expected, we find string correlation between the log disc size and the progression of pattern for both Ac and Dac for all temperature treatments (Figs 3.2 A, 3.3 A and Tables 3.2, 3.3, 3.4 and 3.5). In addition, we find significant differences in the intercepts of the disc growth/Ac pattern regression between discs from wild type larvae at 18˚C and 25˚C (Figure 3.2 A and Tables 3.2 and 3.3). At 29˚C, the slope of the disc

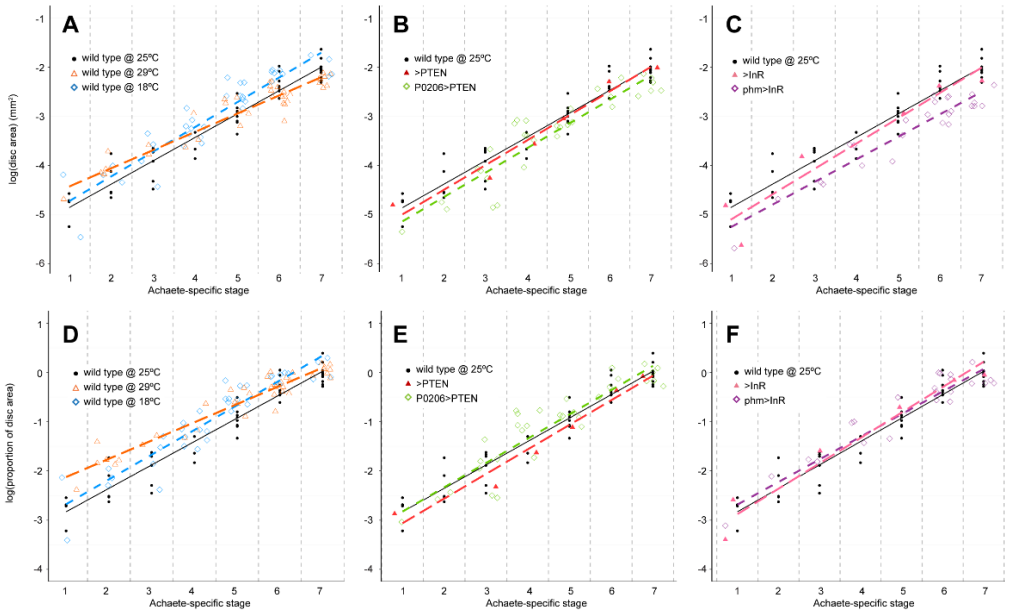


Figure 3.2. Wing imaginal disc size correlates with Achaete-specific stages. Log-transformed wing disc area (A, B, C) and log-transformed proportional wing disc area (normalized by mean size at pupariation, D, E, F) regressed against Achaete stages (1-7). Size is represented in mm^2. The data were fit using linear regression models.

growth/Ac pattern relationship is significantly different from that at 25˚C (Tables 3.2, 3.3 and Supplementary Figure S3.1). We did not observe any distinguishable differences in either the intercept or the slope of the disc growth/Dac pattern regression between any of the temperature conditions (Figure 3.3 A and Tables 3.4 and 3.5). However, the intercept of the relative disc growth/Dac pattern regression at 29˚C is different from that of 18˚C and 25˚C (Supplementary Table S.3.4).

Similar to what we observed for temperature treatments, we found significant correlations between log disc size and patterning stage for Ac and Dac when we altered developmental time by changing ecdysone synthesis (Figs 3.2 B, C, 3.3 B, C and Tables 3.2, 3.3, 3.4 and 3.5). Discs from

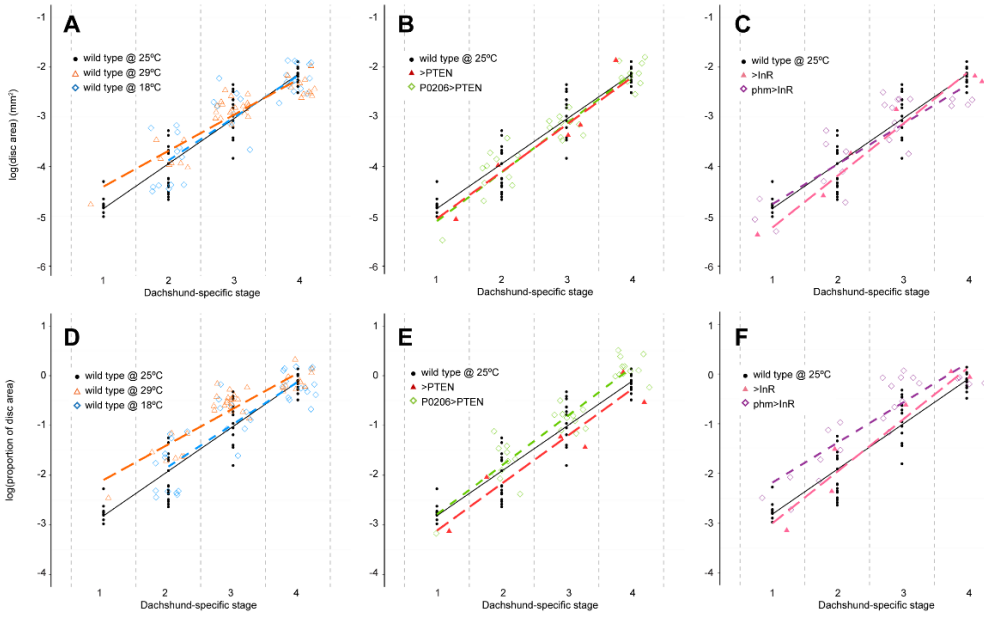


Figure 3.3. Wing imaginal disc size correlates with Dachshund-specific stages. Log-transformed wing disc area (A, B, C) and log-transformed proportional wing disc area (normalized by mean size at pupariation, D, E, F) regressed against Dachshund stages (1-7). Size is represented in mm^2. The data were fit using linear regression models.

P0206>PTEN larvae showed a significant change in the intercept of the disc size/Ac pattern regression when compared to wild type larvae at 25˚C and *>PTEN* (Figure 3.2 B, Table 3.2 and Supplementary Figure S3.1). Discs from both *phm>InR* and *>InR* larvae also demonstrated a significant change in intercept for the disc size/Ac pattern regression when compared to wild type larvae at 25˚C (Figure 3.2 C and Table 3.2). We did not observe significant alterations for any genotype in either intercept or slope for the regression between disc size and Dac pattern (Figure 3.3 B, C, and Table 3.4) however different genotypes showed significant differences in intercepts for the regression between relative disc size and Dac pattern (Figure 3.3 E, F and Supplementary Table S3.4). Taken together, our data suggest that the relationship between disc size and disc pattern is plastic, and we find no evidence for convergence of disc size and pattern at developmental milestones.

Table 3.2. ANCOVA to test for differences in slopes and intercepts among regression lines for log-transformed disc area compared to Ac-specific stage; grey cells highlight p<0.01.

Comparison between conditions		Ac-specific stage	Condition	Ac-specific stage * Condition
wild type at 25°C	wild type at 18°C	<2e-16	0.000598	0.448768
wild type at 25°C	wild type at 29°C	<2e-16	0.756072	0.000382
wild type at 25°C	*P0206>PTEN*	<2e-16	0.00242	0.65883
wild type at 25°C	*>PTEN*	<2e-16	0.606	0.689
P0206>PTEN	*>PTEN*	<2e-16	0.269	0.936
wild type at 25°C	*phm>InR*	<2e-16	2.26e-09	0.667
wild type at 25°C	*>InR*	<2e-16	0.296	0.449
phm>InR	*>InR*	<2e-16	2.26e-09	0.667

Table 3.3. Linear regression lines for log-transformed disc area and log-transformed proportional disc area compared to Ac-specific stage; grey cells highlight p<0.01.

Achaete – Linear regression lines		Coefficients:		Adjusted R-squared	p-value
		(Intercept)	slope		
log disc area	wild type at 25°C	-5.3305	0.4758	0.9274	<2e-16
	wild type at 18°C	-5.2285	0.5033	0.8784	<2e-16
	wild type at 29°C	-4.7977	0.3706	0.9142	<2e-16
	P0206>PTEN	-5.626	0.493	0.8591	1.625e-14
	>PTEN	-5.491	0.499	0.9684	0.0002415
	phm>InR	-5.7135	0.4592	0.8928	7.486e-13
	>InR	-5.6175	0.5159	0.9298	0.001205
log proportional disc area	wild type at 25°C	-3.3041	0.4758	0.9274	<2e-16
	wild type at 18°C	-3.1787	0.5033	0.8784	<2e-16
	wild type at 29°C	-2.4936	0.3706	0.9142	<2e-16
	P0206>PTEN	-3.316	0.493	0.8591	1.625e-14
	>PTEN	-3.558	0.499	0.9684	0.0002415
	phm>InR	-3.1414	0.4592	0.8928	7.486e-13
	>InR	-3.3918	0.5159	0.9298	0.001205

Discussion

Although the problem of organ/body scaling has been studied for almost a century (Huxley, 1924, 1932; Huxley and Teissier, 1936; Thompson, 1917), researchers have paid little attention to the developmental processes that generate variation in final organ size. Even less is understood about the relationship between organ size and organ patterning throughout development, a process that ensures robustness of pattern across a wide range of conditions. In this study, we explored the growth trajectories of wing imaginal discs from larvae reared in environmental or physiological conditions known to alter final wing and body size. We proposed that adult wings could achieve different final sizes if the wing imaginal discs 1) started at different initial sizes, 2) altered their

Table 3.4. ANCOVA to test for differences in slopes and intercepts among regression lines for log-transformed disc area compared to Dac-specific stage; grey cells highlight p<0.01.

Comparison between conditions		Dac-specific stage	Conditions	Dac-specific stage * Conditions
wild type at 25°C	wild type at 18°C	<2e-16	0.771	0.623
wild type at 25°C	wild type at 29°C	<2e-16	0.2008	0.0296
wild type at 25°C	*P0206>PTEN*	<2e-16	0.198	0.310
wild type at 25°C	*>PTEN*	<2e-16	0.445	0.758
P0206>PTEN	*>PTEN*	<2e-16	0.874	0.753
wild type at 25°C	*phm>InR*	<2e-16	0.437	0.335
wild type at 25°C	*>InR*	<2e-16	0.333	0.354
phm>InR	*>InR*	<2e-16	0.706	0.222

growth rates, or 3) altered the length of their growth period. We found that differences in disc size at pupariation appear to result from changes in the growth rate and the length of the growth period. Further, we found that growth rate itself is modulated in the third larval instar at one of two distinct phases of development. Finally, our data suggest that different milestones regulate wing disc size and wing pattern.

Analysing differences in wing size using wing disc growth trajectories

Wing discs from larvae reared at different temperatures or with altered rates of ecdysone synthesis showed differences in all three of processes we proposed: initial size, growth rate and duration of growth. However, not all of these differences resulted in alterations in final size at pupariation. For example, wing discs from larvae reared at 18°C and from *P0206>PTEN* larvae were smaller at the onset of the L3 than from larvae reared at 25°C. The discs from these larvae also grew for longer times than wild type larvae at 25°C. Regardless of these differences, at pupariation wing discs from

Table 3.5. Linear regression lines for log-transformed disc area and log-transformed proportional disc area compared to Dac-specific stage; grey cells highlight p<0.01.

Dachshund – Linear regression lines		Coefficients:		Adjusted R-squared	p-value
		(Intercept)	slope		
log disc area	wild type at 25°C	-5.7362	0.8972	0.8629	<2e-16
	wild type at 18°C	-5.5809	0.8527	0.7654	1.845e-12
	wild type at 29°C	-5.1138	0.7159	0.8354	6.884e-16
	P0206>PTEN	-6.0744	0.9805	0.9097	<2e-16
	>PTEN	-5.987	0.943	0.9504	0.0005985
	phm>InR	-5.5480	0.7966	0.7294	1.324e-07
	>InR	-6.256	1.034	0.924	0.001414
log proportional disc area	wild type at 25°C	-3.7097	0.8972	0.8629	<2e-16
	wild type at 18°C	-3.5311	0.8527	0.7654	1.845e-12
	wild type at 29°C	-2.8097	0.7159	0.8354	6.884e-16
	P0206>PTEN	-3.7650	0.9805	0.9097	<2e-16
	>PTEN	-4.054	0.943	0.9504	0.0005985
	phm>InR	-2.9759	0.7966	0.7294	1.324e-07
	>InR	-4.030	1.034	0.924	0.001414

wild type larvae reared at both 18°C and 25°C and from *P0206>PTEN* and *>PTEN* larvae were indistinguishable in size. This suggests that initial disc size and the length of the growth period did not affect disc size at pupariation under these conditions. Growth period length did play a role in determining disc size at pupariation for discs from *phm>InR* larvae. Discs from these larvae grew at the same rate as their parental controls (*>InR*), but ceased growth at pupariation fifteen hours earlier (Chapter 2 (Oliveira et al., 2014)). Taken together, shortening developmental time limits final disc size, whereas larvae with longer growth periods adjust the growth rate of the disc to reach the same size at pupariation.

Adult wings from larvae reared at 18°C and from *P0206>PTEN* larvae are known to be larger than from those reared at 25°C (De Moed et al., 1997; Debat et al., 2003; James et al., 1995; Mirth et al., 2005; Partridge et

al., 1994; Shingleton et al., 2009). Thus, we found it surprising that at pupariation these discs were not different in size from those from wild type and *>PTEN* larvae reared at 25°C. This suggests that these animals achieve the difference in wing size due to processes occurring after pupariation. Although the size of the disc at pupariation is thought to predominantly determine adult wing size (Day and Lawrence, 2000), growth during the pupal phase also contributes significantly to overall adult size and thus is likely to affect wing size (Okamoto et al., 2009; Slaidina et al., 2009). Furthermore, differences in the final wing size can result from differences in the metamorphosis of the wing disc into a wing during pupal development. The evagination of the wing discs during metamorphosis occurs primarily through cell flattening (Fristrom et al., 1977), which is also partly responsible for final wing size and shape (González-Gaitán et al., 1994). Cell flattening is highly affected by temperature (Kuo and Larsen, 1987). Interestingly, flies reared at lower temperatures have larger wings mostly due to increases in cell area (Partridge et al., 1994). Thus in both conditions that generate animals with larger wings, either growth or morphogenesis during the pupal phase appears to orchestrate differences in final size.

For larvae reared at 29°C and *phm>InR* larvae, small disc size resulted from reduced growth rates both in absolute and relative time. Slow growth rate did not always result in small disc size at pupariation; wing discs from *>InR* larvae compensate for slow early growth rates by accelerating their growth later in development. Thus, even though growth rate in the disc contributes to variation in adult wing size, it can be adjusted through developmental time.

Changes in growth rate: growth constraint or growth milestone?

In all conditions examined, we observed fast-then-slow growth trajectories for the wing disc. We identified two types of these growth trajectories, one that changed to slow growth early (~30% development time) and a second with a later switch to slow growth (~65% development). Discs that switched to slow growth early had higher initial growth rates when compared to those that switched to slow growth late. This change in slope could be due to active regulation of growth rates at developmental milestones, or due to constraints either on the growth of the disc or imposed by larval body size.

Several authors have proposed models suggesting that discs stop growing in response to increased mechanical strain as they approach final size (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007; Shraiman, 2005). Although this may in fact function to induce the cessation of growth, it does not explain the differences we observe in growth trajectory type across treatments. We find that discs that change to slow growth late do so at larger disc sizes than those that switch early.

One could also imagine that these differences in the inflection of the growth curve could be imposed by larval body size. This also appears unlikely, as *P0206>PTEN* larvae grow at faster rates, have proportionally smaller discs (Colombani et al., 2005; Mirth et al., 2005; Mirth et al., 2009), but show early inflections in their growth trajectories.

Shingleton *et al.,* 2008 previously noticed under standard rearing conditions the growth curve of wing discs changes slope around the time of a developmental milestone known as critical weight. This change coincided with a change in sensitivity to nutrition for growth; wing discs from larvae

starved before critical rate grew very little whereas wing discs from larvae starved after reaching critical weight underwent significant growth (Shingleton et al., 2008). Four of our conditions show this early change in growth rates that coincide with the change in slope seen at critical weight. However in the remaining three conditions the change in growth rate occurs later than critical weight. This may mean that the milestones that regulate disc growth rates differ depending on developmental conditions.

Even though disc growth milestones appear to shift in time, both milestones occur between 26 and 68% of development. This is in sharp contrast to the milestones that we previously found to regulate patterning, which occurred at the beginning and the end of the third instar (Chapter 2 (Oliveira et al., 2014)). Thus, the developmental processes that coordinate growth and patterning appear to be different.

Is patterning size-dependent?

Given that the milestones coordinating disc growth and those that coordinate disc patterning occur at different times, how is the correlation between disc size and patterning stage that we observe maintained? We initially proposed that disc patterning stages might depend on a minimal disc size. However, we find no evidence for this as discs size varies significantly for any given patterning stage. Thus we conclude that the larval body coordinates disc size and disc pattern through different developmental processes that result in correctly patterned structures across a broad range of wing sizes.

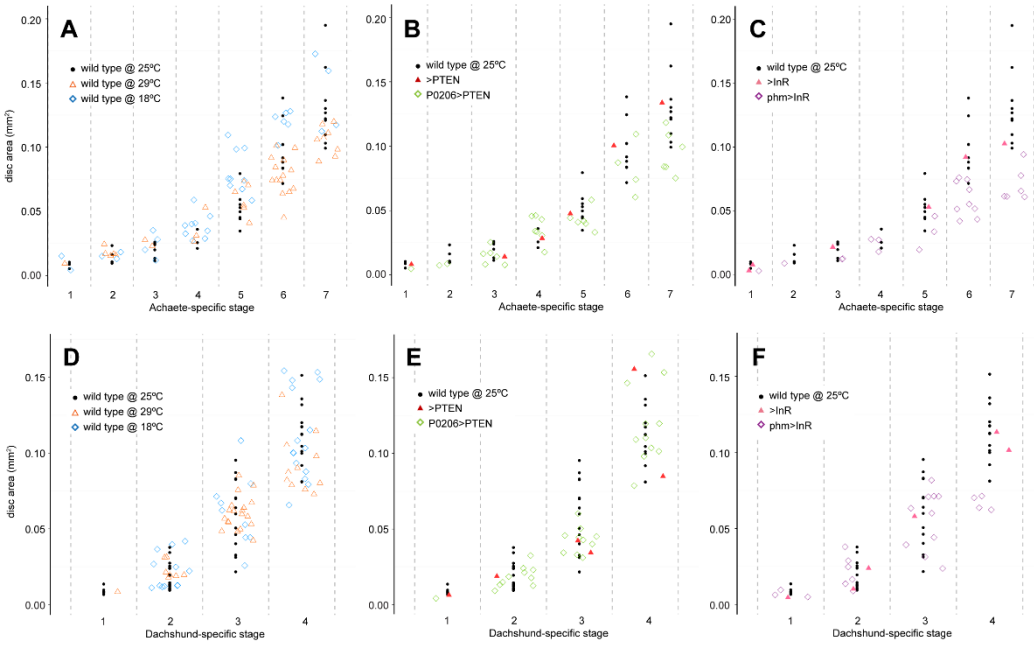
Acknowledgments

I would like to thank Alex Shingleton and especially Christen Mirth for reading and commenting on this chapter.

Publication

The work included in this chapter is in preparation for publication in Development, Genes and Evolution.

Supplementary material to Chapter 3



Supplementary Figure S3.1. Wing imaginal disc area linked to Achaete- (A-C) and Dac-specific stages (D-F). Size is represented in mm^2.

Supplementary Table S3.1. Mean, 95% confidence intervals and number of wing imaginal discs sampled from wild-type larvae at each temperature.

Condition	age (h AL3E)	mean disc size (µm^2)	95% CI	N
wild type at 25°C	0	0.007	0.0005	41
	5	0.013	0.0011	45
	10	0.023	0.0010	28
	15	0.039	0.0041	25
	20	0.046	0.0034	28
	25	0.056	0.0058	26
	30	0.070	0.0031	31
	35	0.091	0.0068	27
	40	0.109	0.0068	30
	45.9	0.107	0.0076	35
	49	0.132	0.0074	34
	all ages	-	-	350
wild type at 18°C	0	0.004	0	1
	10	0.013	0.0011	19
	20	0.024	0.0025	16
	30	0.038	0.0022	15
	50	0.056	0.0038	28
	70	0.083	0.0050	33
	96.4	0.125	0.0062	18
	100.7	0.129	0.0118	19
	all ages	-	-	149
wild type at 29°C	0	0.008	0.0014	3
	10	0.017	0.0018	22
	20	0.029	0.0042	14
	30	0.047	0.0051	14
	35	0.074	0.0070	14
	40	0.076	0.0067	21
	48.2	0.078	0.0097	17
	51.7	0.1	0.0077	18
	all ages	-	-	123

Supplementary Table S3.2. Mean, 95% confidence intervals and number of wing imaginal discs sampled from each condition.

Genotype	age (h AL3E)	mean disc size (µm^2)	95% CI	N
P0206>PTEN	0	0.005	0.0004	3
	10	0.009	0.0016	13
	20	0.018	0.0018	16
	30	0.033	0.0033	19
	40	0.041	0.0033	16
	60	0.049	0.0062	17
	72.9	0.117	0.0128	17
	80	0.099	0.0054	20
	all ages	-	-	121
phm>InR	0	0.005	0.0017	3
	10	0.012	0.0021	18
	20	0.03	0.0036	21
	30	0.057	0.0067	18
	32	0.063	0.0071	16
	35.8	0.076	0.0092	20
	all ages	-	-	96
>PTEN	0	0.007	0.0017	3
	10	0.017	0.0045	2
	20	0.031	0.0059	2
	30	0.045	0.0052	2
	47.9	0.093	0.0153	2
	53	0.145	0.0215	2
	all ages	-	-	13
>InR	0	0.004	0.0006	3
	10	0.009	0.0020	2
	20	0.023	0.0019	2
	30	0.056	0.0046	2
	47.6	0.097	0.0089	2
	51	0.108	0.0104	2
	all ages	-	-	13

Supplementary Table S3.3. ANCOVA to test for differences in slopes and intercepts among regression lines for log-transformed proportional disc area compared to Ac-specific stage; grey cells highlight p<0.01.

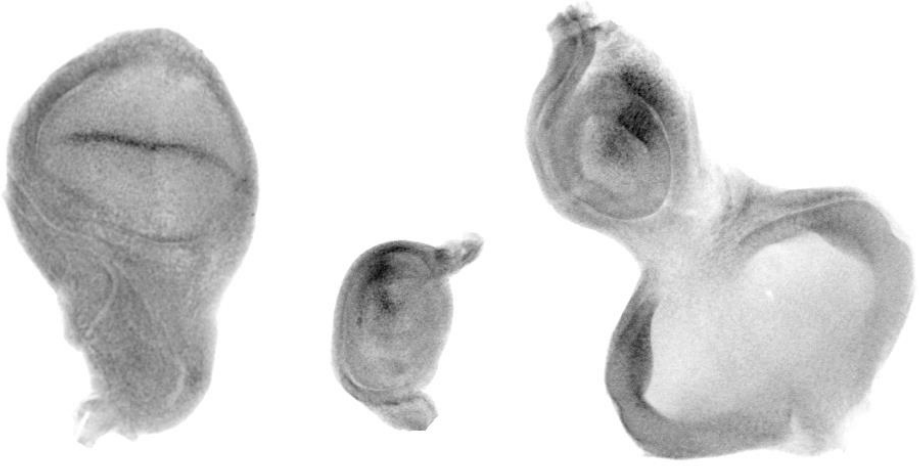
Comparison between conditions		Ac-specific stage	Condition	Ac-specific stage * Condition
wild type at 25°C	wild type at 18°C	<2e-16	0.000172	0.448768
wild type at 25°C	wild type at 29°C	<2e-16	3.11e-07	0.000382
wild type at 25°C	*P0206>PTEN*	<2e-16	0.334	0.659
wild type at 25°C	*>PTEN*	<2e-16	0.188	0.689
P0206>PTEN	*>PTEN*	<2e-16	0.142	0.936
wild type at 25°C	*phm>InR*	<2e-16	0.251	0.667
wild type at 25°C	*>InR*	<2e-16	0.561	0.449
phm>InR	*>InR*	<2e-16	0.964	0.353

Supplementary Table S3.4. ANCOVA to test for differences in slopes and intercepts among regression lines for log-transformed proportional disc area compared to Dac-specific stage; grey cells highlight p<0.01.

Comparison between conditions		Dac-specific stage	Condition	Dac-specific stage * Condition
wild type at 25°C	wild type at 18°C	<2e-16	0.573	0.623
wild type at 25°C	wild type at 29°C	<2e-16	1.26e-06	0.0296
wild type at 25°C	*P0206>PTEN*	<2e-16	0.0204	0.3096
wild type at 25°C	*>PTEN*	<2e-16	0.180	0.758
P0206>PTEN	*>PTEN*	<2e-16	0.00264	0.75321
wild type at 25°C	*phm>InR*	<2e-16	8.57e-06	0.335
wild type at 25°C	*>InR*	<2e-16	0.792	0.354
phm>InR	*>InR*	2.26e-10	0.0446	0.2218

4

Ecdysone coordinates organ growth and patterning with the development of the whole body



Summary

Developmental hormones coordinate the development of the whole body, inducing switches in developmental programs and controlling the duration of developmental phases. Several developmental hormones are known to play pivotal roles in regulating final body size by modulating growth rate and the duration of the growth period of the entire animal. But because different organs show different scaling relationships with the body, it is not clear how these hormonal signals are integrated to produce an organism of the appropriate size and proportions.

In *Drosophila*, the steroid moulting hormone ecdysone determines the timing of developmental transitions, like the larval moults and the onset of metamorphosis. Moreover, ecdysone negatively regulates the growth of the larval body (Colombani et al., 2005; Mirth et al., 2005). Ecdysone represses growth, at least in part, by acting in the fat body to both reduce the size of this tissue and systemically affect the growth rate of the larval body (Colombani et al., 2005; Delanoue et al., 2010). However, when the rate of ecdysone synthesis is repressed, the wing imaginal discs, the precursors of the adult wing and thorax, are smaller and show delays in patterning when compared to age-matched controls (Mirth et al., 2009). Thus, we hypothesized that ecdysone regulates the development of imaginal tissues and larval body in opposing manners.

To test this, we eliminated ecdysone synthesis completely by ablating the glands that produce it, the prothoracic glands, specifically in the third instar. We found that in the absence of ecdysone, imaginal discs are smaller and not properly patterned while larval body weight does not seem to be affected. We could rescue both the wing size and wing patterning phenotypes by feeding larvae an active form of ecdysone. Moreover, we revealed that the size of the wing disc is a result of growth promoted by nutrition-dependent signalling and of growth promoted by ecdysone. Potentially, the balance between these two signalling pathways tunes growth to maintain proportions with the whole-body size. By tackling the interactions between growth, patterning and nutrition, we hope to generate a more comprehensive view of the coordination between the development of the whole body and the growth of tissues.

Introduction

Organisms regulate the size of their organs in relation to one another to ensure that they are correctly proportioned. Although we have a limited understanding of how organs communicate information about growth to one another, several development hormones appear to play key roles. Work in *Drosophila* has identified insulin/target of rapamycin (TOR) and ecdysone signalling pathways as cascades that respond to and transduce systemic signals to regulate tissue and body growth (Koyama et al., 2013; Mirth and Shingleton, 2012). Both insulin and TOR signalling respond to the nutritional status of the organism and share several key regulators therefore are often referred as the insulin/TOR signalling pathways. The steroid moulting hormone ecdysone interacts with the insulin/TOR pathways to control the timing of the body development. Ecdysone is released in pulses and circulates around the body to control the timing of developmental transitions like the larval moults and metamorphosis (Riddiford, 1993). Thus, a compelling hypothesis is that the interaction between ecdysone and insulin/TOR pathways allows tissues to coordinate their development with that of the body.

The nutritional environment is communicated to the body systemically via amino acid sensing in the fat body which in turn regulates the secretion of insulin like peptides (DILPs). The fat body detects the availability of amino acids via the TOR pathway (Colombani et al., 2003; Géminard et al., 2009) and secretes a signal (fat-body-derived signal) that stimulates the insulin producing cells (IPCs) in the brain to release DILPs. The fat body also detects the presence of dietary sugars and fats and, by releasing Unpaired 2, which inactivates the GABAergic neurons in the brain that normally

inhibit the release of DILPs by the IPCs (Rajan and Perrimon, 2012). *Drosophila* have eight DILPs that are expressed in different larval and adult tissues, suggesting that they have non-redundant functions (Brogiolo et al., 2001). In response to a rich nutritional environment, insulin-producing cells (IPCs) in the brain secrete DILPs 2, 3 and 5 into the haemolymph (Grewal, 2009). DILPs bind to the Insulin Receptor (InR), resulting in the activation of kinases such as Akt (Verdu et al., 1999) that positively regulate growth. Insulin signalling then stimulates tissue growth and fat storage (Grewal, 2009), and regulates the duration of the larval growth phase (Shingleton et al., 2005).

The steroid moulting hormone ecdysone also regulates body growth and developmental timing (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). During larval development, ecdysone is produced and released from the prothoracic glands (PG) (Gilbert et al., 2002) and converted into its active form, 20-hydroxyecdysone (20E), by the fat body. Ecdysone production in the PG is stimulated by DILPs through insulin signalling (Colombani et al., 2005; Mirth et al., 2005), amino acids through TOR signalling (Layalle et al., 2008), and the prothoracicotropic hormone (PTTH) through PTTH/RAS/MAPK pathway (McBrayer et al., 2007; Rewitz et al., 2009). Increasing insulin/TOR signalling in the PG accelerates the timing of the ecdysone pulses, thus accelerating development, and decreases larval growth rates. *Drosophila* larvae synthesize PTTH in neurosecretory cells in the brain (McBrayer et al., 2007) in a photoperiodic cycle, imposing a circadian control over ecdysone synthesis. Further, in larvae with damaged discs PTTH transcription is delayed (Halme et al., 2010) revealing that PTTH also integrates cues from the developing imaginal tissues to regulate ecdysone synthesis.

In addition to its role in regulating the timing of development, ecdysone also negatively regulates the growth rate of larval body (Colombani et al., 2005; Delanoue et al., 2010; Mirth et al., 2005). The effect is mediated by the ecdysone receptor (EcR) in the larval fat body. Reducing ecdysone signalling specifically in the fat body (EcR RNAi) enhances pupal size.

Although ecdysone negatively regulates the growth of some tissues, such as the larval fat body, it appears to enhance the development of other tissues such as the wing imaginal disc. Larvae with delayed ecdysone synthesis have small discs that pattern slowly (Mirth et al., 2009). Partially activating ecdysone signalling in the imaginal tissues causes premature patterning in starved animals.

Both the timing of the ecdysone pulse and the concentration of ecdysone produced appear important for coordinating disc development with other tissues and the whole body. Growth-perturbed wing discs slow the development of non-affected discs and of the whole body (Parker and Shingleton, 2011) by releasing the *Drosophila* insulin-like peptide 8 (Dilp8). Dilp8 delays ecdysone synthesis in the PG (Colombani et al., 2012; Garelli et al., 2012). Feeding ecdysone to these larvae restores the growth of the non-affected discs and the timing of whole-body development. This suggests that exogenous application of ecdysone disrupts the coordination between the growth-perturbed discs with the other discs and the body and indicates that ecdysone is limiting for their growth.

Thus it seems that ecdysone positively regulates the growth and patterning of imaginal tissues while suppressing larval tissue growth. Our aim is to characterize the contribution of ecdysone to the growth and patterning of the discs and to reveal the mechanism that allows ecdysone

to regulate the growth of body and imaginal discs in opposing manners. To do this, we ablated the PG (PGX) and examined the growth and patterning of discs and the growth of the whole body. Discs from fed PGX larvae show slow growth rates and delayed progression of pattern. This suggests that nutrition-dependent signalling sustains part of the disc growth in the absence of ecdysone, although it cannot rescue pattern. In contrast, PGX larvae grow faster than controls. Feeding ecdysone reduces the size of PGX larvae, induces growth in their discs and rescues the delays in patterning. Taken together, our results show that both ecdysone and nutrition-dependent signalling contribute to the growth of the disc whereas ecdysone alone promotes wing disc patterning.

Materials and methods

Fly stocks and rearing conditions

To genetically ablate the PG, we used the following flies: *w; UAS grim*; *w; phm GAL4, tub Gal80^{ts}* and the isogenic strain *w^{1118}*. Flies were raised from timed egg collections (6-8 hours) on standard cornmeal/molasses medium (standard food) at low density (200 eggs per 60 x 15 mm Petri dish) unless stated otherwise.

Prothoracic gland ablation (PGX)

To genetically ablate the prothoracic gland, we used larvae resulting from crosses between *w; UAS Grim* females with *w; phm GAL4, tub Gal80^{ts}* males (the progeny are referred as PGX). As controls we crossed females from the isogenic strain *w^{1118}* with males from either *w; UAS grim* (the progeny are referred as *>Grim*) or *w; phm GAL4, tub GAL80^{ts}* (the progeny

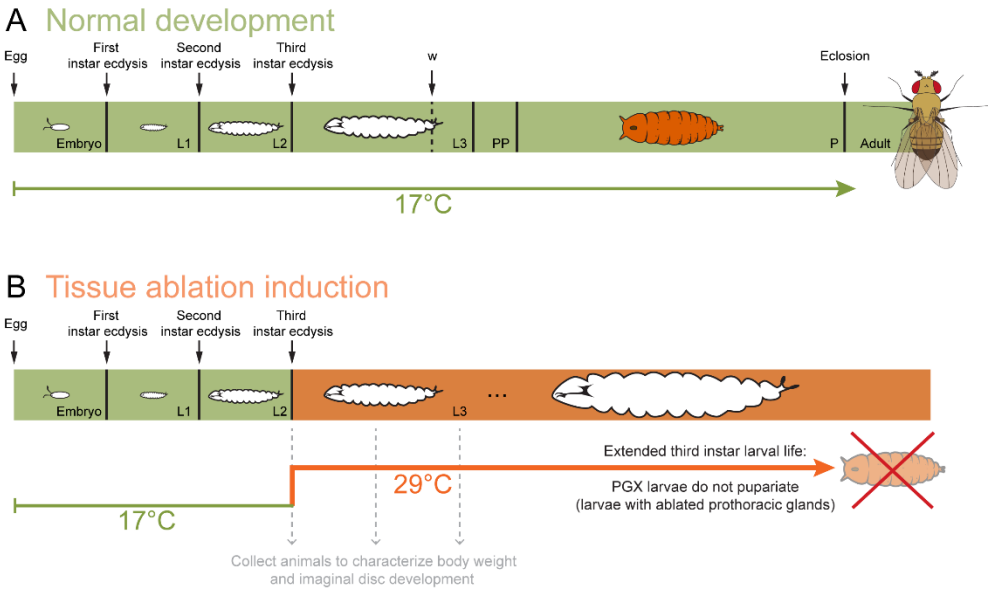


Figure 4.1. Methods used to ablate the prothoracic gland at the beginning of the third instar. To ablate the prothoracic gland, we used a PG-specific GAL4 to drive expression of a cell death-regulatory gene (*>Grim*). To prevent cell death early in development and to restrict the study to where most imaginal tissue growth occurs, the third instar, we controlled the tissue ablation using a temperature sensitive repressor of GAL4 activity (*Tub-GAL80ts*). (A) Tub-GAL80ts is ubiquitously expressed by the tubulin promoter and represses GAL4 at 17°C allowing for normal development. At 29°C Tub-GAL80ts is inactive and, consequently, through temperature shifts we induce ablation of the PG at specific times. (B) We reared all animals at 17°C from egg until the beginning of the L3, collected newly moulted animals at two-hour intervals and shifted them to 29°C for tissue ablation. We measured body weight, imaginal disc size and patterning at different time points.

are referred as *PG>*) parental lines. Tissue ablation was induced by shifting larvae from 17°C to 29°C (Figure 4.1). In all experiments, larvae were maintained at 17°C until the end of the second instar and shifted to 29°C at the moult to the third instar. As an additional control, we allowed larvae to develop continuously at 17°C to confirm that PGX larvae develop normally under these conditions.

Animal staging and developmental time

Larvae were staged into 2-hour cohorts at ecdysis to the third larval instar. At 29°C we weighed larvae and dissected tissue at 0, 12, 24, 36 and 42 (wandering) hours after the third instar ecdysis (h AL3E). PGX larvae do not pupariate and continue as L3 larvae beyond 42 h AL3E (extended larval life). During the PGX extended larval life, we weighed larvae at 48, 72, 96, 120, 144, 168, 216, 240 h AL3E and dissected wing discs at 96 h AL3E.

We measured the average time to wandering and pupariation by counting the number of larvae wandering/pupariating within a cohort every 4 h for 29°C and every 6 h for 17°C. To measure the average eclosion time, we checked for adult eclosion every 12 h.

Feeding treatments

For exogenous application of 20-hydroxyecdysone (20E), either 0.15 mg of 20E dissolved in ethanol, or an equivalent volume of ethanol, was added to 1 ml of standard food. Ethanol/20E-supplemented food was allowed to sit at room temperature for at least 4 hours to evaporate off excess ethanol before use. Twelve larvae were transferred to one of the two supplemented foods either at 0 h AL3E and left to feed for 42 h or at 42 h AL3E and left to feed for 24 h.

To determine the relative contributions for growth of nutrition-dependent signalling or ecdysone we fed newly moulted larvae 1 ml of starvation medium (1% sucrose with 1% agar) supplemented with either 0.15 mg of 20E dissolved in ethanol, or an equivalent volume of ethanol. Supplemented food was left at room temperature for at least 4 hours to

evaporate excess off ethanol before use. Larvae were collected at 24 h AL3E for tissue dissection.

To determine if DILP2 is secreted at normal levels in PGX larvae we fed newly moulted larvae either standard food or starvation medium for 24 h AL3E. At 24 h AL3E, we dissected the larvae and examined DILP2 expression in insulin-producing cells (ILPs) in the brain (as in (Géminard et al., 2009) by immunocytochemistry (see below).

Dissections and immunocytochemistry

For each time point in each experiment, tissue from 10-30 larvae was dissected in cold phosphate buffered saline (PBS) and fixed for 30 min in 4% paraformaldehyde in PBS. The tissue was washed in PBT (PBS + 1% Triton X-100) at room temperature, blocked in PBT-NDS (2% Normal Donkey Serum in PBT) for 30 min and then incubated in a primary antibody solution overnight at 4°C. We used the following primary antibody solutions: 1:50 dilution of rabbit anti-Caspase 3 (Cell Signalling #9661), 1:100 dilution of mouse anti-Wingless (Developmental Studies Hybridoma Bank, 4D4), 1:100 dilution of mouse anti-Achaete (Developmental Studies Hybridoma Bank), 1:3,000 dilution of guinea pig anti-Senseless antibody (from Hugo Bellen, Baylor College of Medicine, Houston, USA) and 1:400 dilution of rat anti-DILP2 (from Pierre Léopold, Institute for Developmental Biology and Cancer, CNRS/University of Nice-Sophia Antipolis, Nice, France). Anti-Achaete and anti-Senseless antibodies were used simultaneously. After washing with PBT, tissue was incubated with fluorescently-conjugated secondary antibodies overnight at 4°C. Tissues including brains, lymph glands, prothoracic glands, wing, eye-antennal, first leg, second leg, third leg discs or haltere discs, were rinsed with PBT and

mounted on poly-L-lysine-coated coverslips using Fluoromount-G (SouthernBiotech). Samples were imaged using a Zeiss LSM 510 confocal microscope and images were processed using ImageJ.

Quantifications of tissue size and larval weight

We quantified imaginal tissue size using area as a proxy. All quantifications were done using ImageJ. For larval weight, we collected staged larvae, washed them in milliQ water and individually weighed them using a Sartorius SE2 ultra-microbalance.

Quantifications of tissue expression pattern

We quantified Ac, Sens and Wg expression patterns as described in Chapter 2 (Oliveira et al., 2014).

Statistical analysis

All data analyses and statistics were conducted using R (www.r-project.org) (Ihaka and Gentleman, 1996).

Results

Larvae with ablated prothoracic gland (PGX) do not pupariate and show extended larval life

To explore how ecdysone regulates the development of imaginal tissues, we genetically ablated the prothoracic glands (PG). These animals are hereafter referred to as PGX. We used a PG-specific GAL4 (*phm GAL4*) to drive expression of a cell death-regulatory gene, *Grim*. If we ablate the PG early in development animals do not moult from first instar. Therefore, we used a temperature sensitive repressor of GAL4 activity, *GAL80^{ts}*, to

prevent cell death in the PG until the third instar. *The GAL80^{ts}* is ubiquitously expressed by the tubulin promoter and represses *GAL4* at 17°C. At 29°C, *GAL80^{ts}* becomes inactive, thereby allowing us to induce ablation of the PG at specific times through temperature shifts (see Materials and methods and Figure 4.1). We collected newly moulted animals at two-hour intervals and shifted them to 29°C for tissue ablation. As controls, we used larvae from the following crosses: *phm GAL4* with w^{1118} (*PG>*) and *UAS Grim* with w^{1118} (*>Grim*). We reared all animals at 17°C from egg until the beginning of the L3 and then shifted them to 29°C to control for the effects of the temperature shift. We measured imaginal disc size at 0, 24 and 42 h AL3E and larval weight at 12-hour intervals, from 0 h AL3E until the controls pupariated and then at 24-h intervals for the extended larval life of PGX. Control *>Grim* larvae wander for a short time interval and pupariate at 45 h AL3E (Table 4.1), hence, 42 h AL3E was used as the final time point for comparisons between PGX and controls.

PGX animals reared at 17°C developed normally and exhibited developmental times similar to the controls (Table 4.1). However, as

Table 4.1. Developmental times measured after the moult to the third instar (L3) for PGX and *>Grim* controls at the permissive (17°C) and non-permissive (29°C) temperatures of *Tub Gal80^{ts}*.

Temperature from the moult to L3	Measured developmental time	Genotype	Mean (h)	95% CI	N
17°C	L3 moult to wandering	*>Grim*	105	± 1.4	90
		PGX	116	± 2.9	142
	L3 moult to pupariation	*>Grim*	123	± 0.9	109
		PGX	131	± 1.2	122
	L3 moult to eclosion	*>Grim*	370	± 1.9	305
		PGX	360	± 2.6	325
29°C	L3 moult to wandering	*>Grim*	43	± 0.5	109
	L3 moult to pupariation	*>Grim*	45	± 0.6	378
	L3 moult to eclosion	*>Grim*	135	± 1.3	237

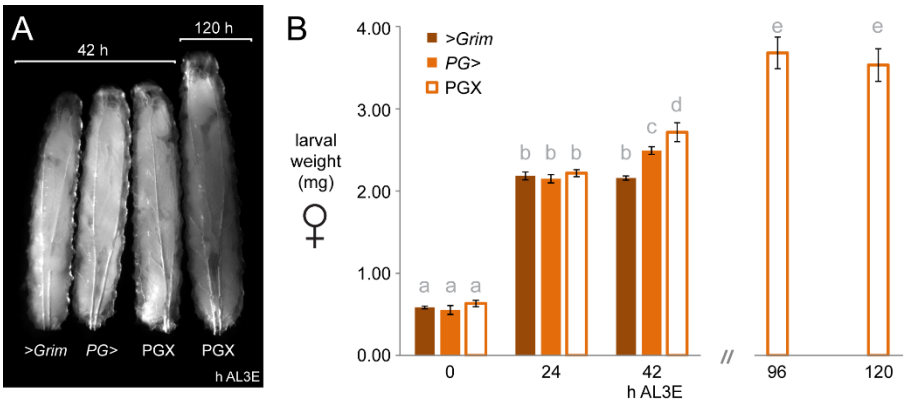


Figure 4.2. PGX larvae grow at faster rates than controls. (A) Control female larvae (*>Grim* and *PG>*) compared to female PGX larvae at 42 h AL3E and PGX at 120 h AL3E (5 days as third instar). 42 h AL3E represents late wandering for controls (*>Grim* and *PG>*). (B) *>Grim*, *PG>* and PGX female larval weight at 0, 24 and 42 h AL3E and PGX at 96 and 120 h AL3E (4 and 5 days as third instar). Measures are characterized by mean and 95% confidence intervals. Letters represent significant differences between genotypes ($p<0.01$, Wilcoxon rank test using Holm's p-value adjustment).

expected, PGX larvae shifted to 29°C at the beginning of L3 did not pupariate, continued to feed as third instar larvae for at least 10 days and exhibited a highly reduced and deformed ring gland complex (the complex comprising the PG, the corpora allata and the corpora cardiaca) at 42 h AL3E (Supplementary Figure S4.1).

PGX larvae show increased larval weight due to continued feeding

To confirm that ecdysone is a negative regulator of whole-body growth, we quantified whole-body weight through time. Both controls, *>Grim* and *PG>*, as well as PGX larvae increased equally in weight from 0 to 24 h AL3E (Figure 4.2 and Supplementary Figure S4.2). Between 24 and 42 hours, PGX larvae show a greater increase in weight than either of the controls (Figure 4.2). *>Grim* larvae stabilize their weight from 36 h AL3E onwards while *PG>*

show a mild increase in weight from 36 until 42 h AL3E (Supplementary Figure S4.2). Between 42 and 45 hours, the control larvae wander then pupariate (Table 4.1). The PGX larvae did not exhibit wandering behaviour, and continued to feed and increase in weight (Figure 4.2 and Supplementary Figure S4.2).

PGX imaginal tissues are smaller than controls

Because we found that PGX larvae grow faster than controls after 24 h AL3E, we addressed if their imaginal discs increased in size in coordination

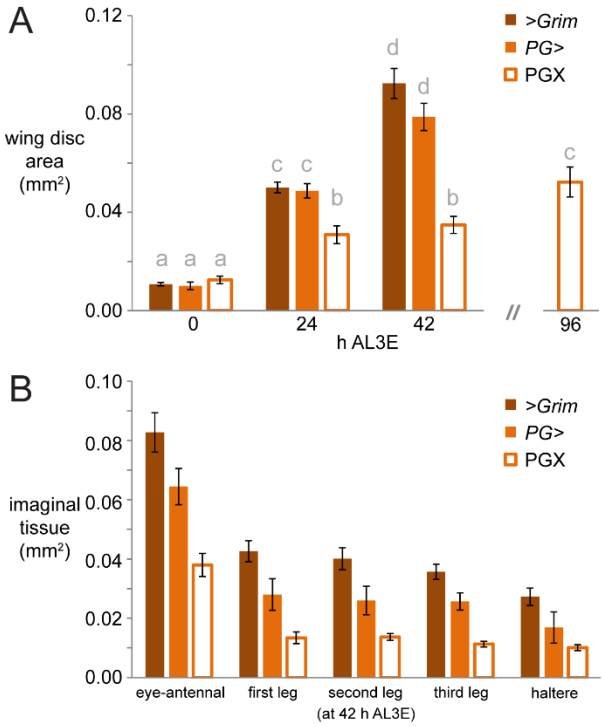


Figure 4.3. PGX larvae have small imaginal tissues. (A) Area of wing discs from *>Grim, PG>* and PGX larvae at 0, 24 and 42 hours after third instar ecdysis (h AL3E) and PGX larvae at 96 h AL3E. (B) Area of eye-antennal, first leg, second leg, third leg and haltere discs from *>Grim, PG>* and PGX larvae at 42 h AL3E. Measures are characterized by mean and 95% confidence intervals. Letters in A represent significant differences between genotypes ($p<0.01$, Wilcoxon rank test using Holm's p-value adjustment).

with whole-body growth. At the moult to L3, we do not see differences in wing disc size between PGX larvae and controls (Figure 4.3 A). From 24h AL3E onwards, discs from PGX larvae exhibit lower growth rates than both controls (Figure 4.3 A). Discs from PGX larvae do not change significantly in size between 24 and 42 h AL3E, which raised the question of whether the growth of PGX discs is arrested after 24 h AL3E. Due to their extended larval life, we measured disc size at 96 h AL3E (more than 2 days extra after the pupariation of the controls) in PGX larvae. We observed a small increase in disc size between 42 and 96 h AL3E. Disc size at 96 h AL3E was equivalent in size to that of control discs at 24 h AL3E. Overall, this suggests that the growth of discs from PGX larvae is dramatically impaired although not completely arrested.

To ask if ablating the PG affected the growth of other imaginal tissues, we dissected and measured the area of eye-antennal, first leg, second leg, third leg and haltere disc. All imaginal tissues from PGX larvae are significantly smaller than those from *>Grim* larvae (Figure 4.3 B). However, the size difference was not statistically significant between PGX and *>PG*.

Because the discs from PGX larvae showed small size and low growth rates, we hypothesized that ecdysone could be necessary for cell survival in the discs and that the reduction in disc size resulted from increased cell death. We used the cleaved Caspase-3 antibody to detect activated Caspase-3 (Casp-3) expression in the wing imaginal disc and used the lymph gland as a positive control. PGX wing discs show mild expression of Casp-3 in the hinge and notum (Figure 4.4), although it is unclear if this would be sufficient to fully explain the reduction in PGX disc size.

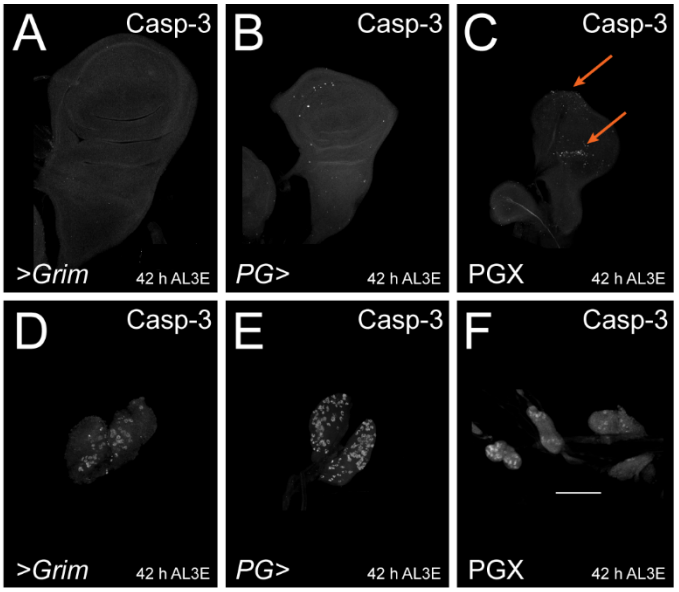


Figure 4.4. Activated Caspase-3 (Casp-3) expression in the wing disc and lymph gland (positive control). Wing discs (A-C) and lymph glands (D-F) from >Grim (A, D), PG> (B, E) and PGX (C, F) larvae, were dissected at 42 hours after third instar ecdysis (h AL3E) and labelled with cleaved-Caspase-3 antibody. (Scale bar 100 µm)

The progression of pattern is delayed in the case of Achaete and arrested in the case of Senseless in PGX wing discs

Previous studies have shown that ecdysone regulates early stages of patterning in the wing disc (Mirth et al., 2009). To access if patterning is affected in PGX discs, we used immunocytochemistry to characterize the expression of three patterning gene products, Achaete (Ac), Senseless (Sens) and Wingless (Wg), at 0, 24 and 42 h AL3E (Figure 4.5). We quantified the expression patterns as described in Chapter 2 (Oliveira et al., 2014), attributing gene-specific stages to each disc. All three gene expression patterns, Ac, Sens and Wg, show little or no progression of pattern from 0 to 42 h AL3E (Figure 4.5 D, H, L) in PGX discs. In contrast, control discs patterned normally.

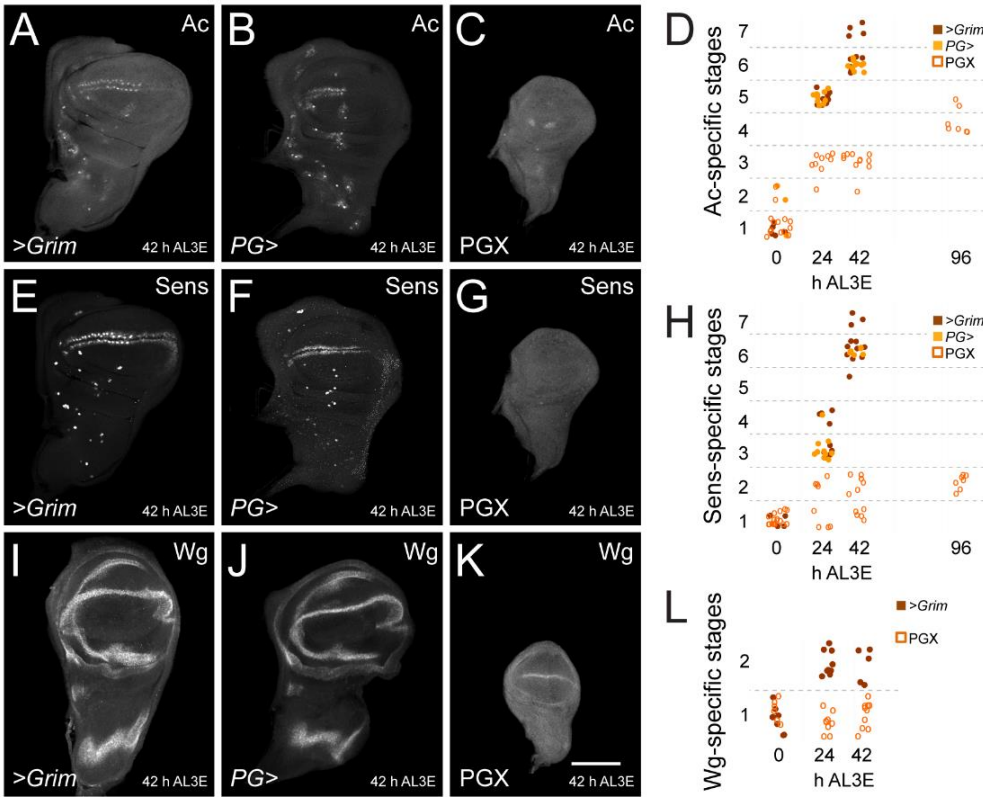


Figure 4.5. Wing discs from PGX larvae show arrested pattern. (A-C) Achaete (Ac) expression patterns and (D) quantification of Ac-specific stages, (E-G) Senseless (Sens) expression patterns and (H) quantification of Sens-specific stages, (I-K) Wingless (Wg) expression patterns and (L) quantification of Wg-specific stages of wing discs from (A, E, I) *>Grim*, (B,F,J) *>PG* and (C,G,K) PGX larvae. Ac and Sens-specific stages were attributed to discs dissected at 0, 24, 42 and 96 h AL3E and Wg-specific stages were attributed to discs dissected at 0, 24 and 42h AL3E. (Scale bar 100 µm)

We observed that PGX discs continue to grow at a slow rate, leading us to ask if the PGX discs were able to also continue to pattern. Surprisingly, Ac pattern showed a slow rate of progression (Figure 4.5 D, H and Supplementary Figure S4.3), although Sens did not.

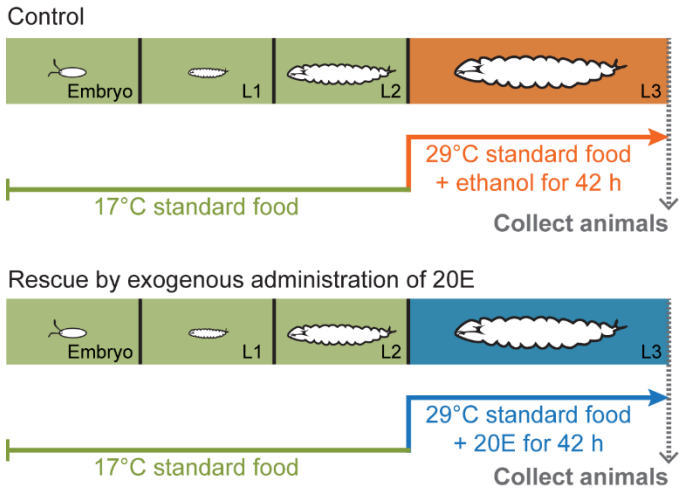


Figure 4.6. Methods to administer the active form of ecdysone, 20-hydroxyecdysone (20E), to control and PGX larvae. Animals were raised from egg to the moult to the third instar (L3) in standard food and from the moult to L3 until 42 hours after third instar ecdysis (h AL3E) in food with either addition of 20E dissolved in ethanol or the equivalent volume of ethanol (control).

Feeding ecdysone to PGX larvae rescues both disc size and patterning

To confirm that the reduced size and arrested pattern observed in PGX discs is due to the absence of ecdysone, we performed rescue experiments by feeding larvae the active form of ecdysone, 20-hydroxyecdysone (20E). We reared both controls, *>Grim* and *PG>,* and PGX from 0 until 42 h AL3E in food to which we either added 20E dissolved in ethanol or the equivalent volume of ethanol (control) (Figure 4.6). As expected, feeding 20E to PGX larvae rescues wandering behaviour, pupariation and body size (Figure 4.7 A). Moreover, feeding 20E completely rescues wing disc size (Figure 4.7 B) and expression patterns of both Ac (Figure 4.7 C-H) and Sens (Supplementary Figure 4.4). These results provide clear evidence that indeed ecdysone is necessary for the development of wing discs and its exogenous administration is sufficient to induce growth and patterning.

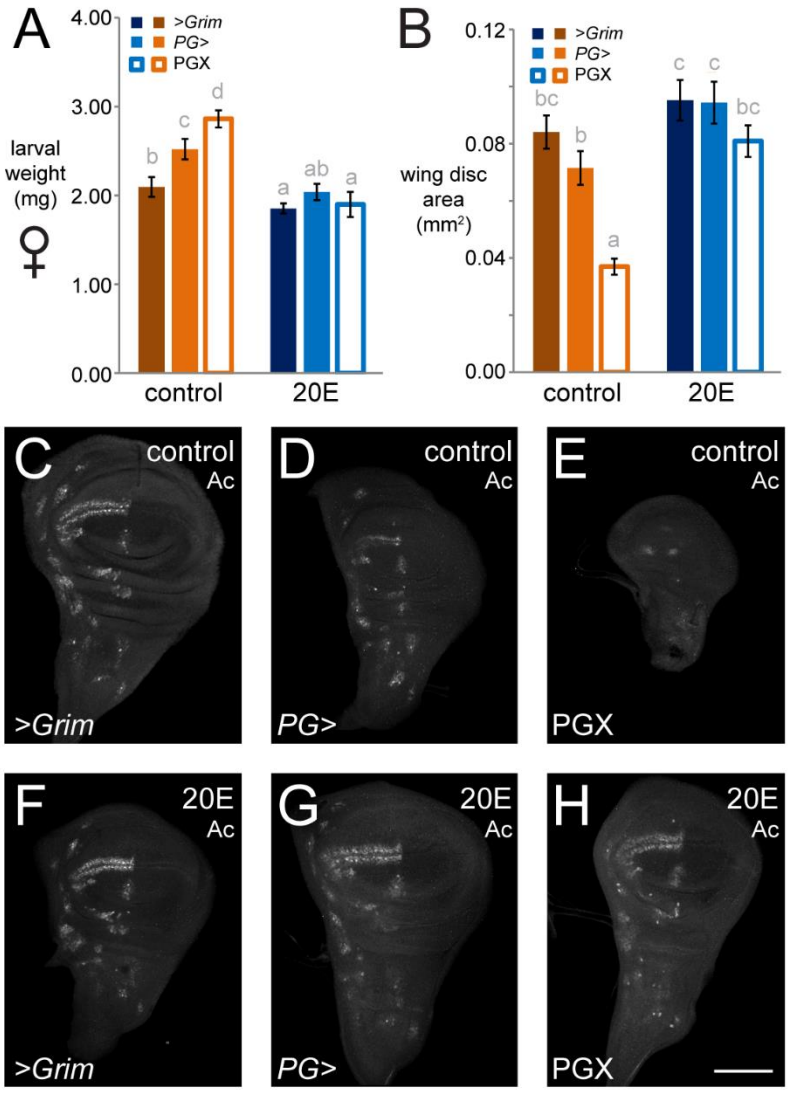


Figure 4.7. Feeding 20E rescues body size at wandering and wing disc growth and patterning. Larvae were raised from the moult to the third instar until 42 hours after third instar ecdysis (h AL3E) in food with the addition of either 20-hydroxyecdysone (20E) or ethanol (control). (A) *>Grim, PG>* and PGX larval weight at 42 h AL3E for 20E or control feeding treatments. (B) Wing disc area from *>Grim, PG>* and PGX at 42 h AL3E for either 20E or control feeding treatment. Measurements are characterized by mean and 95% confidence intervals and letters represent significant differences between genotypes and treatments ($p<0.01$, Wilcoxon rank test using Holm's p-value adjustment). (C-H) Expression of Achaete in wing discs from (C, F) *>Grim*, (D, G) *PG>* and (E, H) PGX larvae from the control (C-E) and 20E (F-H) feeding treatments dissected at 42 h AL3E.

PGX discs at 42 h AL3E show some expression of Casp3. This led us to hypothesize that discs started to degenerate during the 42 h of prolonged absence of ecdysone. If this were the case, feeding 20E to PGX larvae at 42 h AL3E would no longer rescue the development of discs. To test this, we reared PGX larvae in standard food until 42 h AL3E and only then transferred them to 20E or ethanol-supplemented food (Figure 4.8 A). Interestingly, PGX discs retain their ability to respond to ecdysone and both disc size and Ac and Sens expression patterns were rescued within 24 h of development (Figure 4.8 B-E).

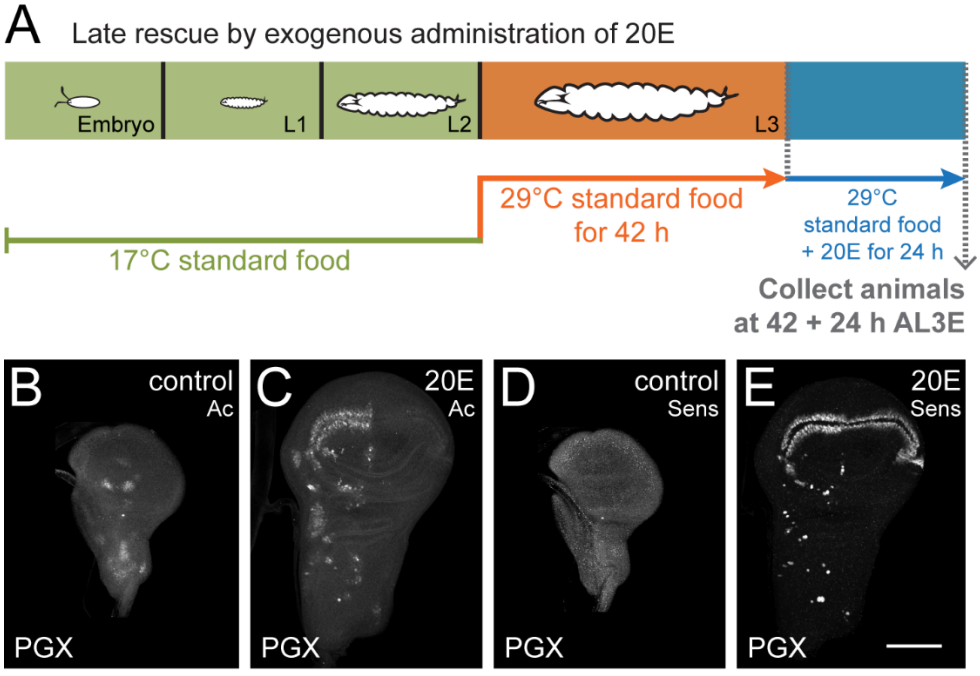


Figure 4.8. Disc size and pattern can still be rescued during the extended larval life of PGX larvae. (A) PGX larvae were raised in standard food until 42 h AL3E and then transferred to standard food with either addition of 20-hydroxyecdysone (20E) or ethanol (control) for an additional 24 h. Wing discs showing Achaete (Ac, B-C) and Senseless (Sens, D-E) expression patterns from PGX larvae fed in the control (B, D) or the 20E (C, E) treatment. (Scale bar 100 µm)

Insulin signalling is not affected in PGX larvae

Increasing ecdysone in the PG affects the growth rates of the whole body by affecting insulin/TOR signalling (Colombani et al., 2005; Delanoue et al., 2010). Thus it seemed possible that PGX larvae may be smaller due to altered insulin/TOR signalling. DILP2, 3, and 5 are produced in the insulin producing cells (IPCs) in the brain and released to the haemolymph when larvae are well fed (Brogiolo et al., 2001; Géminard et al., 2009). Overexpression of DILP2 can rescue most of the effects of IPC ablation on its own (Rulifson et al., 2002). When larvae are starved, DILP2 is no longer secreted and accumulates in the IPCs (Géminard et al., 2009). To determine if insulin signalling is affected in PGX larvae, we checked if DILP2 accumulates in the IPCs by immunocytochemistry. We fed *>Grim, PG>* and PGX larvae for 24 h (starting at the L3 moult) either standard food (fed) or starvation medium (1% sucrose with 0.5% agar, starved). At 24 h AL3E, brains from starved *>Grim, PG>* and PGX larvae show increased DILP2 expression in comparison to the respective fed brains (Figure 4.9). This shows that DILP2 secretion appears normal in PGX larvae.

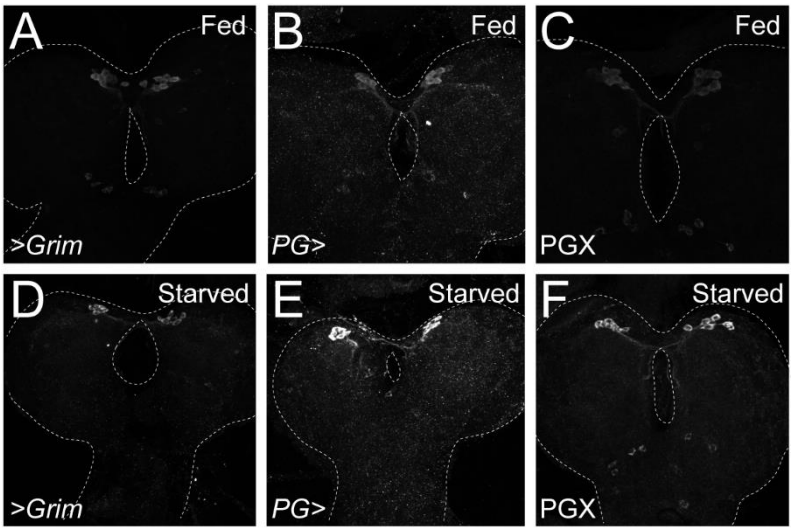


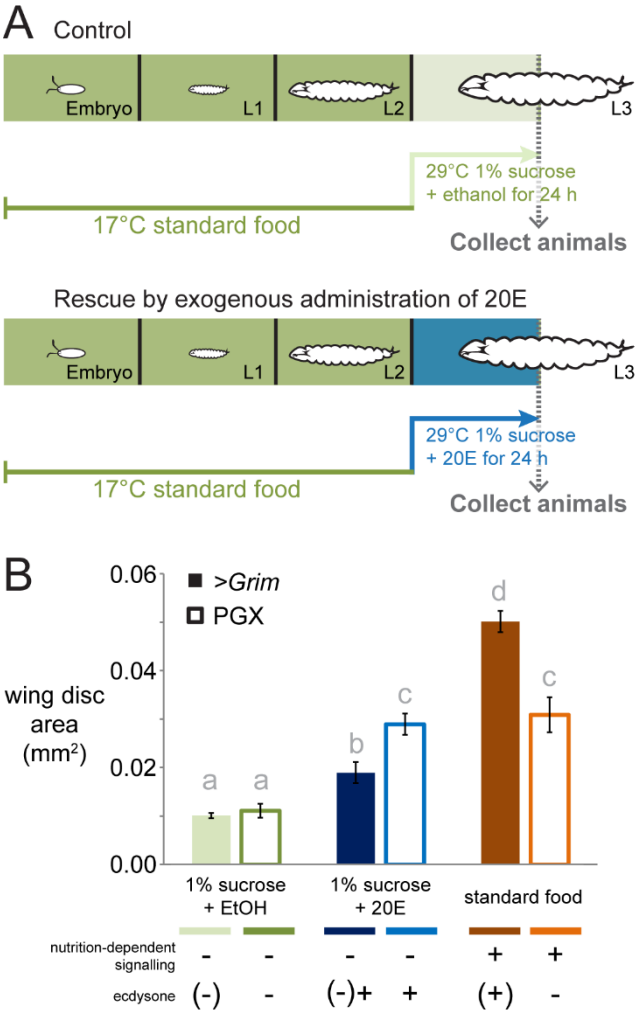
Figure 4.9. Secretion of DILP2 is not affected in PGX larval brains. Newly moulted third instar *>Grim* (A, D), *PG>* (B, E) and PGX (C, F) larvae were raised for 24 h in standard food (fed, A-C) or starvation medium with 1% Sucrose (starved, D-F) and then dissected for immunocytochemistry with anti-DILP2 antibody. Images were collected by confocal microscopy using the same scan settings. DILP2 is expressed in the insulin-producing cells (IPCs). Dashed lines represent brain outlines.

Disc growth is a result from nutrition-dependent and ecdysone-dependent growth, while disc patterning depends primarily on ecdysone

Although PGX discs are small compared to the controls, disc size still increases between 0 and 96 h AL3E. This suggests that in addition to the input from ecdysone on disc growth, continuous feeding can also promote growth *per se*. To determine the relative roles of ecdysone and nutrition-dependent signalling to the growth and patterning of the wing disc, we fed newly moulted L3 *>Grim* and PGX larvae either starvation medium with ethanol or starvation medium with 20E (Figure 4.10 A). We collected the wing discs at 24 h AL3E to measure size and pattern and compared their growth to growth in discs from animals fed from 0 until 24 h AL3E on standard food (Figs 4.10 and 4.11). Interestingly, several of the *>Grim*

larvae collected at 24 h AL3E were outside of the food and seemed small and transparent compared to PGX larvae (Table 4.2).

In starvation medium with ethanol, both *>Grim* and PGX wing discs do not grow (compared to disc size at 0 h AL3E in Figure 4.3). Adding 20E to the starvation medium resulted in increased wing disc size for both genotypes, with PGX discs showing a greater increase in size when compared to *>Grim* (Figure 4.10 B). Interestingly, PGX discs grow to the same size in either the standard food without 20E or in starvation medium



with 20E. This suggests that indeed both nutrition-dependent signalling and ecdysone on their own can contribute to the growth of discs.

Feeding PGX larvae 20E in starvation media rescued both Ac (Figure 4.11 A-D, I) and Sens (Figure 4.11 E-H, J) pattern. In the control *>Grim*, the addition of 20E to the starvation medium fully rescued the Ac pattern and partially rescued Sens pattern. Taken together, this reveals that 20E plays a more important role in regulating patterning than nutrition-dependent signalling.

Discussion

Tissues grow and pattern in response to several hormonal and metabolic cues, all of which interact to determine the final size and shape of the animal. Because not all tissues share the same growth trajectories, we proposed that developmental hormones have tissue-specific effects on growth and patterning.

Figure 4.10. *(preceding page)* Contribution of ecdysone and nutrition-dependent signalling to the growth of the wing disc. (A) *>Grim* and PGX larvae were starved from the moult to the third instar (L3) on starvation medium (1% sucrose) with the addition of either 20-hydroxyecdysone (20E) or ethanol (EtOH). Larvae were collected at 24 hours after third instar ecdysis (h AL3E) for tissue dissection. (B) Wing disc area from *>Grim* and PGX larvae fed on starvation media with either 20E or EtOH, or on standard food for 24 h. Measurements are characterized by mean and 95% confidence intervals and letters represent significant differences between genotypes and treatments ($p<0.01$, Wilcoxon rank test using Holm's p-value adjustment). For the nutrition-dependent signalling, the symbols represent "-" for downregulation and "+" for activation. Ecdysone symbols "(-)" or "(+)"represent the changes in natural levels of ecdysone by starvation or feeding respectively. The ecdysone symbol "-" represents no/low ecdysone while "+" represents high levels of ecdysone due to exogenous administration of 20E.

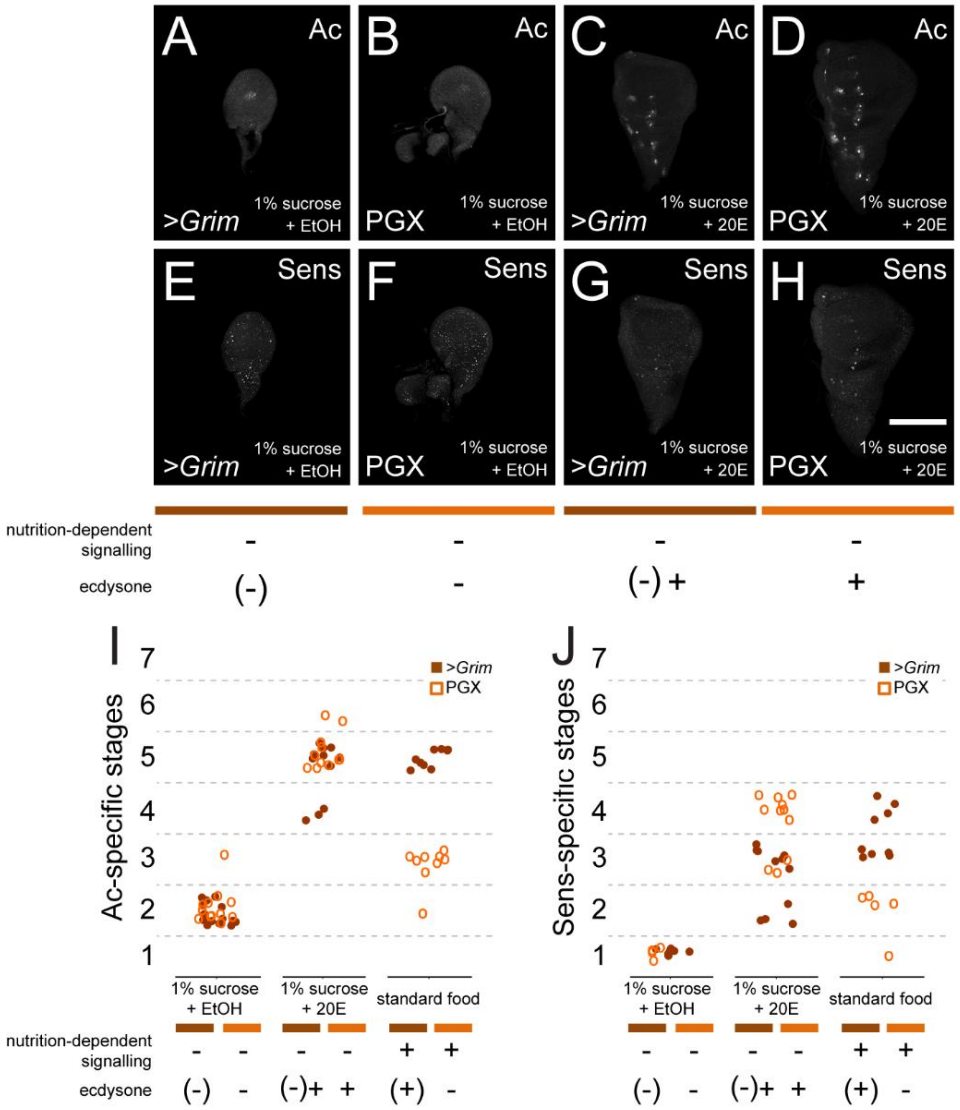


Figure 4.10. Contribution of nutrition-dependent signalling to the patterning of the wing disc. Expression of Achaete (Ac, A-D) or Senseless (Sens, E-H) in wing discs from *>Grim* (A, C, E, G) and PGX (B, D, F, H) larvae fed on starvation media with addition of either ethanol (EtOH, A, B, E, F) or 20-hydroxyecdysone (20E, C, D, G, H) for 24 h. We quantified Ac- (I) and Sens-specific stages (J) for discs from *>Grim* and PGX larvae reared from 0 to 24 h AL3E on starvation media with EtOH or 20E and compared them to standard food. For the nutrition-dependent signalling, the symbols represent "-" for downregulation and "+" for activation. Ecdysone symbols "(-)" or "(+)"represent the changes in natural levels of ecdysone by starvation or feeding respectively. The ecdysone symbol "-" represents no/low ecdysone while "+" represents high levels of ecdysone due to exogenous administration of 20E.

Table 4.2. *>Grim* larvae collected at 24 h AL3E and compared to PGX larvae fed on starvation medium with the addition of either ethanol (EtOH) or 20-hydroxyecdysone (20E).

Temperature from the moult to L3	Genotype	Medium	N	Observations
29°C	*>Grim*	1% Sucrose + 20E	13	12 larvae outside of the food; thin and transparent
		1% Sucrose + EtOH	13	5 larvae outside of the food; thin and transparent
	PGX	1% Sucrose + 20E	13	1 larva outside of the food; fat animals; visibly increased fat body
		1% Sucrose + EtOH	13	4 larvae outside of the food; fat animals; visibly increased fat body

Ecdysone has been implicated in regulating body growth, developmental timing, and tissue patterning (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Mirth et al., 2009; Schubiger et al., 2005; Schubiger and Truman, 2000). In this study, we set out to characterize the extent to which ecdysone contributes to the growth and patterning of the wing disc versus the body, with the aim of elucidating the mechanisms that allow ecdysone to regulate the growth of body and imaginal discs in opposing manners.

The literature suggests ecdysone acts as a negative regulator of body growth (Mirth et al., 2005), due to its action on the fat body (Colombani et al., 2005). Our results show that growth rates in PGX larvae only differ late in the third instar. Clearly, some of this increase in growth results from the fact that PGX larvae continue feeding after control larvae have started wandering. However, because we observe differences in size in the feeding stages and because we can rescue the increase in body size by feeding ecdysone, this suggests that ecdysone negatively regulates growth in the second half of the third instar.

In contrast to its effects on the larval body, we show that ecdysone induces growth in the imaginal discs. Further, our data suggest that nutrition-dependent signalling and ecdysone both contribute to the size of the disc. Based on classical studies in the tobacco hornworm, *Manduca sexta*, Nijhout *et al.* suggested that in *Drosophila*, imaginal discs may have two types of growth: isomorphic and morphogenetic growth (Nijhout et al., 2013). Isomorphic growth is nutrition- and insulin/TOR-dependent, thus reflecting the nutritional conditions and ensuring that the size of the imaginal discs matches the size of the body. In turn, morphogenetic growth is ecdysone-dependent and accompanies differentiation of the imaginal discs into their final adult structure. Similarly, Shingleton et al. (2008) divided the growth the wing discs into two components: nutrition-dependent growth and intrinsic growth (Shingleton et al., 2008). Our data suggests that both nutrition and ecdysone contribute to *Drosophila* wing disc growth. Discs from fed PGX animals also grow, albeit slowly. Conversely in starvation conditions, in which insulin/TOR pathways are donwregulated, feeding 20E to PGX larvae induced growth of the wing disc. During wandering, ecdysone-dependent autophagy of the fat body can provide the nutrients necessary for morphogenetic growth of the imaginal tissues (Rusten et al., 2004). Potentially, 20E promotes the growth of imaginal discs from starved larvae via autophagy of the fat body or larval body. This would explain how ecdysone can reduce fat body and/or whole body growth while promoting disc growth.

When larvae were fed on ecdysone-supplemented starvation medium, the PGX discs show a greater increase in size than the *>Grim* discs. In principle, these larvae differ only in the presence of the PG. This suggests that the presence of the PG promotes negative feedback for growth.

Because ecdysone was exogenously provided, the PG must signal through some mechanism other than ecdysone itself. This feedback could be induced either by signalling to other tissues to regulate the levels of other developmental hormones, by regulating the levels of ecdysone receptors in the tissues, or could promote behavioural changes. Indeed we observed that *>Grim* larvae were outside of the food and their fat body seemed reduced in size. Increased concentrations of ecdysone reduce fat body cell size (Colombani et al., 2005; Delanoue et al., 2010). Thus, we propose that the PG produces a signal that alters disc growth, fat body size and larval behaviour.

Ecdysone concentrations change over time, with defined peaks that correspond to characteristic developmental events (Warren et al., 2006). Although the timing of the ecdysone peaks is essential for whole-body development, whether discs depend on any particular peak of ecdysone for their growth is unknown. Discs could depend on the presence of ecdysone above a minimum threshold for growth but be insensitive to values above this threshold. A second hypothesis is that ecdysone peaks can stimulate different growth rates depending on their timing during development, similar to unleashing waves of tissue growth. These two hypothesis can be addressed using the PGX larvae. By ablating the PG after the known third instar peaks of ecdysone: critical weight (CW), salivary gland secretion (SGS) or wandering (Figure 1.1), we could evaluate the differential contributions of each peak to the growth and development of imaginal discs.

In addition to its effects on growth, we found that the progression of pattern of the gene products Ac, Sens and Wg depended on ecdysone

confirming previous results on the role of ecdysone signalling in the patterning of the disc (Mirth et al., 2009). Surprisingly, nutrition alone promoted part of the Ac stage progression during the extended larval life, although it was insufficient to promote progression of Sens. This shows that the progression of pattern, although mainly dependent on ecdysone, can be induced by nutrition-dependent signalling. Moreover, it shows that gene products rely on ecdysone to different extents for their progression.

Although our data demonstrate that ecdysone regulates growth and patterning in the wing discs, it remains unclear if and how these two processes interact. Morphogens like Wg and Decapentaplegic (Dpp) have received considerable attention as central regulators of both patterning and organ scaling (Baena-López et al., 2009; Ben-Zvi et al., 2011; Hamaratoglu et al., 2011; Neumann and Cohen, 1996; Restrepo et al., 2014; Zecca et al., 1996). Discs where Wg is tethered to the membrane, thereby slowing down its diffusion, produce smaller wings with patterning defects (Alexandre et al., 2014). The progression of Wg pattern is both sensitive to nutrition early in development and depends on ecdysone (Mirth et al., 2009). The Dpp gradient scales with wing size across developmental time (Wartlick et al., 2011) and reducing Dpp signalling reduces wing size (Ben-Zvi et al., 2011; Irie and Kuratani, 2011; Wartlick et al., 2011). In the eye disc Dpp dynamics and levels change the switch between proliferation and differentiation of cells, thereby controlling the size of the organ (Wartlick et al., 2014). Potentially, ecdysone promotes growth through regulation of morphogens like Wg and Dpp. This would provide a means through which the wing disc could coordinate both its patterning state and its size with the development of the whole body.

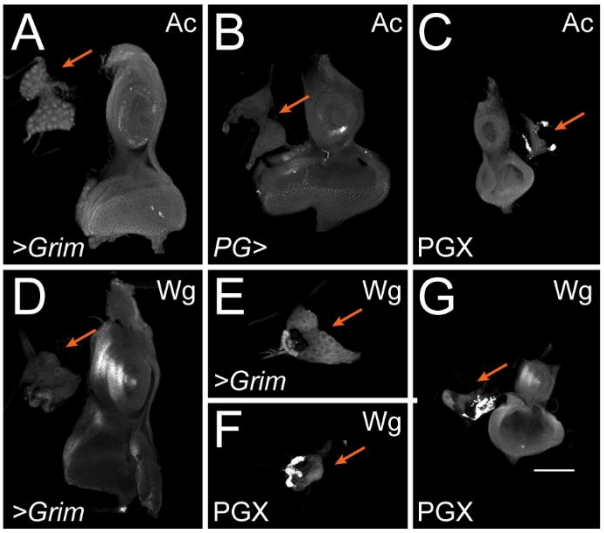
Acknowledgments

I would like to thank Alex Shingleton for giving me the opportunity to start this project in his lab and Christen Mirth for reading and commenting on this chapter. The evo-devo community for the continuous input and the lab for technical help.

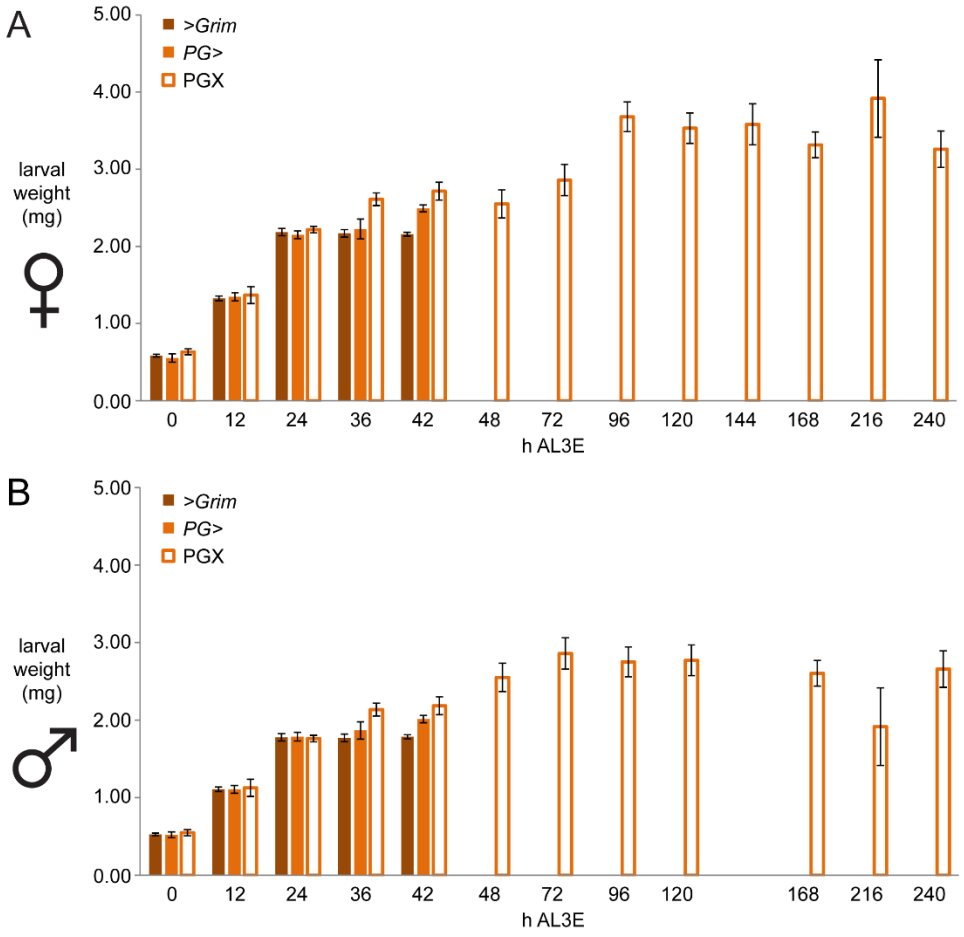
Publication

The data included in this chapter is being prepared for a co-authored paper in collaboration with Rosa Barrio's group.

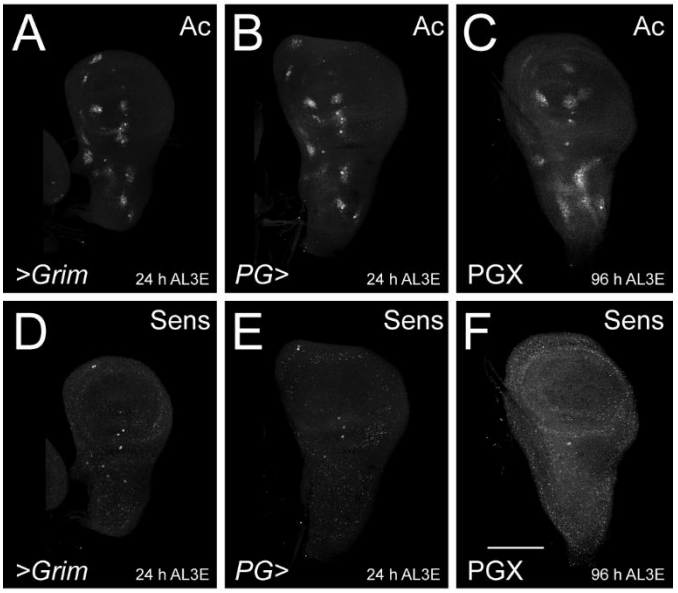
Supplementary material to Chapter 4



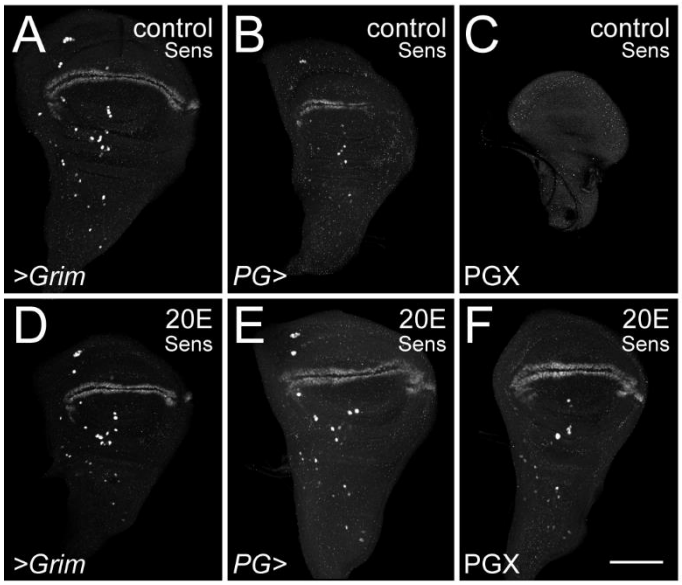
Supplementary Figure S4.1. PGX larvae exhibit a reduced and deformed prothoracic gland. Prothoracic gland (orange arrows) from (A, D, E) *>Grim*, (B) *PG>* and (C, F, G) PGX larvae shifted to 29°C at the moult to the third instar and collected at 42 hours after third instar ecdysis for tissue dissection and immunocytochemistry with Achaete (Ac) or Wingless (Wg) antibodies. (Scale bar 100 µm)



Supplementary Figure S4.2. *>Grim*, *PG>* and PGX body weight time series for females and males. (A) Female larval weight and (B) male larval weight characterized by mean (in mg) and 95% confidence intervals.



Supplementary Figure S4.3. PGX discs at 96 hours after the third instar ecdysis (h AL3E) show delayed Achaete (Ac, C) and Senseless (Sens, F) expression patterns even when compared to the controls *>Grim* (A, D) and *>PG* (B, E) at 24 h AL3E. (Scale bar 100 μm)



Supplementary Figure S4.4. Feeding 20E to PGX larvae rescues Senseless (Sens) expression pattern in the wing disc. Larvae were raised from the moult to the third instar until 42 hours after third instar ecdysis (h AL3E) in food with the addition of either 20-hydroxyecdysone (20E) or ethanol (control). Expression of Sens in wing discs from (A, D) *>Grim*, (B, E) *PG>* and (C, F) PGX larvae from the control (A-C) and 20E (D-F) feeding treatments. (Scale bar 100 µm)

5

Integrated discussion

At the outset of this work, we sought to understand the developmental processes that allow organisms to produce correctly patterned structures across a wide range of organ and body sizes. For fruit flies, having two correctly patterned and shaped wings, proportional to their body size, allows for flight and affects numerous life history traits. Despite considerable work revealing the mechanisms that regulate the development of the wing disc, those responsible for these fundamental characteristics of the animal are still open to debate. With this project, we examined the processes and mechanisms through which wing size coordinates with patterning and with the development of the whole body. To do so, we integrated the interactions between the autonomous development of the wing disc with the whole-body development in changing environment (Figure 5.1).

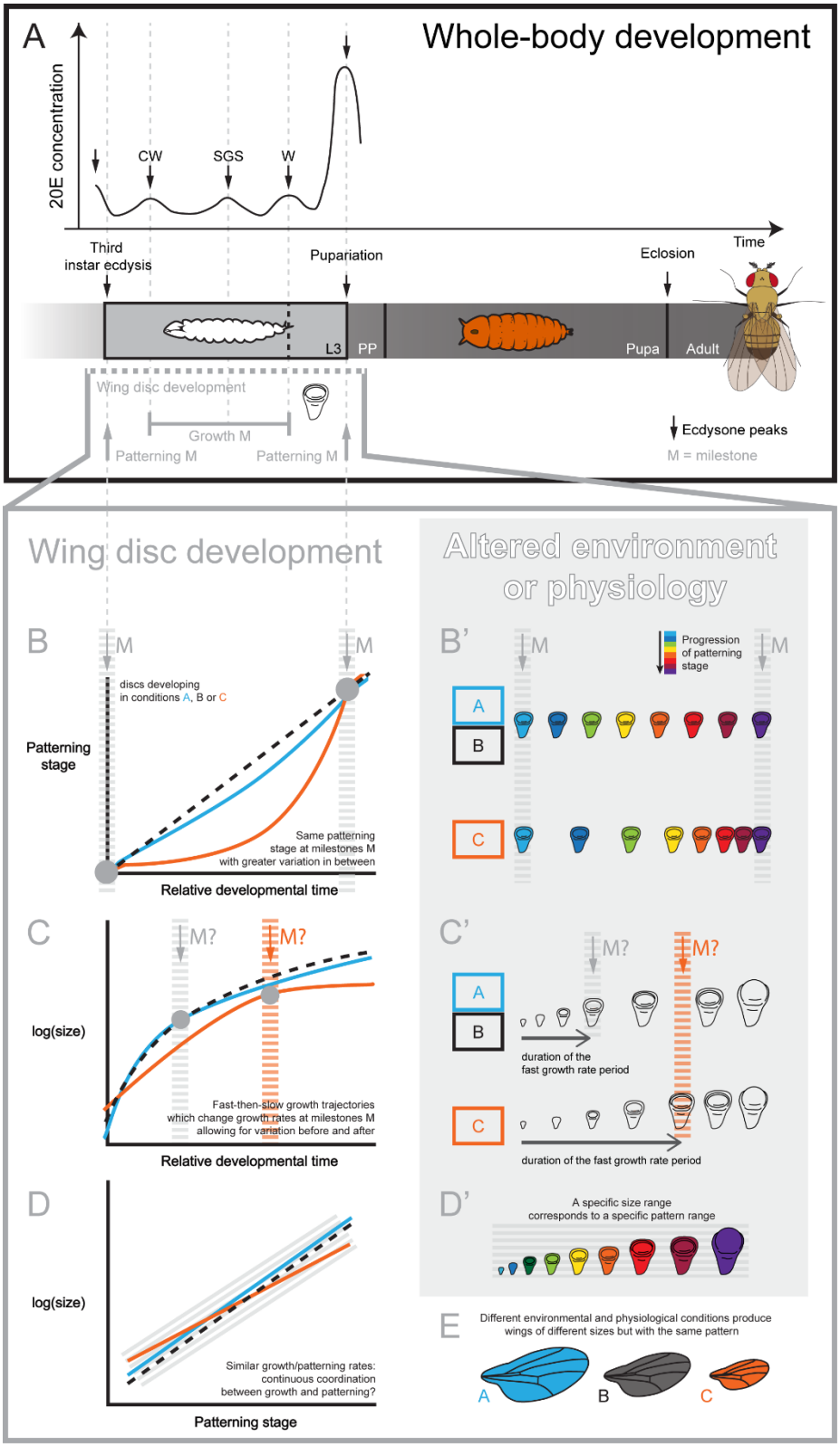
Our environment-body-organ integrative view was divided in three main approaches. First, we studied the development processes that allow patterning to remain robust against environmental and physiological perturbations. In order to do so, we developed a staging scheme based on the expression patterns of canonical gene products from the main patterning cascades that characterized wing disc development.

We hypothesized that to ensure robustness during development, patterning coordinates with whole-body development either continuously or at developmental milestones. Second, under the same variation of environmental and physiological conditions, organ size can change proportionally to changes in body size. This led us to explore how growth trajectories are altered to generate wings with different sizes. Additionally, to understand how patterning remains robust while growth trajectories are

variable, we studied the correlation between organ size and patterning. Last, we hypothesized that the moulting hormone ecdysone is necessary for and coordinates organ size with patterning. By exploring the contributions of ecdysone and the nutritional-dependent insulin/TOR signalling pathways to organ size and pattern, we have provided insights into the mechanism allowing milestones to coordinate organ with whole-body development.

On our first approach, to distinguish between our two hypotheses, we developed a staging scheme for the wing imaginal discs using the expression of patterning genes. This scheme was further used to quantify patterning on chapters 3 and 4. Using the staging scheme, our results revealed that although the progression of pattern varied depending on conditions, milestones coordinated patterning with the development of the whole body (Figure 5.1 B). Two developmental events, the moult to the third instar and pupariation, act as developmental milestones at which pattern is aligned independent of environmental or physiological alterations (Figure 5.1 B-B').

Although our studies only identified these two events as milestones, it is possible that many more exist during *Drosophila* development. For instance, we know that some patterning gene products become nutritional-independent after a developmental event early in the third instar known as critical weight (CW) (Mirth et al., 2009). Consequently, it would be reasonable to suggest that patterning may be coordinated at CW. CW in *Drosophila* larvae results from a small pulse of ecdysone early in the third instar whose timing is sensitive to nutrition and other environmental stimuli ((De Moed et al., 1999; Ghosh et al., 2013; Mirth et al., 2005)).


A
Whole-body development
20E concentration
CW
SGS
W
Third instar ecdysis
Pupariation
Eclosion
Time
L3
PP
Pupa
Adult
Wing disc development
Growth M
Patterning M
Patterning M
Ecdysone peaks
M = milestone
Wing disc development
Altered environment or physiology
B
M
M
discs developing in conditions A, B or C
Patterning stage
Same patterning stage at milestones M with greater variation in between
Relative developmental time
B'
M
M
Progression of patterning stage
A
B
C
C
M?
M?
log(size)
Fast-then-slow growth trajectories which change growth rates at milestones M allowing for variation before and after
Relative developmental time
C'
M?
M?
A
B
duration of the fast growth rate period
C
duration of the fast growth rate period
D
log(size)
Similar growth/patterning rates: continuous coordination between growth and patterning?
Patterning stage
D'
A specific size range corresponds to a specific pattern range
E
Different environmental and physiological conditions produce wings of different sizes but with the same pattern
A
B
C

To confirm whether CW acts as a developmental milestone for patterning, we would have to identify the time of CW for the tested temperatures/genotypes and then have sufficient resolution both in the transitions from our patterning map and in the developmental times sampled to observe if patterning aligns. The sensitivity of tissues to hormonal pathways may change in multiple ways. For example, either

Figure 5.1. *(preceding page)* Whole-body development coordinates wing disc development by process-specific milestones. (A) The active form of ecdysone, 20-hydroxyecdysone, regulates the four whole-body developmental events that take place during the third larval instar (L3): critical weight (CW), salivary gland secretion (SGS), wandering (W) and pupariation, through which the larva becomes a pre-pupa (PP). With our work, we propose that the wing disc that develops inside the larval body coordinates either pattern or growth at the milestones (M) specific to each process. These milestones may be regulated by the different peaks of ecdysone. (B) Wing discs from larvae developing under three different rearing conditions, for example different temperatures, may exhibit difference in the progression of pattern. We suggest that milestones coordinate patterning of the wing disc to achieve robust wing disc patterning regardless of the rearing conditions. To so, the progression through the patterning stages, represented in (B') by the change in disc colour from blue to purple, may progress either linearly, where the transitions from stage to stage happen at a constant speed (as in conditions A or B) or non-linearly, where rates of transitions between stages are variable but are always aligned at the milestone at pupariation. Similarly, (C) discs show a wide range of sizes but may coordinate their development at growth milestones. We observed that across all conditions, discs show fast-then-slow growth trajectories. The timing of the switch between the fast and slow growth rate is condition dependent (C'), which may indicate that either the regulation of the growth milestone itself depends on the condition or that there are alternative growth milestones. Regardless of the variation found for disc size, disc size correlates tightly with disc patterning within a condition (D). Between conditions, we observe plasticity in the disc size/patterning relationship. (D') Milestones may ensure that wing size variation under several conditions may maintain patterned wings. (E) Coordination between disc patterning and whole-body development at milestones, between disc growth and whole-body development at milestones and plasticity in the wing size/wing patterning relationship result in correctly patterned wings of a broad range of sizes.

increasing insulin receptor or increasing FOXO expression specifically in imaginal discs, increases the sensitivity of the wing disc to insulin signalling so that wing size becomes hyperallometric with body size (Shingleton and Tang, 2012). Decreased expression of FoxO results in hypoallometry of male genital arches with respect to wing size (Tang et al., 2011). This difference in the expression of components of the insulin pathways permits organs to respond differently to the same systemic signal.

Similarly, the four pulses of ecdysone that occur between the moult to third instar and pupariation regulate four distinct developmental events (Figs 1.1 and 5.1 A). Tissues can differ in their sensitivity to ecdysone concentration, and often tissues respond to only some of these pulses (Andres and Cherbas, 1992; Talbot et al., 1993). Presumably, the response of a tissue to a given ecdysone pulse is dependent on its function explaining why developmental milestones may be tissue-specific.

It is likely that multiple signals promote a given milestone. The PG synthesizes ecdysone in response to both prothoracicotropic hormone (PTTH) and insulin/TOR signalling to induce pupariation (Gibbens et al., 2011). Affecting signalling from either of these pathways significantly delays the development of the animal (Caldwell et al., 2005; Colombani et al., 2005; McBrayer et al., 2007; Mirth et al., 2005). However, either one of these signals on their own eventually promotes pupariation (Gibbens et al., 2011; Mirth and Shingleton, 2012). This integration between signalling pathways allows developmental milestones to be tuned to environmental or physiological conditions.

Despite the obvious importance of milestones in coordinating organ patterning, it is unclear whether the patterning state of the discs can

feedback to regulate the timing of the milestone in the event that patterning is delayed. Myriad examples in the literature show that larvae can undergo metamorphosis and form incorrectly patterned wings when patterning cascades are dramatically perturbed (De Celis, 2003; Quijano et al., 2010). Further, when Wg signalling is altered throughout the body, using a tethered version of Wg, larvae take longer to pupariate (Alexandre et al., 2014). If Wg is only tethered in the imaginal tissues, the resulting adult wings show missing sensory bristles in their margins but larvae do not take longer to pupariate. One would expect that if the patterning state of the disc feedback to regulate developmental milestones, slowing down the progression of Wg-mediated disc patterning would also slow down the attainment of the milestone, unless the attainment of the milestone is through some action of Wg that is now blocked. This data suggests that this feedback indeed may exist, perhaps depend on signalling through Wg.

In our second approach, we explored how development generates discs of different sizes under varying physiological and environmental conditions and how disc size coordinates with patterning. We proposed that differences in disc size can be attributed to 1) differences in the initial size, 2) altered growth rates or 3) altered lengths of their growth period. We found examples of alterations in all three processes, however only changes in growth rates and the length of the growth period resulted in differences in disc size at pupariation. Further, discs can use different trajectories to attain the same final size (Figure 5.1 C-C').

Despite the differences in growth trajectories observed, we observed several common, yet unexpected features. We observed an inflection point in all growth trajectories revealing that disc growth can be described by at

least two different growth rates. This inflection point characterizes a fast-then-slow trajectory in which the time a disc spends in each growth period is dependent on the condition. The wing disc proliferates extensively during the third instar (Madhavan and Schneiderman, 1977) and cell proliferation trajectories (Bryant and Levinson, 1985) resemble the ones we found for wing disc growth (fast-then-slow). Thus, this change in growth rate may be due to a regulatory change at the level of cell proliferation.

Several lines of evidence support the existence of two phases of disc growth. Nijhout et al. suggested that in *Drosophila*, imaginal discs have isomorphic and morphogenetic growth (Nijhout et al., 2013), which correspond to nutrition-dependent and ecdysone-dependent growth respectively. Moreover, Shingleton et al., (2008) deduced from published data on disc growth that the growth rate of discs changes from fast to slow growth around CW. Using mathematical modelling, Shingleton *et al.* (2008) showed that this change in disc growth rate results from a change in the relative contribution of nutrition- and insulin-sensitive growth and intrinsic (or morphogenetic) growth (Shingleton et al., 2008). Both from their model and from experimental data, they show that before CW, wing disc growth is regulated primarily by nutrition-sensitive growth. Because of this, wing discs from starved larvae before they have reached CW do not grow. After CW, intrinsic growth of the wing contributes more to the increase in disc size such that even discs from starved, post-CW larvae show considerable increase in size. Taken together, this suggests that CW acts as a growth-controlling milestone for the disc.

Our data show some support for the existence of a growth rate-regulating milestone at CW. Four of our conditions/genotypes showed an

early switch from fast-to-slow growth consistent with the time at which CW is reached. However, for the remaining three conditions/genotypes this switch occurred much later. It is unlikely for any of these conditions that the timing of CW shifts this late in development, as the genotype in which we expect CW to be delayed the most, *P0206>PTEN* (Mirth et al., 2005), shows early switch in growth rate. Possibly, two alternative milestones can regulate growth rate: CW and a later milestone. Which of the two milestones induces the shift in growth rate depends on the larval environmental/physiological conditions.

We also find differences in growth regulation between discs that will generate large versus small wings. For both genotypes that produce large wings, the disc size at pupariation was not significantly different from controls. Thus, wing size in these large wings increases in response to processes occurring during pupal development. Smaller wings, however, can result from changes in disc size in larval development. This suggests that the mechanisms that regulate small wing size are different those controlling size in large wings.

These findings suggest that larvae regulate maximum growth rates very tightly, possibly to avoid organ overgrowth. One could imagine that this tight regulation is merely a limitation of how fast cells in the wing can proliferate. However, our data show that larvae can extend the fast phase of their growth, and doing so would cause wing discs to attain a larger size at pupariation. Further, larvae are known to have mechanism to prevent overgrowth of the discs. Cell competition and apoptosis counteract the induction of overgrowth of wing discs in larvae, leading to wings of normal size (de la Cova et al., 2004). Thus, the maximal increase in disc size

appears to be under tighter regulation than the minimal increase in disc size.

To address the nature of the relation between size and patterning, we compared wing disc size with patterning during the third instar. Both disc size and disc patterning increase over time, thus wing disc growth and patterning are strongly correlated (Figure 5.1 D-D'). In Chapter 2, we showed that there are at least two patterning milestones, the moult to the third instar and pupariation (Oliveira et al., 2014). Growth milestones occur either at around 30% or 60% development. How can a tissue have different patterning and growth milestones? Hypothetically, alternating between coordinating growth and coordinating patterning may be a mechanism to allow for a broad range of disc sizes with correct pattern. Patterning in the second instar wing disc separates broad regions of the disc such as wing blade from notum (Ng et al., 1996). At this stage, Wg is expressed throughout the entire wing blade region (Ng et al., 1996). In the third instar, the first patterning milestone shows Wg restricted to the wing margin and a ring around the wing pouch. This change in expression may act to establish the patterning field for third instar discs. Secondly, a growth milestone regulates disc growth so that they reach sufficient size to build a wing and continue the next phase of patterning. Lastly, the final patterning milestone ensures that at pupariation discs can vary in sizes but not in patterning stage (Figure 5.1).

In our third and last approach, we explored the roles of ecdysone and insulin/TOR signalling pathways in coordinating organ size with pattern. We showed that ecdysone is necessary for the growth and patterning of wing imaginal discs. Exogenous administration of ecdysone was sufficient to

induce patterning even in discs from starved larvae. Previous work showed that ecdysone signalling at CW induces disc patterning to become nutrition independent. Together with our data, this highlights the role of CW as both a growth and patterning milestone for the wing disc and as a coordinator of disc and whole-body development.

Additionally, by comparing the disc growth rates in fed PGX larvae to those from starved PGX larvae supplemented with ecdysone, we could

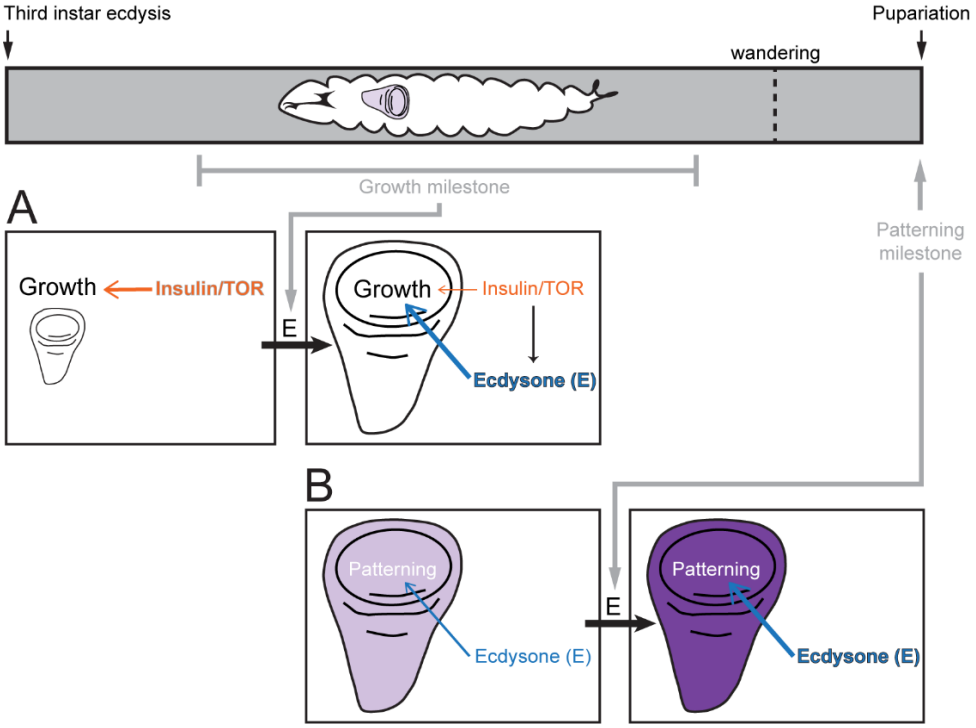


Figure 5.2. Contributions of ecdysone and nutrition-dependent signalling for growth and patterning of the wing imaginal disc. (A) Growth milestones act during the mid-third instar and may regulate size of the wing disc by switching their growth from nutrition-dependent (insulin and TOR) to morphogenetic growth (ecdysone and nutrition-dependent growth). (B) The patterning milestones occur at the beginning and end of the third instar and depend on ecdysone. Patterning may be coordinated at these milestones, and not other, as a result of a change in tissue sensitivity to ecdysone.

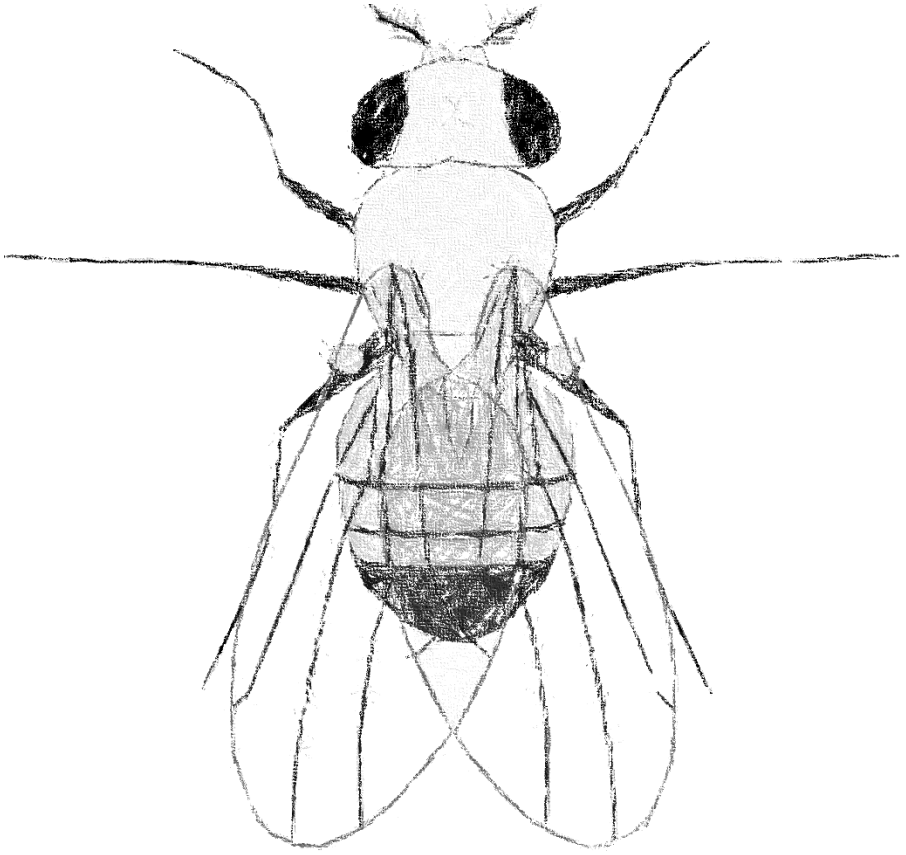
disentangle and quantify nutrition-sensitive and ecdysone-sensitive growth rates respectively. We showed that nutrition-dependent signalling, in the absence of ecdysone, sustains part of the growth of the wing disc. This increase in size due to nutrition-dependent signalling alone presumably results from growth induced early in development (Figure 5.2 A). The growth induced by ecdysone alone occurs in the later phases, corresponding to the switch from primarily nutrition-dependent to primarily morphogenetic growth at CW (Shingleton et al., 2008). Thus it would seem that ecdysone induces morphogenetic growth.

Interesting questions arise as to how developmental milestones change with altered environmental conditions? Based on our results, we suggest that nutrition-dependent signalling, presumably mediated through insulin/TOR, and ecdysone might interact throughout larval development to coordinate developmental milestones. Previous work shows that insulin/TOR act to regulate the timing of ecdysone synthesis at CW (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). In different environmental conditions the whole body may differentially modulate the levels of insulin/TOR signalling and, consequently, tune the timing and levels of ecdysone production. This would ensure that tissue development would take place at the appropriate times in coordination with the development of the whole-body. The differential contributions of ecdysone and insulin/TOR signalling to disc growth seems an efficient way to allow for a range of different disc sizes by pupariation, while relying on one main input for patterning may ensure that patterning stage is invariant at pupariation (Figure 5.2).

Taken together, our data revealed that the robust development of an organ in face of environmental and physiological perturbations can be achieved by many ways. Process-specific milestones within an organ ensure coordination between many developmental processes, including growth and patterning. Furthermore, we suggest that organ development coordinates with whole-body development at milestones, and that there are tissue-specific milestones to allow for coordination between the different organs and the body at different times. Moreover, we propose that the differential contributions of ecdysone and insulin/TOR signalling for the growth and patterning of organs can determine organ-specific milestones, hence, ensuring organ-body coordination.

6

Conclusions and perspectives



With this work we propose a new paradigm for thinking about organ-organ and organ-body coordination during development. Our work suggests that these types of coordination are achieved not by continuous but rather by discrete communication focused on developmental milestones.

Wing discs show the same final pattern in all conditions, despite growing and patterning with different dynamics. This suggests that there is more to coordinate at the organ level than pattern and size. Indeed, wing shape changes with temperature (Debat et al., 2003). Perhaps, differences in the dynamics of growth and patterning dynamics accommodate for other processes such as alterations in wing shape. Relative growth of wing pouch, hinge and notum, changes in the folding of the disc epithelium, and disc and pouch shape should be coordinated with the different patterning cascades of the tissue. This opens a new interesting avenue, to explore how imaginal tissues coordinate these developmental processes – do milestones play a role?

Despite considerable effort from many labs, what defines an organ's size is still a mystery. We proposed that growth milestones ensure an organ's minimum size, but it is still unknown how milestones are perceived by the organ. Moreover, we did not observe growth rates higher than those observed in discs reared in standard conditions, highlighting the existence of limitations for the growth of tissues, which, in turn, can constrain organ size *per se*.

Patterning is differently coordinated compared to size, so that at the end of development wings can have different sizes but only one correct pattern. It is surprising, however, the dramatic changes in the rate of

patterning that we observed in the various environmental/physiological manipulations of larval development. How can a disc pattern faster than it would in standard rearing conditions and given that it can pattern faster, why not always? What are the constrains or trade-offs of fast patterning?

Last, we propose that nutrition-sensitive signalling interacts with hormonal pathways to coordinate tissue-specific milestones. Despite all the knowledge of these signalling cascades, it is still largely unknown how they interact throughout development: what are the changes in the relative levels of hormone signalling and how do different tissues autonomously modulate their sensitivity to perceive each of the signals? Further, we have explored the role of nutrition-dependent signalling and ecdysone in regulating milestones, are there other hormones involved?

In conclusion, we feel our work provides insight into how the development of tissues is coordinated with the physiology of the body. Moreover, it highlights the importance of a temporal perspective of development and exploring how developmental processes change with environment. Last, science asks questions to find out how the world works. The answers always generate even more questions and we are certain that we have contributed ours.

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