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Determination of organ size: a need to focus on growth rate, not size

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ABSTRACT The regulation of growth and the determination of organ-size in animals is an area of research that has received much attention during the past two and a half decades. Classic regeneration and cell-competition studies performed during the last century suggested that for size to be determined, organ-size is sensed and this sense of size feeds back into the growth control mechanism such that growth stops at the "correct" size. Recent work using *Drosophila* imaginal discs as a system has provided a particularly detailed cellular and molecular understanding of growth. Yet, a clear mechanistic basis for size-sensing has not emerged. I re-examine these studies from a different perspective and ask whether there is scope for alternate modes of size control in which size does not need to be sensed.

KEY WORDS: *size sensing, size control, growth control, imaginal disc*

"Well, I should like to be a little *larger, sir, if you wouldn't mind", said Alice, "three inches is such a wretched height to be". "It is a very good height indeed!" said the caterpillar angrily, rearing itself upright as it spoke (it was exactly three inches high).*
Chapter V, Alice in Wonderland, by Lewis Caroll.

Being the right size is an important developmental outcome to most animals and plants and helps an individual of a species successfully occupy an ecological niche. Size is regulated at the level of both the organ and the whole organism. Whole organism growth would require the coordination of growth of individual organs and is known to be influenced by levels of circulating nutrition and hormones (see Nijhout, 2003). In addition, growth needs to be coordinated with the developmental programme (see Boulan *et al.,* 2015). It is well established that organs have an intrinsic ability to regulate their size and will do so even when transplanted into a different environment (see Bryant and Simpson, 1984). How intrinsic organ size is determined remains a mystery despite the extensive studies on organ growth that have been performed particularly over the past two decades. Detailed experimental and theoretical studies have been performed using the fruit fly *Drosophila melanogaster* as a model organism. These studies enable an analysis of the question from a number of different angles. Therefore, this article will focus on the fly and in particular the wing imaginal disc. The working hypothesis used to understand how individual organs grow towards a desirable target size is that there exists an organ-size check point or that size *per se* feeds back onto growth and regulates it (see Bryant and Simpson, 1984; Day and Lawrence, 2000). In this article I will attempt to re-examine the arguments that have been used in support of the hypothesis that organ size is sensed. This article is not intended to be an exhaustive review of the literature on growth studies. Instead I focus on work that relates to whether size is sensed and whether there is an organ-size check point. I briefly propose at the end an alternative mode of size control that does not depend on an organ's ability to sense size. I suggest that the target of the growth control mechanism is to reach a suitably slow growth rate by the end of the growth period.

Drosophila melanogaster as a system to study growth regulation

Drosophila melanogaster is a holometabolous Dipteran insect and is a classic example of an animal that undergoes determinate growth, where growth occurs during a defined period (see Hariharan *et al.,* 2016). The adult appendages and most of the adult cuticle are derived from sac like epithelial structures called imaginal discs. These grow within the larva and metamorphose during pupation.

Abbreviations used in this paper: CDK4, cyclin dependent kinase 4; Dilp8, Drosophila insulin like peptide 8; dMyc, Drosophila homolog of Myc proto-oncogene family; Dpp, decapentaplegic; En, engrailed; Hh, hedgehog; JNK, c-Jun N-terminal kinase; PI3Kinase, phosphoinositide-3-kinase; PIP3, phosphoinositol (3,4,5) trisphosphate; Rn, rotund; Rp, ribosomal protein; UAS, upstream activating sequence; Wg, wingless; Yki, yorkie.

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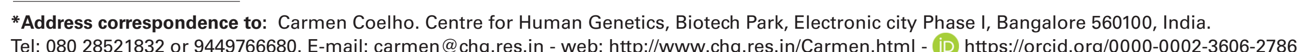
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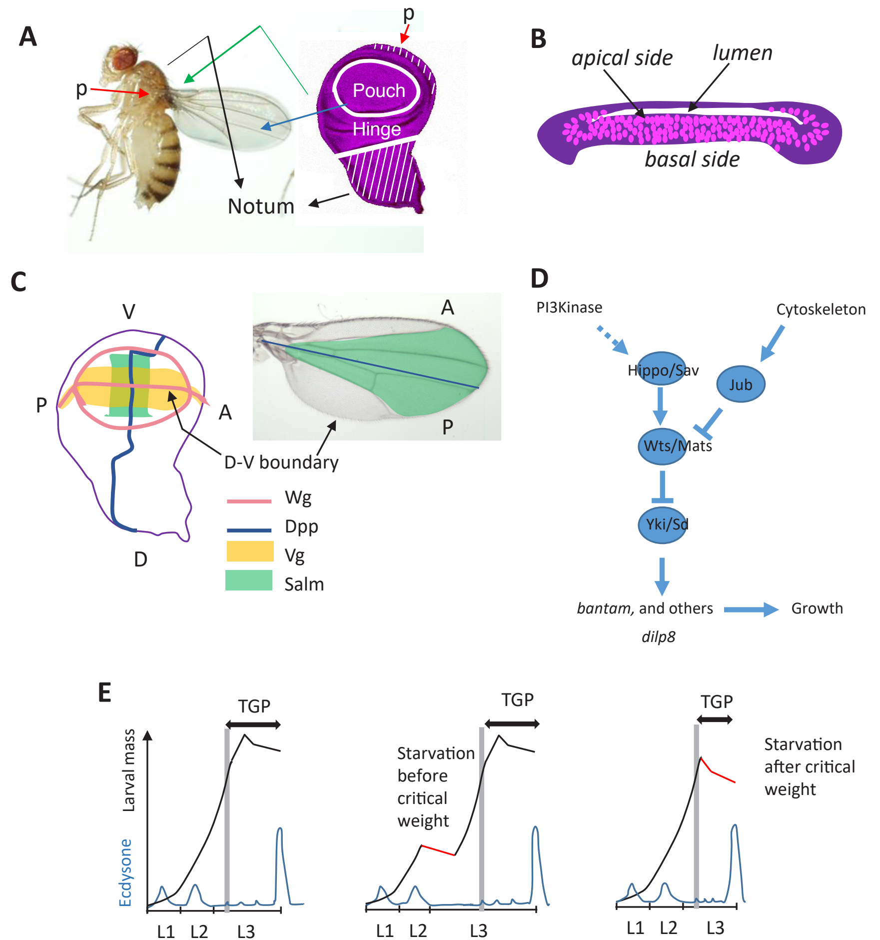

Fig. 1. Wing imaginal discs in *Drosophila melanogaster* as a system to study organ size control. **(A)** *image of a female adult fly on the left displaying the derivatives of the wing imaginal disc on the right; the central oval, flat region (labelled Pouch) contributes to the adult wing blade (blue arrow), the folded hinge region around the wing pouch gives rise to the hinge (green arrow), that connects the wing to the thorax, most of which consists of the notum (black arrows). The hatched region in the disc contributes to the thorax, with the larger region on the dorsal side giving rise to the notum and narrower region on the ventral side giving rise to the pleura (red arrow, p).* **(B)** *Schematic of a cross-section of a disc showing the positions of the nuclei (magenta circles) in the pseudo stratified epithelium (known as the disc proper), the basal and apical sides of the disc proper and the lumen between the disc proper and the peripodial membrane above.* **(C)** *Schematic of a wing disc in the same orientation as the disc in (A), ventral (V) is above, dorsal (D) is below, posterior (P) is on the left and anterior (A) is on the right. A picture of an adult wing is also shown. The schematic shows simplified expression patterns of Dpp (blue), Wg (pink) and Vg (yellow) and Spalt major (Salm, green, also indicated in the adult wing as the derivative of this part of the wing pouch). The region of synthesis of Dpp is shown as a line (blue), the protein diffuses to form a gradient that is shallower in the lateral regions of the disc (not shown). This line marks the Anterior-posterior boundary (shown as a blue line in the picture of the wing). Peak Wg expression is at the Dorso-ventral boundary (pink curve perpendicular to the Dpp stripe, indicated with an arrow in the disc and in the picture of the wing) and in two concentric rings around the wing pouch (only one is shown here). The point where the Dpp stripe and the Wg stripe intersect is the most distal region of the disc.* **(D)** *Simplified version of the Hippo signalling pathway (Sav, Salvador; Sd, Scalloped; Wts, Warts) showing the cellular inputs it responds to and the downstream target Yorkie (Yki) a transcription factor that drives growth.* **(E)** *A schematic adapted from Tennessen and Thummel (2011), showing the influence of starvation on final size and developmental time. The duration of three larval instars (L1, L2, L3) is indicated on the x axis. The black curves indicate larval size during the growth period. If larvae are starved early in development (size during the starvation period indicated in red), larvae take a longer time to reach critical weight (grey vertical bar indicates time when critical weight is reached), thus lengthening developmental time. When larvae are starved after reaching critical weight, the terminal growth period (TGP) is shortened (Stieper* et al., *2008). Thus, the length of the growth period is affected by nutritional input before and after critical size is reached. Critical size is a poorly understood genetically determined trait reflecting the nutritional status and/or size of the larva, rather than the imaginal discs and ascertains the composite state of readiness for completion of metamorphosis (reviewed in Boulan* et al., *2015). The final size of the adult is affected by growth during the TGP.*

The size of the adult is largely determined by the amount of growth that occurs during the larval stage although there have been studies that have revealed a minor extent of growth of imaginal tissue during the early pupal phase (Shingleton *et al.,* 2005).

Larvae undergo two moults that allow transitions through the three instars (see Fig. 1E). The first two instars are of equal length, approximately 24 hours each at 25°C, the third is double this length. Shortly after the moult from the 2nd to the 3rd larval instar, hormonal events are initiated that result after a specific time period in the termination of feeding, and the initiation of metamorphosis (see Nijhout, 2003; Tennessen and Thummel, 2011). Ecdysone is the key hormone that is responsible for pupation and is secreted by the Prothoracic gland, which is part of the Ring gland situated on the top (dorsal side) of the brain. Ecdysone is released throughout larval life, but increases in pulses with low pulses detected during the 1st and 2nd larval instars and a major pulse just before pupation, driving pupation (see Thummel, 2001). The length of the larval period is modulated by environmental inputs such as nutritional status (see Fig. 1E and legend for details). The release of hormones by the neuroendocrine system is delayed if larvae have not reached a certain critical weight that they normally reach early in the third larval instar (Stieper *et al.,* 2008; Nijhout, 2015). Both larvae and imaginal discs undergo a rapid increase in size (volume or mass is estimated in larvae, area or cell number is estimated in wing discs) during the second instar and beginning of the third instar (Tennessen and Thummel 2011; Bryant and Simpson, 1984; Wartlick *et al.,* 2011a). When the y-axis depicting size (as volume in larvae or total cell number in discs) is converted to a log-scale, there is a slow-down in growth clearly observable at mid-3rd instar for both larvae and wing discs (Layalle *et al.,* 2008; Bryant and Simpson, 1984).

Definition of terms used to depict growth

The term "growth" is often used to describe an increase in cell number, mass or volume of a tissue and sometimes cell size (reviewed in Coelho and Leevers, 2000). Historically growth was

used to denote proliferation, with the assumption that cell size did not vary. With the recognition that cell size can vary during certain developmental stages or due to certain genetic manipulations, the term "cell growth" was introduced to emphasise that tissue volume is being considered and not the number of cells (Leevers *et al.,* 1996; Neufeld *et al.,* 1998; Johnston *et al.,* 1999; Böhni *et al.,* 1999). In wing discs, during the first one-eighth of the growth period, cell size increases without cell division (Madhavan and Schneiderman, 1977). During the remainder of the larval period, cells divide. This paper is concerned largely with these later stages of development, when cells are dividing. A decrease in cell-size has been observed during the last quarter of the larval growth period (Neufeld *et al.,* 1998). This decrease in cell-size is likely to reflect a gradual decrease that had likely begun once disc-cells started to divide during the first instar. But the decrease in cell-size is not sufficient in magnitude to suggest that cell number cannot be used as an indicator of growth.

There are certain genetic manipulations that alter cell size because they have a larger impact on cell growth than on cell division (see Table 1, columns 1 and 2). Other genetic manipulations affect tissue volume without affecting cell size (Table 1, columns 3 and 4), because of a co-ordinate effect on cell division and cell growth. Mutations in Ribosomal proteins (Rp) known as the *Minutes* show a characteristic delay in development with little effect on cell-size or final body size ((Morata and Ripoll, 1975; Lambertsson, 1998; Coelho *et al.,* 2005; Marygold *et al.,* 2007). However, in the interests of accuracy, it must be pointed out that certain *Minutes* ($RpL38^{+/-}$, $RpL5^{+/-}$) show a mild increase in cell size and a larger increase in body size (Marygold *et al.,* 2005), because of a larger effect of the mutation on developmental time and cell division than on growth rate (rate of volume increase).

To keep the text simple, the term 'growth' will be used to denote increase in tissue volume, area or cell number. When growth is monitored *in situ,* within a tissue, cell division markers are used to detect proliferation by immunofluorescence or immunohistochemistry. This is because monitoring a local increase in tissue volume or area is not possible, if the regions being studied are not labelled. When these experiments are discussed, the terms "proliferation", "cell-division" or "mitotic-index" will be used, only to indicate that the method detects cell division.

A description of wing imaginal discs

Wing discs give rise to the adult wing and a large part of the thorax. Fig. 1A shows an image of the adult fly and a wing disc with the different regions labelled. The pouch region of the disc gives rise to the adult wing. It is surrounded by a folded region which forms the hinge connecting the adult wing to the thorax. The triangular region (larger hatched region in Fig. 1A) on the dorsal side of the disc forms the notum, while a small portion on the ventral side forms a part of the ventral thorax (the pleura, indicated with a p in Fig. 1A). Using markers to detect cells in the various phases of the cell cycle, only a proportion of cells are labelled at any given time during larval development suggesting that wing disc cells divide asynchronously (Milan *et al.,* 1996). When such methods are used, disc cells appear to divide at a spatially uniform rate through most of larval life. However, other studies examining the size of labelled mitotic clones in different regions of the disc, demonstrate that pouch cells divide at a 10% slower rate than the rest of the disc (Coelho *et al.,* 2005; Johnston and Sanders, 2003). There are temporally dynamic non-uniformities that are observed within the pouch particularly during the fast phase of growth, and these even out towards the end of larval life (see le Goff *et al.,* 2013 and Mao *et al.,* 2013 and references therein). The cells remain undifferentiated and essentially plastic until late in the third instar, when their capacity to regenerate on damage decreases (Halme *et al.,* 2010). Thus, the growth of wing disc cells is not subject to feedback from differentiated progeny as is the case in many developing organs. The question of size control here boils down to the question of how an undifferentiated tissue reaches the appropriate size at the end of the growth period. Hence the wing discs are a good system to understand the fundamental principles of intrinsic size control.

Although wing imaginal discs do not differentiate during larval life, they acquire patterning information, such that by the end of larval life, the territories that will form vein and intervein regions are clearly marked by the expressions of specific genes (reviewed in Crozatier *et al.,* 2004; Tabata and Takei, 2004). These regions get specified by the actions of the morphogens Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg) (reviewed in Tabata and Takei, 2004). The secreted products of these genes form two perpendicular gradients spanning the wing pouch and surrounding regions, with Dpp forming a gradient in the anterior-posterior direction and Wg in the dorso-ventral direction (see Fig. 1C). The highest level of Dpp is at the middle of the wing pouch (see blue line in Fig. 1C) and the highest level of Wg is at the dorso-ventral boundary that runs through the centre of the wing pouch (indicated with an arrow in Fig. 1C).

The posterior side of the Dpp stripe marks a boundary between the anterior (A) and posterior (P) sides of the disc. This boundary is known as a compartment boundary because cells do not traverse this boundary. The P compartment is marked by the expression of the homeobox gene *engrailed (en).* En expressing cells do not mix with non-En expressing cells. Hh is secreted by the P cells and forms a short range gradient along the A-P axis, inducing the synthesis of *dpp* in the cells just anterior to the P compartment (Guillén *et al.,* 1995). The size of the compartments can be deter-

TABLE 1

EFFECTS OF VARIOUS GENETIC MANIPULATIONS ON CELL SIZE OR CELL NUMBER

Genes/proteins affecting cell size	Reference	Genes/proteins affecting cell number, but not cell size	Reference
Members of the Insulin signalling pathway	Böhni, et al. 1999; Leevers, et al. 1996	microRNA *bantam*	Hipfner, et al. 2002
dMyc	Johnston, et al. 1999;	Members of the Hippo signalling pathway	Udan et al. 2003
eukaryotic translation initiation factor binding protein (4E-BP)	Miron, et al. 2001;	*cyclinD* along with *CDK4* (referred to as *cyclinD/CDK4*)	Datar, et al. 2000
ras	Prober and Edgar, 2000	*wingless (wg)*	Baena-Lopez, et al. 2009
		decapentaplegic (dpp)	Martin-Castellanos and Edgar, 2002

mined independently from each other and therefore compartments are considered to be the units of size control (Crick and Lawrence, 1975; see Day and Lawrence, 2000). In the embryo, when the discs are first formed, the P compartment is smaller than the A compartment (Lawrence and Morata, 1977). This discrepancy is reduced during larval life such that by the end of larval life the P/A ratio is 0.66 (Martin and Morata, 2006) and the A-P boundary bisects the wing pouch into two nearly equal halves. In the adult wing blade, the P/A ration is 1.16 (Martin and Morata, 2006; Martin *et al.,* 2009).

The morphogens Wg and Dpp are required for growth and patterning of the disc. Besides these morphogens, the insulin and mTOR signalling pathways drive growth in response to nutrition (see Leevers and McNeill, 2005 for a review). The Hippo pathway suppresses growth in response to planar polarity, mechanical forces and signalling through the insulin signalling pathway (reviewed in Yu and Guan, 2013; Irvine and Harvey, 2015; see Fig. 1D for a summary of the components of the Hippo signalling pathway that are relevant to this paper).

Organ-size is determined intrinsically

Organs grow in an environment that consists of circulating growth hormones and nutrition that ensure that the individual organs grow in a coordinated fashion meeting the needs of the entire animal. As mentioned above the *Drosophila* larva utilises the neuro-endocrine system to regulate the larval period in response to nutrient availability (see legend to Fig. 1E for further details). Thus, if the growth period is fixed by the animal's neuro-endocrine system, the size of an organ would be determined by the rate at which it grows during this period. When the growth period is extended organ size should increase. Or, if organs are made to grow in a foreign milieu, their growth should be altered. If neither of these outcomes result, then it would indicate that organ size is constrained intrinsically by the organ itself and not by the length of the growth period or by the surrounding milieu.

These possibilities have been tested through transplantation experiments both in vertebrate and invertebrate systems (reviewed in Bryant and Simpson, 1984). Using *Drosophila* wing imaginal discs, it is found that when immature discs are transplanted into adult abdomens, they grow to their normal size (Bryant and Levinson, 1985; Garcia-Bellido, 1965). In the experiments by Garcia-Bellido, head regions of newly hatched first instar larvae were transplanted into adult hosts. The associated eye-antennal discs grew to their normal sizes whether the transplant contained brain tissue or not. This is important as the presence of neuroendocrine secretions from the brain and ring gland would have re-created at least in part, the larval environment. In the experiments by Bryant and Levinson, wild-type discs were transplanted into adult abdomens at a stage when they measure approximately 20,000 cells (mid-3rd instar stage). These discs would have grown in the larva to a final size of about 48,700 cells in approximately 24 hours. Thus, their doubling time during this period is approximately 19 hours, characteristic of the slow-down phase that occurs during the middle of the 3rd instar. It was found that on transplantation, the discs grew very slowly and reached an average cell number of 44,600 after 21 days of culture, after which cell number decreased possibly because of a deterioration of conditions inside the host. This slow growth contrasted well with that of mutant discs that drastically overgrew under the same treatment, emphasizing that the host environment in these experiments did support growth and even allowed overgrowth in mutant discs; but the wildtype discs did not overgrow. These results together with those by Garcia-Bellido show that wildtype imaginal discs grow to their normal size even when removed from their normal larval environment in the absence of larval stage hormones and growth factors and even when the growth period is not defined by the length of the larval period. However, one could argue that in a foreign environment, the growth of wildtype discs might be hampered such that they grow very slowly and so despite the extended developmental time, they do not have the opportunity to overgrow. Thus, we need to examine situations in which larval developmental time is extended.

Achieving allometry in genetic mosaics

There are two ways of lengthening the larval developmental period. One is by making use of the *Minute* phenotype. Rp loci show haplo-insufficiency, the effect being on rate of growth. Thus, a strain that is deficient in one copy of an Rp gene grows slowly, but gives rise to close to normal sized flies at the end (Lambertsson, 1998). These strains are known as *Minutes.* Genetic techniques can be used to generate wild type discs in *Minute* larvae. Now we can ask if these discs overgrow. The other way of lengthening developmental time is by generating slow-growing or damaged discs in wildtype larvae. This is known to extend the larval period as the presence of damaged discs delays pupation (Simpson and Schneiderman, 1975; Simpson *et al.,* 1980; Halme *et al.,* 2010; Stieper *et al.,* 2008). Such studies are performed using genetic mosaics in which some imaginal discs are mutant for a slow-growing mutation and the remaining are wildtype. Thus, here again, we can ask if the wild type discs overgrow. In all these studies we are in essence addressing the question of whether and how allometry is achieved in animals that contain both wildtype and growth impaired tissue.

These findings reveal two different mechanisms by which allometry is achieved in genetic mosaics. Martin and Morata (2006) found that wildtype discs generated in *Minute* larvae did grow faster than *Minute* discs in *Minute* larvae. In these experiments all the imaginal discs were wildtype and their growth is compared to wildtype discs growing in wildtype larvae and also *Minute* discs growing in *Minute* larvae. Wildtype discs in *Minute larvae* did not overgrow despite the extended larval life and produced wings of the same size as the controls. Martin and Morata (2006) also made mosaics in which one compartment of each disc was wildtype in *Minute* larvae. They found that wildtype compartments grew faster than the *Minute* compartments such that the posterior to anterior compartment ratio (P/A ratio) was altered during larval development but the appropriate ratio was reached at the end of larval life, just before pupariation. The surprising finding here was that at the end of larval life both the faster growing wild type discs and compartments showed a similar mitotic index to the slower growing *Minute* discs and compartments (Martin and Morata, 2006). This shows that even when discs or compartments decelerate their growth at different stages, they do not stop growing _ the faster wildtype discs and compartments have grown faster and so would have started to decelerate their growth earlier. Thus, it is likely that the faster discs do not overgrow, not because an organ-size check point has stopped their growth, but because after they have completed their fast period of growth, they slow-down to a sufficiently slow rate.

The second mechanism by which allometry is achieved involves

growth retardation of the wildtype tissue during phases when it should be undergoing fast growth. This mechanism has been revealed by using the Gal4-UAS system. The Gal4-UAS system drives the expression of target genes in tissues of choice along with reporter genes (such as GFP) that label these regions. Using this technique, a subset of discs are genetically manipulated (henceforth referred to as mutant) in wild type larvae. The presence of these mutant discs causes the larvae to extend their developmental time. But, since only some of the discs are mutant, one can ask if the other wild type discs overgrow. In the experiments by Parker and Shingleton (2011) Rp function was specifically reduced in the pouch region of wing discs by driving the expression of gene-specific (ribosomal protein S3, *RpS3*) RNA interference (RNAi). In contrast to the experiments by Martin and Morata (2006) these experiments show quite clearly that indeed the remaining leg and eye discs that are wildtype because they don't express the RNAi, slow their growth in a co-ordinated fashion such that at any stage in development, all discs are of a similar stage in development from the point of view of their size. Similar results have been obtained by Boulan *et al.,* (2019) expressing RNAi against RpS3 and RpL7 in the wing pouch (see Fig. 2A).

Boulan *et al.* (2019) further show that the remainder of the wing disc, in which RNAi is not expressed is also growth retarded, maintaining the normal shape of the disc throughout larval life. Such growth retardation within the disc to maintain shape was also shown by Mesquita *et al.,* (2010; summarised here in Fig. 2B). Mesquita *et al.,* used many more lines expressing Gal4 in different regions of the wing disc (Mesquita *et al.,* 2010). Some of the expression patterns define compartments, but the others don't. Three different growth altering transgenes were expressed using these Gal4 drivers. The first was cold sensitive Ricin; Ricin being an inhibitor of 28SrRNA and hence translational activity. The second was $4EBP^{AA}$ a form of eukaryotic-translation-initiator-4e binding-protein (4EBP) that cannot be inhibited by phosphorylation. The third was PTEN (phosphatase and tensin homolog), a lipid phosphatase and PI3Kinase antagonist. These genetic manipulations result in increased cell death in the regions where the inhibitors are expressed, but also a decreased proliferation in the rest of the disc. In these experiments by Mesquita, *et al.,*2010), a reduction in total adult wing size is observed but shape is conserved because of the growth retardation in regions where the Gal4 is not expressed. Thus, this preservation of allometry (or proportionate size) is restricted to within the organ and not between organs.

Thus, in the above sets of experiments, mutant and wildtype wing discs achieve allometry by the end of the growth period. But, the mechanism used differs. Importantly, neither of the two mechanisms demonstrate that an organ size check point stops growth at a specific target size. In the experiments from Morata's lab (2006) the wildtype discs and compartments in *Minute* larvae continue growing, albeit slowly. In the experiments by Parker and Shingleton (2011), Boulan *et al.,* (2019) and Mesquita *et al.,* (2010), the wild type tissue has slowed its growth in coordination with the mutant tissue.

The above experiments present an interesting paradox that should be pointed out at this stage, at the risk of digression. Both the experiments by Martin and Morata (2006) and the experiments by Parker and Shingleton (2011) and Boulan *et al.,* (2019) involve reductions in Rp function. Yet, the mechanism by which allometry is achieved differs. In one, growth of the wild type tissue is co-ordinately retarded; in the other kind of manipulation the wild type tissue grows at its normal rate. To examine whether wildtype cells indeed grow at their normal wildtype rate in the *Minute* larvae clonal analysis has been utilized. In 2009, Martin *et al.,* generated wildtype clones in *Minute* discs in *Minute* larvae. They found that the wildtype clones grow at their autonomous rate whether they are surrounded by wildtype cells (in control animals) or *Minute* cells (in the experimental animals). This was shown earlier by Morata and Ripoll in 1975. Thus, the surrounding slow growing *Minute* cells do not alter the rate of growth of wild-type cells. Because of the extended developmental time of the *Minute* larvae, wildtype clones grows extensively and overtake the *Minute* compartment (illustrated here in Fig. 4C', lower row of discs). One observation that could help resolve this paradox is that in the experiments by Boulan *et al.,* (2019) and Parker and Shingleton (2011), the wing pouch expressing RNAi against Rp genes is surrounded on all sides by wild type tissue. Whereas, in the discs when one compartment is wildtype or when a clone of wildtype cells is generated in a *Minute* disc, a large part of the free edge of the disc consists of slower growing *Minute* cells (Martin and Morata, 2006; Martin *et al.,* 2009; compare the green region surrounded by white regions in discs in Fig. 2A with the blue clone surrounded by the black region in Fig. 4C' - lower row of discs). This latter configuration would perhaps impose less mechanical stress on the centre of the disc. See below for a discussion on the role of mechanical forces in disc growth.

It is important to mention the experiments performed by Simpson (1976). Simpson made genetic mosaics using a temperature sensitive recessive slow-growing mutant (*l(1)ts1126*) that has not been mapped molecularly as yet and thus, the gene that is mutated is not known (Flybase, FBal0007828). The technique used induced mosaicism in early embryos and did not allow control of which tissues were mutant and which were not. Neither did it allow precise control of when the mosaicism was induced. Thus, the mosaics had differing degrees of mutant tissue, depending on how early the mosaicism was induced. Developmental delay was observed only when at least 47% of the whole larva was mutant and these animals displayed approximately 50% extended developmental time. Some of the mosaics were half-body mosaics with one wing mutant and one wing wild type. When there was no developmental delay, the mutant wing was smaller than the control. When Simpson observed developmental delay, the mutant wing had grown to the same size as wildtype, revealing that the extended developmental time allowed growth to a normal size. This means that just as in the *Minutes,* these slow growing mutant discs can grow to a normal size if provided with sufficient time before pupation. Simpson mentions in the discussion of this paper, quoting unpublished data, that when there was developmental delay, the wildtype discs grew to their full extent and then waited for the mutant ones to grow, supporting her argument that there exists an organ-size check-point. It would be important to repeat these experiments and find out if, just as in the experiments by Martin and Morata (2006, mentioned above), cell division persists in the wild type discs until the end of the extended larval life.

Does the size of the discs regulate the length of the larval period?

Since discs grow within larvae, it might be possible for discs themselves to regulate when the larval period ends. This would

ensure that pupation occurs when the discs have reached the right size. The experiments mentioned above by Parker and Shingleton (2011) and Simpson (1976) are examples of situations where the presence of slow-growing discs results in extended developmental time. Though it should be pointed out that in Simpson's experiments, developmental delay is observed only when at least 47% of the larva is mutant. One cannot rule out that in these larvae the delay in pupation was due to the prothoracic gland itself being mutant. The question that needs to be addressed now is whether the size of the mutant discs needs to be sensed for the delay in development to occur. And, whether such size sensing occurs only in the presence of slow-growing mutant discs or whether it occurs in other growth perturbed situations and also during normal development. Such coordination of growth of imaginal and larval tissue has received much attention during the past two decades and the molecular pathways involved have been reviewed elsewhere (Boulan *et al.*, 2015). The results will only be discussed here from the point of view of whether size needs to be sensed for growth coordination to occur.

Theoretically, discs could signal to the neuro-endocrine system using either positive or negative signals. A positive signal could be secreted from discs and could accumulate in for example the circulating haemolymph as discs grow and could induce pupation when the signal has reached a threshold level. On the other hand, a negative signal secreted from discs could act to delay pupation and it would have to be cleared away from the system to allow pupation to occur. There is evidence that supports the idea that there is no positive signal involved. This evidence is that pupation can occur even when discs are absent. Simpson *et al.,* (1980) generated mosaic larvae containing tissue bearing a temperature sensitive cell-lethal mutation. Shifting the mosaic larvae to the restrictive temperature caused cell death in the mutant tissue. Occasionally entire discs were missing, but this did not cause a delay in pupation. Hence, absence of discs does not cause delay. This suggests that the presence of disc tissue is not sensed by the neuroendocrine system through a positive signal that is relayed in proportion to the amount of disc tissue that exists.

There is evidence of negative signals secreted from discs that delay pupation. A number of experiments from Hadorn's work in 1937 to recent experiments by Boulan *et al.,*2019) have shown that mutant discs signal to the neuroendocrine system making the prothoracic gland secrete Ecdysone at a lower rate. Ecdysone is not only required to induce pupation, but also for the growth of the imaginal discs (Martin and Shearn, 1980; Mirth *et al.,* 2009). Thus, when Ecdysone levels are low, larval and imaginal tissue grow slowly and pupation is delayed. Parker and Shingleton (2011) found they

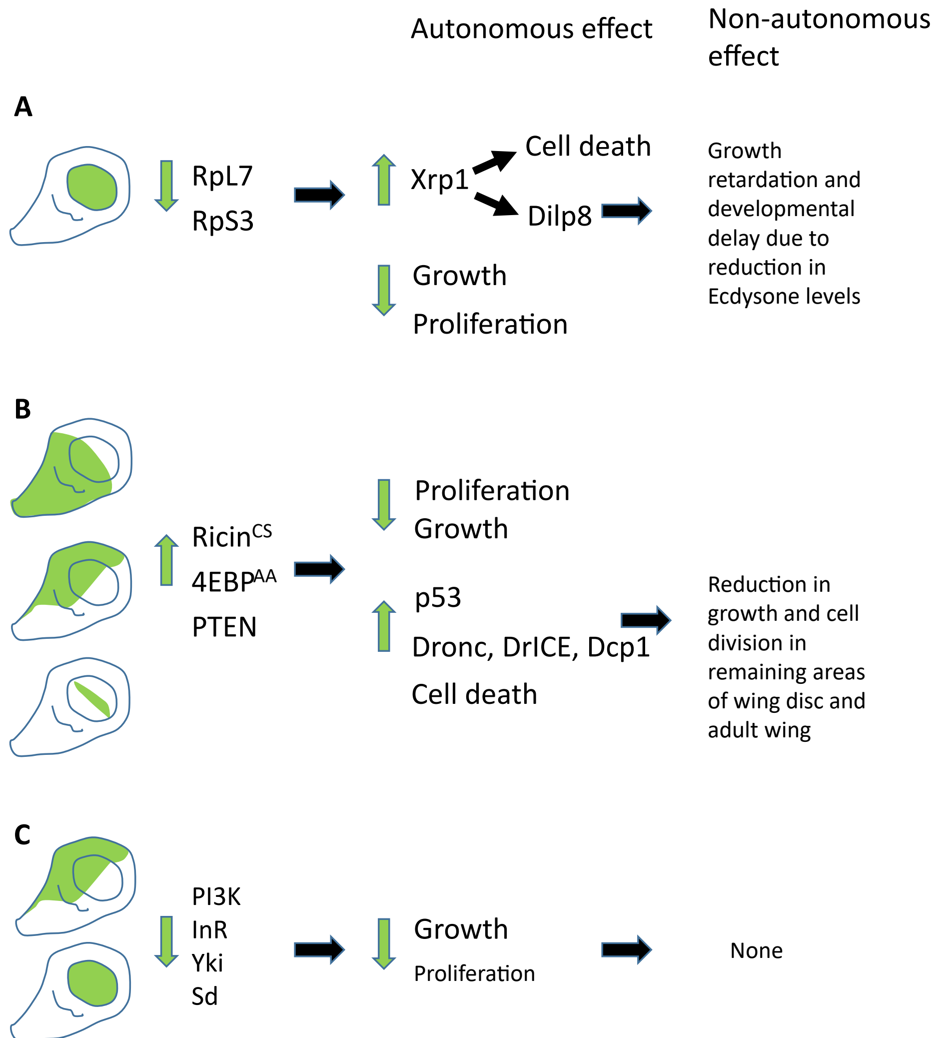

Fig. 2. Growth perturbation and growth coordination. *Using the Gal4-UAS system, the expression levels of specific genes was altered in specific regions (shown here in green, representing the co-expressed green fluorescent protein, GFP) of the wing imaginal disc. The schematics here summarise the main findings of Parker and Shingleton (2011) and Boulan* et al., *(2019) in* **(A)** *Mesquita* et al., *(2010) in* **(B)** *and Weinkove* et al., *(2000) and Boulan* et al., *(2019) in* **(C)**. *Expression of RNAi against rpl7 or rps3* **(A)** *results in an autonomous increase in cell death mediated in part by expression of the transcription factor Xrp1. Xrp1 drives expression of Dilp8 which non-cell autonomously drives growth retardation of other regions of the disc and larva by reducing Ecdysone synthesis. Expression of Ricin, PTEN and constitutively activated 4EBP in different regions of the wing disc* **(B)** *induces cell death and a net decrease in size in these regions, but also a non-autonomous growth retardation of the remaining regions of the disc. This non-autonomous effect on area reduction is dependent on p53, the non-autonomous reduction in cell division is dependent on activity of the effector Caspases diap1 and DRIce. In contrast, modulating the activity of insulin signalling components such as PI3kinase, the insulin Receptor inR, and other growth regulators such as Yorkie (Yki) and Scalloped (Sd) show only autonomous effects on the region of expression, but no non-autonomous effects* **(C)**.

could rescue the developmental delay and make the non-mutant discs to grow at their normal rate by providing Ecdysteroids extraneously. This extraneous treatment with Ecdysteroids now results in a breakdown of allometry between the mutant and non-mutant discs. Thus, the signal from imaginal discs to the neuroendocrine system is a negative one, aimed at delaying pupation when discs are not "ready". This negative signal not only delays pupation, it results in allometric growth retardation of the rest of the larva.

Experiments during the last decade have identified these signals emitted from the mutant discs. These experiments have been summarised schematically in Fig. 2. The transcription factor Xrp1 is synthesized by cells in which Rp gene function is reduced (Lee *et al.,* 2016; Lee *et al.,* 2018). Boulan *et al.,* (2019) expressed RNAi against RpS3 in the wing pouch and found that Xrp1 drives expression of the relaxin-like secreted factor, Dilp8 (*Drosophila* insulin like peptide 8) (Colombani *et al.,* 2012; Garelli *et al.,* 2012). Dilp8 is secreted into the haemolymph and binds to its receptor Lgr3 (leucine-rich repeat G protein-coupled receptor 3) that is expressed in two pairs of symmetric neurons on the dorsal side of the brain (Vallejo *et al.,* 2015). These neurons arborize with prothoracicotropic hormone (PTTH) secreting neurons and the insulin producing cells (IPCs) that secrete Dilp2, 5 and 6. PTTH induces Ecdysone synthesis by the prothoracic gland; Dilp2,5 and 6 promote growth and the synthesis of juvenile hormone (JH). These neurons are inhibited by increased Lg3 signalling leading to decreased JH synthesis, reduced Ecdysone synthesis and reduced growth of the larva and imaginal discs (Vallejo *et al.,* 2015). Thus, Dilp8 is one of the negative signals emitted from the imaginal discs, signalling that they are not ready for pupation. Halme *et al.,* (2010) found that when cell death is induced by X-rays or by the expression of the pro-apoptotic gene *reaper* in wing discs, secretion of Retinoic acid from the damaged discs resulted in lower secretion of Ecdysone by the neuro-endocrine system and delayed pupation. Thus, Retinoic acid is another negative signal delaying pupation when discs are not ready.

Does the secretion of these negative signals require disc-size to be sensed? The growth retardation of wild type tissue and delay in pupation described above appear to be in response to stress. There is no indication that the size of the mutant tissue directs the response. For example, when both Xrp1 and RpS3 expression are co-inhibited (in Boulan *et al.,* 2019), cell death is reduced, suggesting that Xrp1 expression is partly responsible for the cell death associated with reduced Rp function. Interestingly, the wing pouch region remains small (because of the reduced Rp function), but the non-autonomous effects on growth coordination and developmental delay are no longer observed. Thus, reduced disc size *per se* does not cause developmental delay. It is the function of Xrp1 and its downstream target *dilp8* that causes the delay. Further, *dilp8* expression is induced in a number of stress induced contexts including during regeneration in response to wounding (Katsuyama *et al.,* 2015) and also in discs undergoing abnormal growth and tumour formation (Colombani *et al.,* 2012; Garelli *et al.,* 2015). The Jun N-terminal Kinase (JNK) and JAK/STAT signalling pathways induce the synthesis of *dilp8* in these cases (Colombani *et al.,* 2012; Garelli *et al.,* 2015; Katsuyama *et al.,* 2015).

The above findings suggest that stress causes coordinate retardation of growth and developmental delay, but reduction in size does not. However, one indication that growth coordination might involve size-sensing during normal development is the finding that *dilp8* expression is a target of the Hippo signalling pathway (Boone *et al.,* 2016). Dilp8 is normally expressed in wing discs during the 2nd and 3rd instars and the levels go down during normal development around the middle of the third instar, which is when growth begins to slow (Colombani *et al.,* 2012) and the neuroendocrine system has initiated the events that will lead to pupation. Thus, *dilp8* expression is poised to play a role as a sensor that senses whether discs have reached an appropriate size, then allowing pupariation. Consistent with this possibility, *dilp8* and *lgr3* mutants show fluctuating asymmetry in adult wing size (approximately 15% difference in size). The occurrence of fluctuating asymmetry in these mutants raises the question of whether disc growth is normally error prone, resulting in differences in size during normal development. The differences in size eventually even out. The dilp8 and lgr3 signal would then inform the neuroendocrine system when the differences in size between bilateral discs is eliminated. Removing the Yki responsive element upstream of the dipl8 open reading frame through genome editing also induces fluctuating asymmetry (Boone *et al.,* 2016) (see Fig. 1D for components of the Hippo signalling pathway). On the other hand, mutations in the JNK kinase *hemipterous,* which is responsible for stress induced *dilp8* expression does not induce fluctuating asymmetry. This suggests that the response to Hippo signalling occurs during non-stressed conditions. Hippo signalling is considered to play a role in organ-size sensing (Rauskolb *et al.,* 2012). Put together these findings might suggest that growth coordination during normal development is regulated by organ-size sensing. There is a caveat here. *dilp8* mutants or the depletion of *lgr3* affect developmental timing slightly (8 hours earlier for depletion of *lgr3,* Vallejo *et al.,* 2015; 4 to 5 hours for *dilp8* mutants, Colombani *et al.,* 2012). It is not clear whether this small difference in developmental timing is sufficient to consider Dilp8 to be a negative signal delaying pupation during normal development. Nevertheless, this issue will be discussed further later in this paper.

Correlation between time of pupation and disc size

Another argument against the possibility that disc-size drives the decision to pupate is the absence of a strict correlation between final disc size and the extent of delay. These observations are once again made in mosaic larvae, in which the discs are mutant and the neuroendocrine system is wild type. In the experiments by Mesquita *et al.,* (2010), in which Ricin is expressed in regions of the wing disc, pupation is delayed by 24 hours, but the delay did not result in normal final disc-size. Conversely, Parker and Shingleton (2011) report that modulating insulin signalling specifically in wing discs using the A9-Gal4 driver (drives expression in the wing pouch, see Fig. 2C) does not induce developmental delay. It causes a reduction in size of adult wings specifically without affecting the size of the rest of the body. Boulan *et al.,*2019) also show that reduction in insulin signalling components in the wing pouch do not elicit a non-autonomous effect on growth of the remainder of the disc and larva (see Fig. 2C). It was reported in the year 2000, that modulating class 1A PI3Kinase activity using the Gal4-UAS system specifically in the posterior compartment of wing discs altered its size. This manipulation also altered the P/A compartment ratio because the size of the corresponding anterior compartment remained normal (Weinkove *et al.,* 2000).

Put together these results indicate that non-autonomous growth coordination and delay in pupation is elicited by a certain subset

of growth regulators. When it is elicited, allometry of organ size is achieved as in the case of decrease in Rp function in discs (in the experiments by Parker and Shingleton (2011) and Boulan *et al.,* 2019). But, growth retardation does not result in allometry in certain instances such as in the case of Ricin expression in discs. Why the insulin signalling pathway does not elicit the non-autonomous growth coordination response, is unclear. One possibility is that growth coordination is elicited in response to stress and altering the activity of the insulin signalling pathway does not stress the system as much as the above mentioned growth perturbation pathways.

How might size be sensed?

From the above discussion, there is no clear indication that disc-size is sensed during larval development. Nevertheless, we need to examine if there is a means by which size can be sensed. There is evidence that disc- cell number is not counted. Altering the rates of cell division by altering the activity of cell cycle promoting genes, can give rise to small cells and large cells without changing the area occupied by these cells (Neufeld *et al.,* 1998). In these experiments, disc compartments were generated consisting of many small cells or fewer large cells but the total size of the compartment remained unaltered. This suggested very strongly that the size of imaginal discs is not determined by counting cell number, but by determining dimensions. However it is not clear whether total volume is somehow estimated, or dimensions along a particular axis. Models have been put forth suggesting how the development of morphogen gradients in growing discs could act as read outs of dimension and feedback onto growth. These models have been reviewed extensively and therefore will not be mentioned here (Restrepo *et al.,* 2014; Wartlick *et al.,* 2011b). Briefly, it has been postulated that either the steepness of a morphogen gradient or the concentration of a morphogen at any point in the gradient could evolve with developmental time and size. If this steepness determined the rate of growth, then growth would stop when the steepness reached below a certain threshold (Day and Lawrence, 2000). If the rate of change in the concentration at any point in the gradient determined the rate of growth, then growth would finally stop when the concentration changed at a very low rate (Wartlick *et al.,* 2011a). Experimental support for these models have not been obtained as yet and there is much debate on the relationship between morphogen gradients and growth control (Schwank *et al.,* 2008; Hamaratoglu *et al.,* 2009; Schwank and Basler, 2010; Schwank *et al.,* 2012; Restrepo *et al.,* 2014; Akiyama and Gibson; 2015; Bosch *et al.,* 2017; Baena-Lopez *et al.,* 2009; Zecca and Struhl, 2007a and b). More recently, mechanical forces have been suggested to play a role in size sensing and growth control (Hufnagel *et al.,* 2007; Aegerter-Wilmsen *et al.,* 2007). I will list some of the experimental findings below pertaining to the control of growth by morphogens and then discuss the role of mechanical forces.

Rogulja and Irvine (2005) generated large clones in wing discs that expressed an activated form of Thick-vein, the receptor for Dpp. This generated a steep juxtaposition of high and low Dpp signalling activity in neighbouring cells, stimulating proliferation non-autonomously, but only temporarily. These experiments showed importantly that a sharp juxtaposition of high and low signalling levels of a morphogen can stimulate growth and proliferation, but some form of negative feedback stops it. However, in whole discs, reducing the steepness of the Dpp gradient does not prevent growth (Schwank *et al.,* 2008). More recently it has been shown that a low level of *dpp* expressed uniformly throughout the disc is sufficient for normal size of the wing disc, but not normal pattern, thus, un-coupling these two outcomes of Dpp signalling (Bosch *et al.,* 2017). Nevertheless, there could be spatial and temporal differences in the levels of Dpp or Dpp signalling in these discs that have not been detected in these experiments. Similarly, low levels of uniformly expressed Wg are suggested to be required in a permissive fashion to promote growth, while higher and graded levels are required for patterning (Baena-Lopez *et al.,* 2009; Zecca and Struhl, 2007a and b). These data put together suggest that morphogens are required for growth and can stimulate growth. But it is not clear whether it is their absolute levels that regulate growth, or whether it is their temporally evolving spatial differences that regulate growth. Logically, evolving spatial and temporal differences could provide information about dimensions, but absolute levels cannot. Thus, it is not clear whether morphogens play a role in sensing size.

Mechanical forces have received much attention for their ability to modulate growth in response to contact inhibition in cultured cells and cytoskeletal tension in imaginal discs (reviewed in le Goff and Lecuit, 2016; Yu *et al.,* 2015). Models have been proposed involving an interplay between morphogen signalling and mechanical forces in the control of growth and organ size. Wing imaginal discs at the start of their development have an elongated tear drop shape without morphogenetic folds. By the end of the third instar there exists a complex geometry that is highly likely to be regulated by both mechanical forces and morphogens. There are prominent morphogenetic folds in the region that gives rise to the hinge and there is a considerable expansion of the entire disc along the x and y axes (see schematics in Fig. 4C here). Wing pouch cells become longer and thinner in the centre of the wing pouch leading to an expansion here along the z-axis (Le Goff *et al.,* 2013).

The model by Aegerter-Wilmsen *et al.* (2007) takes into account a scaled *Dpp* gradient and will be briefly mentioned here. It is proposed that as morphogens drive growth faster in the centre of the disc where they are at a higher concentration, the periphery becomes stretched in a tangential direction compressing the central region. This stretching causes further growth. The growth decreases the stretching, but some stretching remains and continues to cause compression of the centre until finally growth stops. Growth stops because the morphogens in the centre can no longer drive growth because of the inhibition caused by compression. In support of this model, it is known that compression increases in the centre of the wing pouch around the middle of the third instar (Nienhaus *et al.,* 2009; Rauskolb *et al.,* 2014; le Goff *et al.,* 2013). In addition, cells at the proximal edges of the wing pouch region start to elongate at the end of the 2nd instar and orient themselves perpendicular to the proximal-distal axis (Mao *et al.,* 2013).

The Hippo signalling pathway could provide the molecular basis for this model. Stretching can cause a change in localization of the protein Ajuba to the adherence junction where it associates with αCatenin and inhibits Hippo signalling. Inhibiting Hippo signalling activates Yki and growth (Rauskolb *et al.,* 2014). Further it has been shown that Hippo pathway signalling can read the state of mechanical tension in genetically manipulated disc cells and respond to negative feedback from the surrounding cells. *bantam* is downstream of Yki (Thompson and Cohen, 2006; see Fig. 1D here)

and can drive overgrowth even in the absence of Yki (see Yu and Guan, 2013). Pan *et al.* (2016) overexpressed *bantam* in clones and showed that they caused the surrounding slower growing wild type cells to stretch. This stretching activates a negative feedback on the faster cells, which is dependent on Hippo signalling in the faster cells and results in their decreased cytoskeletal tension, decreased Yki in the nucleus and therefore slower growth. Thus Hippo pathway signalling can read the state of mechanical tension in genetically manipulated disc cells and respond to negative feedback from the surrounding cells. Whether Hippo pathway signalling is sensitive enough to respond to the rate at which cytoskeleton tension changes during normal development is left to be seen.

Nevertheless, the above mentioned control of dilp8 expression by Yki (Boone *et al.* 2016) leads one to re-examine the role of size sensing in growth coordination. As mentioned above, Dilp8 levels in wing discs drop during the middle of the third instar (Colombani *et al.,* 2012). The higher level of Dilp8 expression during the second instar and early third instar could be due to higher cytoskeletal tension and therefore higher Yki activity.

And, the drop in dilp8 levels in the wing disc during mid-third instar (Colombani *et al.,* 2012) could signal to the neuro-endocrine system that the discs have passed the phase of fast growth. If the two bilateral discs reach the slow phase at slightly different times, the neuroendocrine system would start the events leading to pupation only when both of them have done so, avoiding fluctuating asymmetry. If the decrease in cytoskeletal tension in the wing pouch occurs at a defined size as is expected by the theoretical models and simulations (Aegerter-Wilmsen *et al.,* 2007), then *dilp8* decrease would also occur at a defined size. But if, for example, earlier rates of growth change the size at which the transition from fast to slow growth occurs, then *dilp8* decrease would correlate not with size but with the transition from fast to slow. However, it is left to be seen how accurate such systems of communication are and it would be necessary to test the above trend in ideas experimentally. In summary, it is not clear if normal growth coordination occurs (if it does occur) in response to size-sensing of discs or a certain change in disc-growth rate.

Regeneration of damage

Regeneration experiments of the previous century suggested that sensing of size was somehow linked to patterning information. This mode of patterning information elaborates on the concept of positional values as proposed by Wolpert (Wolpert, 1971, 1989). The positional values consist of polar coordinates that give cells a sense of where they are in a field and thereby influence their getting determined to adopt a particular fate. On injury, these coordinates are disrupted and coordinate values or positions that were originally further away from each other, now come closer. This juxtaposition of disparate positional values is expected to promote intercalary growth, such that the missing values are now restored. This model described normal development by suggesting that as an organ grows, the size of the field increases and hence the number of positional values increase. The fewer positional values in early discs promote growth because they are positional values that in a mature disc would be farther apart, for example the positional values that represent the centre and edge of a disc (see Fig. 3A). As the tissue grows and the number of positional values increase the differences between neighbouring positional values decrease and growth stops when a continuum is reached (Wolpert, 1971; Bryant and Simpson, 1984). If positional information is specified by patterning information, the positions in the morphogen gradients of Dpp and Wg should specify positional information (Wolpert, 1989). In Fig. 3A, the Dpp gradient is used to illustrate the working of this model, based on the schematic representation used by Bryant and Simpson (1984). Importantly, these models depend on the coupling of patterning information and growth of the disc. As mentioned above, the relationship between morphogen gradients and growth control is under much debate. Nevertheless, it is worth examining whether the process of regeneration could provide scenarios where the slowing of growth and the emergence of pattern are uncoupled.

When a wound is inflicted, regeneration may require proliferation to generate new tissue that can then be patterned to form the missing tissue (known as epimorphosis) or the existing tissue may get remodelled to generate the missing tissue (known as morphallaxis; see Agata *et al.,* 2007). Wells and Johnston (2012) found that in response to X-irradiation, the rate of proliferation in imaginal discs remained unchanged. But, in response to other forms of insults, for example, over-expression of cell-death inducing genes or excision of discs, proliferation is increased at the site of injury (O'Brochta and Bryant, 1987; Bosch *et al.,* 2008; Smith-Boulton *et al.,* 2009; Bergantinos *et al.,* 2010; Herrera *et al.,* 2013; Diaz-Garcia and Baonza, 2013). Thus, we can now ask what stops or slows down the increased growth. In principle, there are three possibilities. One is that growth is accompanied by and driven by pattern as it gets restored, the other is that growth precedes restoration of pattern and stops before pattern is complete, the third is that growth precedes pattern restoration, but this growth is in excess and pattern restoration eliminates the excess cells. The first possibility would be in accordance with intercalary growth. If all regeneration processes follow this first possibility, then these observations would add considerable weight though still not prove that patterning information directs the extent of growth.

In both the classical experiments (reviewed in Bryant and Simpson, 1984) and the recent ones, regeneration was studied after inducing extensive cell death or after cutting the imaginal disc into pieces and allowing the pieces to regenerate. I will focus more on the recent experiments in which the emergence of pattern was studied during regeneration.

Induction of cell death

As mentioned above in the section about damaged discs and developmental delay, temperature-sensitive, recessive cell-lethal mutations were used in the previous century to ablate regions of the leg, wing and other discs in mosaic larvae (Simpson and Schneiderman, 1975; Simpson *et al.,* 1980; Russell, 1974 are some examples of these experiments). Because the mutations were temperature sensitive, cell death could be induced at the restrictive temperature at specific periods during larval development and then regeneration could be allowed by bringing the cultures back to the permissive temperature. In these experiments, the mutant tissue could not be traced during larval stages, as the cell markers affected the adult cuticular hairs and not larval cells. In more recent years, the Gal4 system has been used to drive cell death inducing genes coupled with a temperature sensitive version of the Gal4 inhibitor, Gal80. (Smith-Boulton *et al.,* 2009; Bergantinos *et al.,* 2010; Martin *et al.,* 2017; Martin and Morata, 2018). Here, cell death is induced when larvae are at the restrictive temperature of Gal80. When brought

back to the permissive temperature of Gal80, Gal4 is shut down, cell death inducers are no longer expressed and the damaged tissue is allowed to repair. GFP is used to mark the areas where the cell death genes were induced and also allows one to determine how much of the targeted domain is actually killed and how much the un-killed cells contribute to the process of repair. The unkilled cells could be expected to retain some of the patterning information that existed in the region before cell death was induced. The extent of damage to the tissue depended on the cell death inducer that was expressed. Using the *rotund*-Gal4 driver, which is expressed

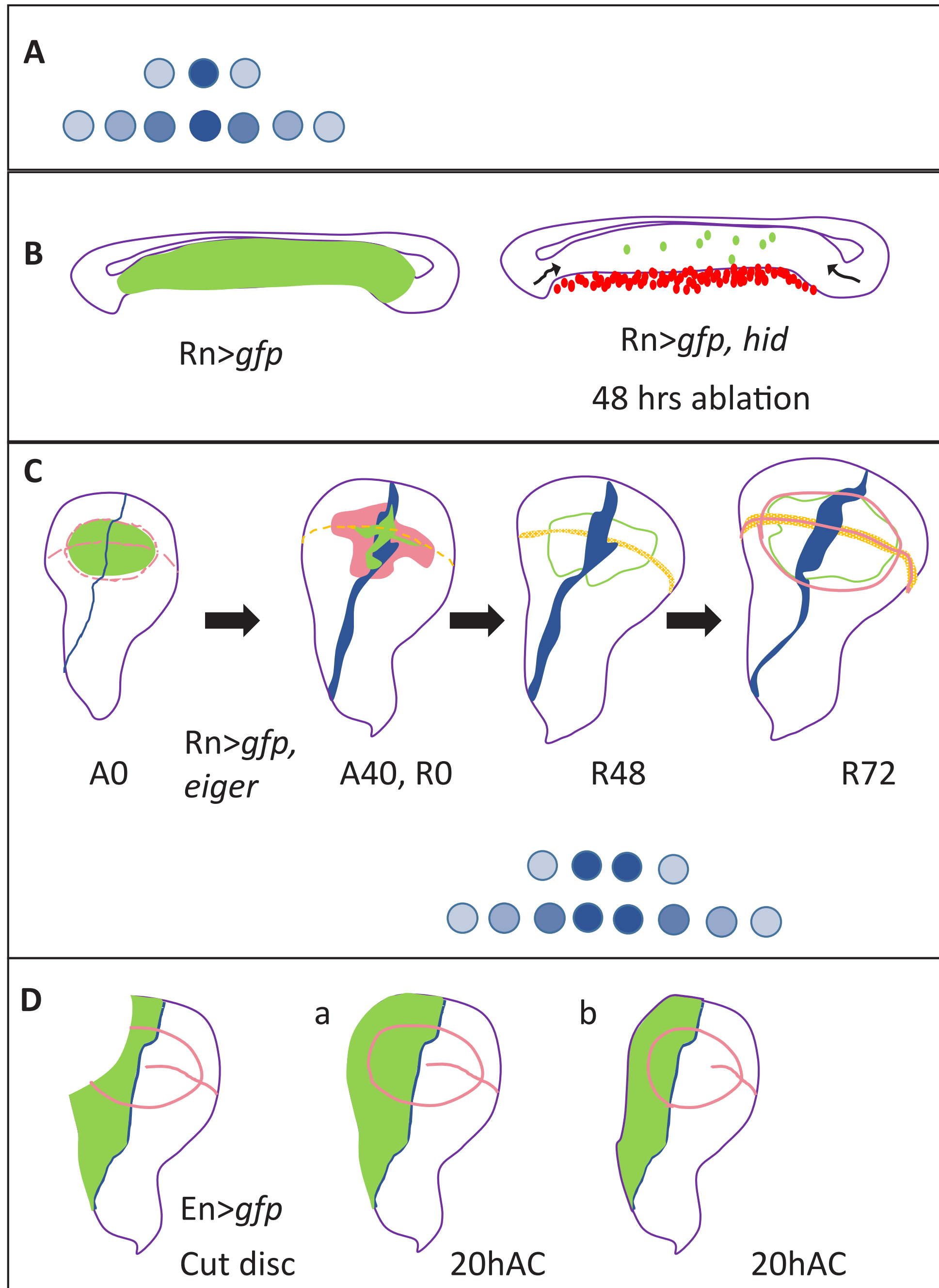

Fig. 3. Models of regeneration using *Drosophila melanogaster* wing discs as a system. *In all schematics, pink represents Wg; yellow, Vg; green, GFP. The Dpp protein gradient is represented by blue circles in (A,C), and the synthesis domain (marked by dpp-lacZ) as a blue stripe in (C).* **(A)** *A schematic adapted from Bryant and Simpson (1984) illustrating the intercalation of positional values model. Here dark blue represents the centre of the disc where the Dpp protein gradient is at its highest, light blue represents the edge of the wing pouch, where the Dpp gradient is at its lowest. At early stages (upper row), or when different positional values are juxtaposed due to injury, the juxtaposition of these disparate values promotes proliferation such that intermediate values can be generated (lower row). In* **(B,C),** *the Gal4-UAS system is used to drive expression of death inducing genes in the wing pouch in combination with the temperature sensitive Gal80ts that inhibits Gal4 at its permissive temperature and so allows temporal control of the expression of target genes.* **(B)** *A cross section of a wing disc showing the domain of expression of* rotund*-Gal4 (green) on the left and the result of 48 hours of ablation by expressing* hid *in this region on the right. The dead cells (red) have extruded out of the epithelium on to the basal side, leaving the epithelial integrity intact and pattern undisturbed (not shown). A small proportion of the* hid *expressing cells (green dots) still remain in the epithelium (Herrera* et al.*, 2013). The arrows indicate the direction of migration of cells from outside the damaged domain.* **(C)** *When Eiger is expressed in the* rotund *domain (Smith-Boulton* et al.*, 2009), the wing pouch region is heavily folded 40 hours after ablation and pattern is disturbed and gradually restored. At the start of ablation (A0) the discs are at early 3rd instar stage, the Wg expression pattern is just emerging (dashed pink lines). At 40 hours (A40, R0) after ablation and the start of regeneration, Wg is heavily upregulated (pink zone), Vg is downregulated (dashed yellow line) and the* dpp-lacZ *stripe is slightly expanded (dark blue distorted stripe). After 48 hours of the regenerative period (R48), Wg is downregulated in a proportion of discs, Vg expression is slightly higher and the* dpp-lacZ *stripe is expanded. After 72 hours of regeneration (R72), Wg expression looks close to normal and Vg expression has increased, though the* dpp-lacZ *stripe is still expanded and the wing pouch looks still folded. The blue circles below show how the expanded zone of* dpp *synthesis could influence positional information in the regenerating disc. Since there are extra positions of high Dpp levels (two dark blue circles in the upper row), there is no scope for intermediate values to emerge and so the broad band of Dpp high values remain in the lower row despite proliferation.* **(D)** *In the experiments by Diaz-Garcia and Baonza (2013), en-Gal4, UAS-GFP marks the posterior domain (green) that is excised by physical pressure (disc on extreme left, cut disc) during the 3rd instar. 20 hours after the cut (20hAC), the excised region is regenerated and proximal elements of Wg patterning is restored (pink ring). Distal expression at the D-V boundary is restored more gradually. In a proportion of discs (disc b), the proximal ring of Wg expression defines a smaller wing pouch region.*

in the wing pouch, Herrera *et al.* (2013) compared the effects of cell death induced by different genes. When the proapoptotic gene *hid* (head involution defective) is expressed, the dying cells extrude out from the basal side, whereas when the TNFα homolog, Eiger is expressed, dying cells are found both on the apical and basal sides of the epithelium. When the pro-apoptotic gene *reaper* is induced dying cells are initially only found basally, but later (48hours after induction) are found both apically and basally. Interestingly, expression of all these cell death inducers does not destroy the integrity of the epithelium as assessed by immunostaining for the apical epithelium marker Crumbs (Herrera *et al.,* 2013; Tepass and Knust, 1993; Morais-de-Sa *et al.,* 2010). But tissue architecture is severely disrupted with the latter two, resulting in folds in the normally flat wing pouch.

When Hid is expressed (Herrera *et al.,* 2013; see Fig. 3B here for schematic representation), dying cells extrude out onto the basal side, the epithelial integrity and tissue architecture is retained intact and patterning information consisting of components of the Wg and Dpp signalling pathways and also Nubbin and Rotund expression (molecular markers of the wing pouch) remains undisturbed. The proportion of dead cells increases with time of ablation and regeneration proceeds alongside ablation. At the end of 40 hours of ablation, about 30% of the regenerated tissue is made up of un-killed cells from the wing pouch region and the remaining 70% consists of cells that migrate to this region from the neighbouring undisturbed region. Proliferation is increased throughout the disc. Since ablation is gradual and is accompanied by simultaneous proliferation, this form of regeneration does not test the model of intercalary regeneration sufficiently. Morphogens can be expected to play as much of a role in normal growth as they do here (see Herrera *et al.,* 2013 for discussion). Interestingly in these experiments, RNAi against *dpp* was shown to impede regeneration, but RNAi against *wg* was shown not to affect regeneration. Since 70% of the repopulating cells were from outside the targeted domain, this system displays the remarkable ability of cells to migrate to and replace the dead cells without disturbing pattern or the integrity of the epithelium (Herrera *et al.,* 2013).

When Reaper is expressed for just 5 hours or 10 hours in a narrow domain using the *ptc*-Gal4 driver or the *salm*-Gal4 driver both of which include the region where *dpp* mRNA is normally expressed, a gap is created in the epithelium (see Fig. 1C for depiction of *salm* expression domain). This gap is resealed by proliferation and migration of cells from the unablated region (Bergantinos *et al.,* 2010). In this example of regeneration, *dpp* expression in the wing pouch is lost until the region is restored. *dpp* is not required for the immediate increase in proliferation in the region of death. The re-synthesis of *dpp* occurs due to signalling through Hh from the neighbouring posterior compartment. Once the *dpp* domain is restored, proliferation continues just as in a normal disc. Thus, although Dpp is required for the remainder of the growth period, the information gathered from these experiments about its requirement is not different from the information gathered about the requirement for Dpp during normal growth.

When Eiger is induced for 40 hours, the extent of cell death is massive and pattern is severely disrupted, with deep folds visible in the region of cell death (Smith-Boulton *et al.,* 2009). The relationship between patterning information and regeneration appears complex (Smith-Boulton *et al.,* 2009). Aspects of the pattern are lost and re-emerge during the process of restoration. It is not clear from these experiments when proliferation stops or slows-down. It is possible that the deep folds that have arisen are due to excess proliferation and the cells would have to be eliminated after restoration of pattern. Therefore we are unable to assess whether proliferation is guided by pattern information, stops before restoration of pattern or after restoration of pattern. Nevertheless the disruption and then restoration of pattern raises interesting questions regarding the role of positional information.

Wg is heavily upregulated by the end of the ablation phase and its domain of expression fills the wing pouch. Smith-Boulton *et al.,* (2009) suggest that this pattern of expression resembles the normal expression pattern of Wg during an earlier stage of development (2nd instar pattern) and that this shift to an earlier mode of development could aid in the regeneration (see pink zones in discs in Fig. 3C). However, it is likely that the ectopic Wg is induced by dying cells, just as has been observed during compensatory proliferation (Wells *et al.,* 2006; Perez-Garijo *et al.,* 2009). In a proportion of wing discs, Wg expression is downregulated during the restoration period and appears again after 72 hours of restoration (Smith-Boulton *et al.,* 2009; see Fig. 3C here, R48 and R72) also suggesting that a simple re-enactment of the normal dynamic expression pattern of Wg does not drive regeneration.

The stripe of *dpp* expression is maintained during the ablation period, but expands during the restoration period (blue stripe in discs in Fig. 3C) (Smith-Boulton *et al.,* 2009). It is not evident from these studies that the expanded stripe of *dpp* expression causes a specific alteration in the pattern of proliferation. If we were to now relate this process of regeneration to the intercalation of positional information suggested by Bryant and Simpson (1984), the expansion of the *dpp* expression domain, would mean that there is an increase in the number of positions ascribed to high *dpp* (dark blue circles in Fig. 3C). The positional intercalation model states that positions of intermediate values come in between positions of disparate values (see Fig. 3A). Since all the cells expressing high *dpp* would have the same positional values, this model leaves no scope for intermediate values to emerge in this region (Compare dark blue circles in Figs. 3C and 3A) and therefore one would have to look for other mechanisms to correct the expansion in the *dpp* stripe. These mechanisms would be unique to the process of regeneration as the *dpp* stripe does not expand in this manner during normal development. In summary, the restoration of pattern during this mode of regeneration is remarkable and emphasizes quite strongly its ability to reorganize after severe disruption (Smith-Boulton *et al.,* 2009). But, not enough has been revealed about the relationship between proliferation and the reorganization of pattern. Studying this relationship is particularly important since pattern is distorted before is it properly restored.

Excision of discs

The second method of studying regeneration is to cut discs and observe how they grow. In the earlier experiments of the last century, leg and wing discs were cut at different angles generating pieces either of equal or unequal size. The pieces were cultured in adult abdomens. Generally the larger piece regenerated the missing piece and the smaller piece generated a mirror image duplication of itself (Bryant, 1971; 1975). This close correlation between the filling-in of missing patterning information and size of the regenerated tissue suggested the link between pattern and growth (see Bryant and Simpson, 1984). Using this method, more

recent studies (Bosch *et al.,* 2005; Mattila *et al.,* 2005; Bosch *et al.,* 2008) have shown the involvement of the JNK pathway in the healing of the wound. These studies have also examined the patterns of proliferation accompanying wound healing. Diaz-Garcia and Baonza (2013) applied a unique method to break discs inside live larvae and then allow them to regenerate, without removing them from their original positions inside the larvae. They applied external pressure using tweezers to squeeze the larva in the region where the discs are present (Diaz-Garcia and Baonza, 2013). They followed the emergence of pattern during the reconstruction of the ablated regions and therefore these experiments will be discussed here. This kind of ablation does not result in extensive cell death. But, cell proliferation was observed to increase adjacent to the excision, both in the compartment that was excised (forming a blastema) and in the neighbouring compartment. Due to technical difficulties, the discs could be broken only during the third larval instar. The extent of regeneration depended on the relative size of the fragment removed and the time of ablation (Diaz-Garcia and Baonza, 2013).

Interestingly, in response to the excision, pattern is disrupted in a large region adjacent to the break beyond the blastema and even in the neighbouring compartment (Diaz-Garcia and Baonza, 2013). This is observed by the absence of Wg expression and markers of vein and intervein regions. Pattern is gradually restored, but reconstruction of the pattern occurs even when proliferation is inhibited by the expression of the cyclin A inhibitor, Roughex throughout the restoration period. During restoration of the pattern, proximal elements of the Wingless expression pattern (the rings surrounding the pouch region) are restored before the more distal expression at the D-V boundary (see Fig. 3D, disc a). Often the proximal pattern elements are restored when the regenerated region is still small. This region now defines a smaller than normal wing pouch (Fig. 3D, disc b). These scaled-down wing pouches give rise to small normally patterned adult wings (Diaz-Garcia and Baonza, 2013). This is interesting because one could question why these wing pouches did not grow to their normal size. If the wings are normally patterned, one can expect that the morphogen gradients that normally pattern the wing have scaled their gradients down to fit the smaller wing pouch. But a gradient that fits a smaller wing pouch should represent a younger disc and therefore should promote further growth. If growth in these discs is not promoted it could mean that growth has stopped disc-intrinsically through a mechanism that is independent of the patterning machinery. It remains possible however, that the small wing pouches would have given rise to normally sized wings if provided with an artificially extended developmental time (discussed by Diaz-Garcia and Baonza, 2013). Thus, we do not know if they have stopped growth at the small size because of the onset of pupation, whose timing in this case is not controlled by the disc.

Among the above models of regeneration in wing discs, the one by Diaz-Garcia and Baonza (2013), could help establish the role if any of the renewal of pattern information in directing growth. However, these results do leave us with the possibility that pattern information on its own cannot direct the extent of required growth and they provoke a search for alternative modes of growth control that are independent of the patterning process.

Mixing fast and slow growing cells in a compartment

As noted above, when patches of slow growing cells are generated within wing discs, there is compensatory growth retardation in the normal wild type tissue (Mesquita *et al.,* 2010; Boulan *et al.,* 2019). However, in these experiments, Mesquita *et al.,* (2010) observed that when they generated small randomly distributed patches of slow growing cells (using a different kind of driver from the region specific Gal4 drivers), these slow-growing cells are always either eliminated or occupy a much reduced space. The inability of small patches of slow growing cells to survive in the vicinity of faster cells is called cell-competition. Cell-competition was first observed in *Drosophila* imaginal discs using mutations in Rp genes (Morata and Ripoll, 1975; Simpson, 1979; Simpson and Morata, 1981; Moreno *et al.,* 2002; reviewed in Baker, 2017). As mentioned above, larvae that are haplo-insufficient for an Rp gene (*Minutes*) grow slowly with an extended developmental time, but the flies that eclose display a close to normal adult size. However, when small patches of these *Minute* cells are made to grow in otherwise wild type imaginal discs, their representation in adult tissue is much lower than would be expected purely from a difference in growth and cell division rate between wild type and mutant cells. This reduction in size of mutant clones is due to cell death that occurs more frequently at the boundaries between wild type and *Minute* tissue (Simpson and Morata, 1981; Li and Baker, 2007).

Cell-competition has since been observed in a larger range of contexts and also in other organisms (reviewed in Nagata and Igaki, 2018; Vincent *et al.,* 2013). Cells get out competed due to mutations that reduce either the levels of growth regulator signalling, morphogen signalling, protein translation rates or levels of endocytosis (Böhni *et al.,* 1999; Vincent *et al.,* 2011; Lee *et al.,* 2018; Thompson *et al.,* 2005). Over-growing tumour cells get out-competed when surrounded by wildtype cells (Igaki *et al.,* 2009). In contrast, wild type cells get out-competed when they grow in the vicinity of cells that over-express certain growth regulators, such as Myc, activated Ras (Ras^{V12}), Wg and downstream effectors of the Hippo signalling pathway (de la Cova *et al.,* 2004; Moreno and Basler, 2004; Karim and Rubin, 1998; Tsuboi *et al.,* 2018; Vincent *et al.,* 2011). This latter form of competition is called super-competition. Cell-competition is also observed amongst stem cells and cells in culture (reviewed in Johnston, 2009; Senoo-Matsuda and Johnston, 2007).

The mechanisms responsible for cell-competition vary with context and have been reviewed elsewhere (for example, Nagata and Igaki, 2018; Vincent *et al.,* 2013). In this article, I will discuss why cell-competition is important from the point of view of organ size-sensing. Why cell-competition is important in this context is that despite the presence of fast and slow growing cells, the target size is not changed. Martin *et al.,* (2009) argue that the presence of fast and slow growing cells in a tissue should push the limits of the size control mechanism. The implications of cell-competition are that growing cells fill up a defined space and even if the rates of growth of these cells vary, this variability does not impact on that space. If it did impact on that space, then there would be indentations or inward distortions where cells grew slower and bulges or outward distortions where cells grew faster (see Fig. 4A). Thus, faster growing cells must spread out to occupy spaces that are kept open because they are not occupied by the neighbouring slower growing cells. It follows that if cells fill up a space, then this space would appear to be predefined and hence sensed. The observation that cell-competition respects compartment boundaries, meaning that the space that is defined is a compartment and

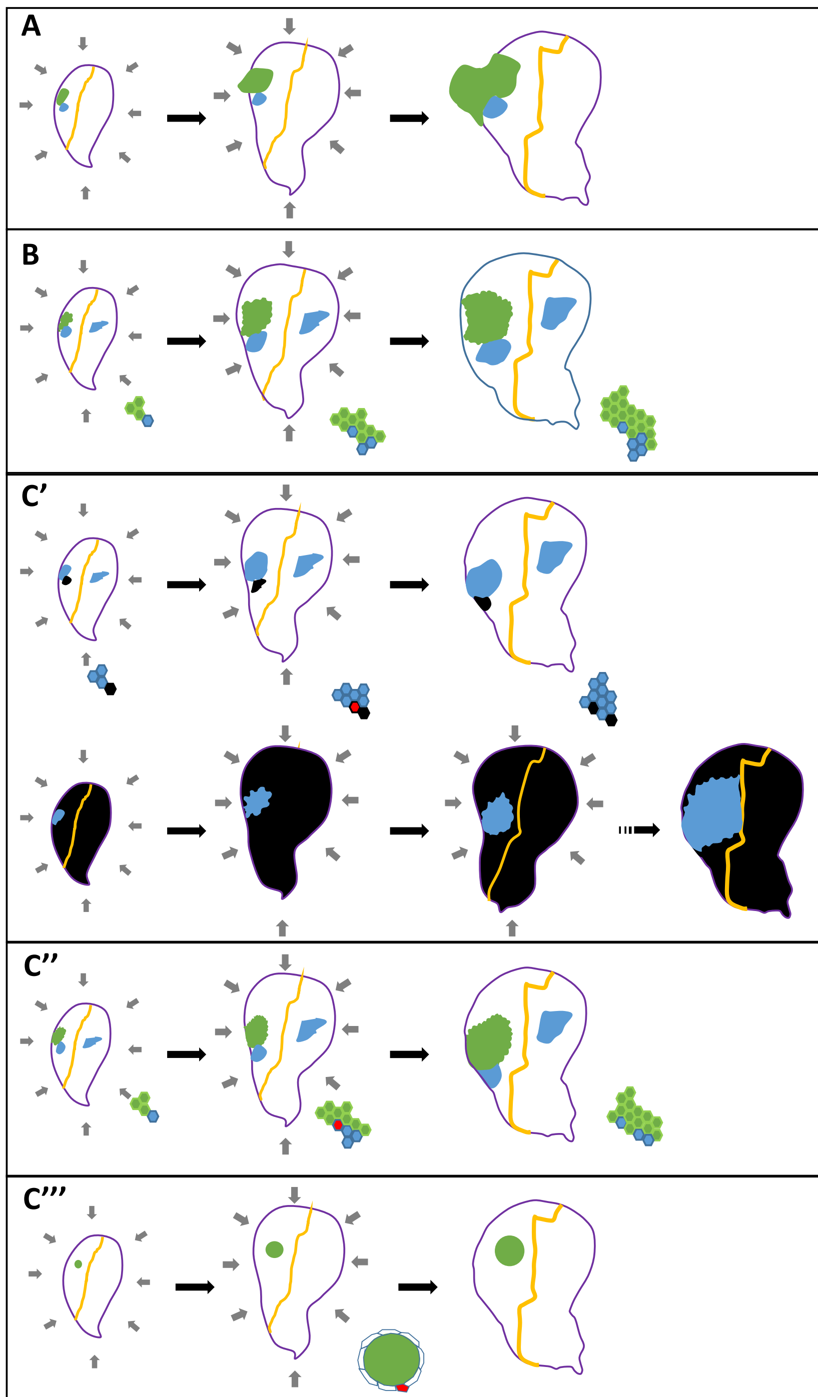

Fig. 4. The consequences of mixing fast and slow growing cells in the same disc. *Each panel represents a different outcome depending on whether the fast growing cells are able to overcome the mechanical forces that normally feedback on faster growing cells in a growing disc. In all panels the disc on the left represents a second instar tear drop shaped stage, the disc on the extreme right represents a disc just before pupation and the discs in the middle represent early to mid-third instar stages. The grey block arrows surrounding the younger discs represent mechanical forces that are involved in maintaining the shape of the disc, preventing distortion. The yellow line represents the A-P compartment boundary, with the posterior compartment on the left in all discs. The discs contain clones induced during the second instar. In the green clones genes are overexpressed, the blue clones represent wild-type and the black clones represent mutant cells.* **(A)** *Situation in which overexpressing clones are able to overcome the mechanical forces maintaining the shape of the disc and bulge out.* **(B)** *Situation exemplified by over expression of PI3Kinase or CyclinD/CDK4 where the clone (green) does not bulge out of the disc, is able to expand by infiltrating into the disc without killing the neighbouring cells, thus causing a net increase in volume. Note the expansion in the area of the P compartment. The wild type clone (blue) in the anterior compartment is shown to indicate that wild type clones in both compartments are growing at a similar rate. The sketch below shows details of the faster green cells intercalating between the slower wild type cells.* **(C', C'', C''')** *Situations where cell-competition occurs and disc shape and size is not altered.* **(C')** *Two situations wherein, wildtype cells grow next to slow growing mutant cells, exemplified by mutations that reduce Rp function and insulin signalling mutants. A mutant clone is outcompeted, but the wildtype sister clone grows at its normal rate in the discs above. The discs below describe the growth of wildtype clones (blue) in slow growing* Minute *discs (black region in discs). The discs have an expanded developmental time (dashed arrow) and allow the wildtype clone to overtake the compartment even though it is growing at its normal rate. The sketch of cells show the intercalating wildtype cells and death of the mutant cells (red), resulting in jagged boundaries and fragmentation of mutant clones (see text for literature references).* **(C'' , C''')** *Super-competition.* **(C'')** *dMyc overexpressing cells intercalating between wildtype cells causing cell death at the boundaries.* **(C''')** *Yki or Ras^{V12} overexpressing clones that sort out and round up resulting in mechanical strain on the neighbouring wildtype cells (up to 15μm). These cells are stretched in an anisotropic manner, such that their long edges align with the clone border. This anisotropy results in cell death (red cell).*

not the entire imaginal disc, added emphasis to the view that the unit of size control is the compartment and not the entire disc (Crick and Lawrence, 1975; see discussion in Simpson, 1979).

However, to establish that size-sensing enables cell-competition, we have to provide evidence that the mechanisms that drive cell-competition sense size. There are two reasons to argue that is not the case. One is that cell-competition is driven by local cell interactions. As discussed below there is no evidence that these interactions are influenced by size-sensing. Secondly, the space occupied by a disc is itself not rigid. Both these arguments are illustrated in Fig. 4, which illustrates the different outcomes of fast and slow cells growing simultaneously in wing imaginal discs.

Reconciling local cell interactions with global space constraints

The experiments by Simpson and Morata (1981) showed that *Minute* cells needed to be in contact with non-*Minute* cells to be eliminated. In keeping with this observation, the mechanisms uncovered so far for the various kinds of cell-competition, reveal local cell interactions (reviewed in Nagata and Igaki, 2018; Vincent *et*

al., 2013), though the distance at which the cell death occurs may vary with context (see Levayer, 2018). In the rest of this discussion and in keeping with current trends, the faster growing cells will be referred to as the winners and the slower cells as the losers.

First let us understand the nature of the space defined by a disc. It is reasonable to expect that the mechanical properties of the organ help define that space during development. The question that is important here is to what extent do these mechanical properties allow distortion. The A-P compartment boundary is clearly defined throughout development and the D-V boundary from the middle of the third instar onwards. These boundaries establish clear demarcations in space as cells do not traverse them. But, the remaining boundaries of the disc, those that establish the shape cannot be rigid. They need to be flexible to allow growth. We can ask how rigid disc space is, by analysing what kinds of manipulations deform this space.

It is useful to first review what is known about the mechanical strains experienced by disc cells as they grow. There have been experiments and theoretical analyses on the mechanical forces acting within a disc (Shraiman, 2005; Farhadifar *et al.,* 2007). The mechanical properties of an epithelium are in part determined by the adherens junctions near the apical surfaces of cells and the actin cytoskeleton that connects these junctions. The disc consisting of a pseudostratified epithelium has defined mechanical properties that determine the average number of edges (neighbour to neighbour contact surfaces) of the constituent cells and the state of mechanical tension (see Farhadifar *et al.,* 2007; Gibson *et al.,* 2006 and references therein). The cells of the wing disc epithelium have a disordered geometry that allows asynchronous divisions, but generally minimal movement (Gibson *et al.,* 2006). At the same time, global mechanical forces play a role in orienting cells and cell division in the periphery of the wing pouch region (Mao *et al.,* 2013; le Goff *et al.,* 2013). It is known that the compartment boundaries are under increased strain when compared to the rest of the disc (Landsberg *et al.,* 2009).

If disc rigidity was high, the forces that maintain rigidity would not be overcome by any kind of fast growing cells. In Fig. 4, the forces that maintain overall shape are denoted by grey block arrows surrounding the entire disc. The implication of these arrows is that there is a tension on the edges of a tissue that prevents distortion along the x and y axes. However, this is not meant to imply that the tension is greatest along the edge of the disc. But, if tension along the x and y axes are not dissipated within the disc, they would impinge on and distort the edge. Fast growing cells that easily overcome local mechanical forces, create a bulge as depicted in Fig. 4A. Tissue distortions are observed through certain kinds of gene manipulations. Wg overexpressing clones form bulges in the hinge and periphery of the wing pouch (see Fig. 2B in Vincent *et al.,* 2011). Cells expressing Ras^{V12} bulge out of the wing disc epithelium (Karim and Rubin, 1998). The growth promoting microRNA *bantam,* which is a target of Yki, creates invaginations in disc epithelia when over-expressed in clones (Pan *et al.,* 2016). Overexpression of Yki in clones also causes local distortion in the epithelium (le Goff *et al.,* 2013; Mao *et al.,* 2013). As mentioned above, Yki is downstream of the Hippo signalling pathway and thus responds to the state of mechanical tension. *bantam* overexpression can drive overgrowth even in the absence of Yki and so is no longer responsive to mechanical feedback received through the Hippo signalling pathway. Thus, this is a good example of distortions arising from fast growth that simultaneously overcome the mechanical forces that maintain the shape of the disc. Levayer *et al.,* (2015) showed that reducing levels of Myosin II heavy chain in cells at the A-P boundary was sufficient to increase contact between cells on either side of this boundary, making the boundary wavy. These findings together show that disc-shape can be distorted under certain conditions. In some of the above examples, the distortion is due to loss of control by mechanical forces (*bantam* and Yki overexpressing clones) and in some due to decreased mechanical tension (reduction in Myosin II heavy chain in cells at the A-P boundary).

Certain fast growing cells can expand the disc in the x and y axes without distorting shape or the integrity of the compartment boundaries. This is illustrated in Fig. 4B where fast growing cells exert in theory at least, only a gentle pressure enough to counteract the mechanical forces maintaining the shape of the tissue. This pressure is sufficient to displace or push the surrounding wildtype cells, causing an overall expansion of the total space occupied, but without creating any distortion in shape (see Fig. 4B). This gentle pressure on the surrounding cells could take the form of a mechanism that allows fast growing cells to intermingle and spread amongst slower growing cells. If the pressure of fast growth is dissipated by cell intercalation, then both fast and slow growing cells can co-exist in the same tissue in the same plane. The number of cells or the total mass of the tissue will increase because the slower wild type cells are not dying and the fast cells add more space. Clones of cells overexpressing CyclinD/CDK4 activity or the class 1A PI3Kinase, Dp110 in wing imaginal discs do not alter the growth or survival of their neighbours and increase disc and wing size (de la Cova *et al.,* 2004). Levayer *et al.* (2015) have shown that differences in Phosphoinositol (3,4,5) tris-phosphate (PIP3) levels facilitate intercalation, but do not give rise to death of the cells with lower PIP3 levels. Thus, the discs are larger at the end of larval life and they give rise to larger wings than do the control discs (de la Cova *et al.,* 2004). Thus, the presence of faster growing cells can alter the target size in these situations. The growth of these clones exemplify another mode of distortion of disc space where size is altered, but shape is not.

Cell competition on the other hand does not distort disc size or shape. Cells involved in cell-competition display two kinds of behaviours. One in which the faster cells are able to intercalate between the slower cells as shown in Fig. 4C' and 4C'', resembling the cells in Fig. 4B. The other kind of behaviour is illustrated in Fig. 4C''', where faster cells sort out from the remaining slow cells forming round clones. In both these kinds of behaviours, loser cell elimination compensates for the increased space occupied by the winners. In the case of super-competition, the cell death would prevent an increase in organ size. Consistent with this, when cell death is reduced in dMyc dependent super-competition, the discs overgrow (de la Cova *et al.,* 2004). It is not known if, in the second form of cell competition listed above, suppression of cell death would lead to overgrowth (Tsuboi *et al.,* 2018).

The first form is exemplified by Rp dependent cell-competition (Fig. 4C'; Simpson and Morata, 1981; Li and Baker, 2007; Levayer *et al.,* 2015) and dMyc dependent super-competition (Fig. 4C''; Johnston *et al.,* 1999; de la Cova *et al.,* 2004; Moreno and Basler, 2004; Levayer *et al.,* 2015). Here too, the ability to intercalate between the slower growing cells is due to higher levels of PIP3 expression in the faster cells (Levayer *et al.,* 2015). However, in addition to expressing lower levels of PIP3, the slower cells also

upregulate other genes that lead to their cell death, making them losers. Loser cells are found to upregulate *brinker* _ *brinker* is normally inhibited by Dpp signalling and drives cell death in regions of high Dpp signalling (Martin *et al.,* 2004; Moreno *et al.,* 2002; Moreno and Basler, 2004). Loser cells also upregulate Toll-receptor signalling and are subject to *spätzle* expression by winner cells (these are components of the innate immunity pathway and drive cell death in Rp and dMyc subjected losers; Meyer *et al.,* 2014). The $Flower^{Lose}$ isoforms of the Ca^{2+} channel are also upregulated in loser cells and are responsible for their death (Rhiner *et al.,* 2010; reviewed in Nagata and Igaki, 2018). There is no evidence that upregulation of these genes require long-range cell interactions. Indeed Levayer *et al.,* (2015) have shown that increasing cell sorting between Rp and dMyc dependent winners and losers by driving expression of E-Cadherin or a constitutively activated form of the regulatory light chain of Myosin II, decreased cell contact between the winners and losers. This decreased cell contact decreased elimination of the losers.

The second form of cell-competition is exemplified by Ras^{V12}, Yki and *bantam* dependent super competition (Levayer *et al.,* 2016; Pan *et al.,* 2016; Tsuboi *et al.,* 2018). The round shape and faster growth of these clones exert a force on their neighbours. This force increases the anisotropy of the slow growing neighbours up to a distance of 15 μm from the fast clone boundary and this increased anisotropy kills the neighbours (Tsuboi *et al.,* 2018, also see Shraiman, 2005; le Goff *et al.,* 2013; Mao *et al.,* 2013).

As elaborated above, the mechanisms that drive cell-competition consist of essentially local cell interactions. Either loser cells succumb to the effects of anisotropic stretching in the vicinity of fast growing winner clones, or loser cells express molecules that lead to their death. There is no evidence for an influence of global space-sensing on these interactions. The idea that cell competition reveals the existence of a pre-defined space is not supported. Moreover, the space occupied by a disc is not rigid, but reveals a certain degree of flexibility. The degree of rigidity appears sufficient to maintain shape, while allowing growth, as illustrated by the ability of PI3Kinase and CyclinD/CDK4 over-expressing clones (de la Cova *et al.,* 2004) to alter size but not shape.

de la Cova *et al.* (2004) suggested a different perspective on what cell-competition tells us about size control. They proposed that cell-competition is a disc intrinsic mechanism of size control. They showed that supressing cell death in otherwise wild type discs through the expression of the pan-Caspase inhibitor, p35 did not alter the average final size of wing discs, but increased the variance, in other words the deviation from the mean. There is a little cell death that occurs during normal development (approximately 1.4% TUNEL labelled cells per disc at any given time and higher levels just after the larval moults; Milan *et al.,* 1997). It is not clear why there is this cell death and also whether cell-competition is responsible for it as it is not clear if cell-competition occurs during normal development. However, since cell-competition results in cell death, supressing cell death should supress cell-competition. In the same work, de la Cova *et al.,* (2004; also see Johnston *et al.,* 1999) had observed that discs that contained dMyc overexpressing clones were of a normal size because of cell-competition. But, supressing cell death in these discs increased their size. Putting all these findings together they argued that cell-competition was necessary to achieve appropriate size.

The above findings imply that supressing cell death in otherwise wildtype discs allows the propagation of slower growing cells and faster growing cells. We would have to assume that variation in final size is increased because at the time of pupation, the faster and slower growing cells would have grown to different extents. A co-assumption here is that disc size has not determined the time of pupation. A disc containing a higher proportion of slower growing cells, would give rise to a smaller disc. The larger than normal

TABLE 2

A RE-EXAMINATION OF THE ARGUMENTS MADE SUPPORTING THE EXISTENCE OF AN ORGAN SIZE CHECK-POINT

Experiments performed or question addressed	Observation	In support or not	Why?
Experiment: Transplantation of immature imaginal discs into adult abdomens	Discs do not over grow	Not clear	Because there is the possibility that discs grow too slowly to overgrow (Bryant and Levinson, 1985)
Experiment: Observations on the growth of wildtype discs inside slow-growing larvae	Wildtype discs do not overgrow	No	Wild type tissue (discs and compartments) generated in *Minute* larvae grow faster than *Minute* discs, but do not stop growing even towards the end of the extended larval life (Martin and Morata, 2006) When only the wing pouch is *Minute,* wild type larval and imaginal disc tissue are co-ordinately growth retarded (Parker and Shingleton, 2011; Boulan, et al. 2019)
Question: Is disc size sensed to enable growth coordination	1) Absence of discs does not prevent pupation.	No	1) There is no positive signal secreted in proportion to disc size Simpson, et al. (1980)
	2) Damage to discs delays pupation and the delay is mediated by the release of Dilp8 from damaged discs, acting as a negative signal that delays pupation when discs are not "ready"		2) Dilp8 is released as a response to stress (Colombani, et al 2012; Garelli, et al 2012; Boulan, et al. 2019)
	3) Normal Dilp8 levels in wing discs decrease during mid third instar; *dilp8* mutants show fluctuating asymmetry (FA); removing response to Yki induces FA.	Not clear	3) It is not clear if *dilp8* synthesis under the control of Yki is dependent on discs reaching a particular size or a particular growth rate (Boone, et al. 2016).
Experiments: Examining the correlation between the emergence of pattern and patterns of proliferation during regeneration	The associations are complex and dependent on the nature of injury.	Not clear	In excised discs, following the growth of the smaller than normal wing pouches defined by the proximal rings of Wg expression (Diaz-Garcia and Baonza, 2013) should help resolve the issue of whether growth slows down before pattern is restored.
Experiments: Mixing slow and fast growing cells	Certain fast growing clones that do not induce cell competition (overexpressing PI3Kinase or CyclinD/CDK4) can increase disc size (de la Cova, et al. 2004).	No	Cell competition is a local cell-cell interaction phenomenon. There is no evidence that it requires size sensing (References in text).

This table provides a summary of the arguments made in this paper. These arguments are elaborated in the text.

disc would have had more of the faster growing cells. The purpose of cell death during normal development would be then to maintain cells that have a similar average growth rate. The mechanisms of cell-competition that have been unravelled so far explain how slower cells can be eliminated, but not how faster cells can be eliminated (reviewed in Nagata and Igaki, 2018). Thus, it is not clear whether the suppression of cell death in these experiments represents the suppression of cell-competition or something in addition to cell-competition. Thus, the role played by cell-competition in determining size through the reduction of size variation is not evident.

An alternative model for size control that does not require size to be sensed

The arguments made in this paper on whether size is sensed or not are tabulated in Table 2. While the jury might still be out, it is worthwhile considering the possibility that size is indeed not sensed. Can size be controlled without it being sensed? An alternate means of thinking of growth control might be to consider the target of the organ-intrinsic growth-control mechanism to be simply the appropriate and timely slowing of growth. In such a model, growth is expected to be regulated at the level of cells, such that a single cell or a group of cells slow down their growth in a timely manner, with no awareness of the size of the disc.

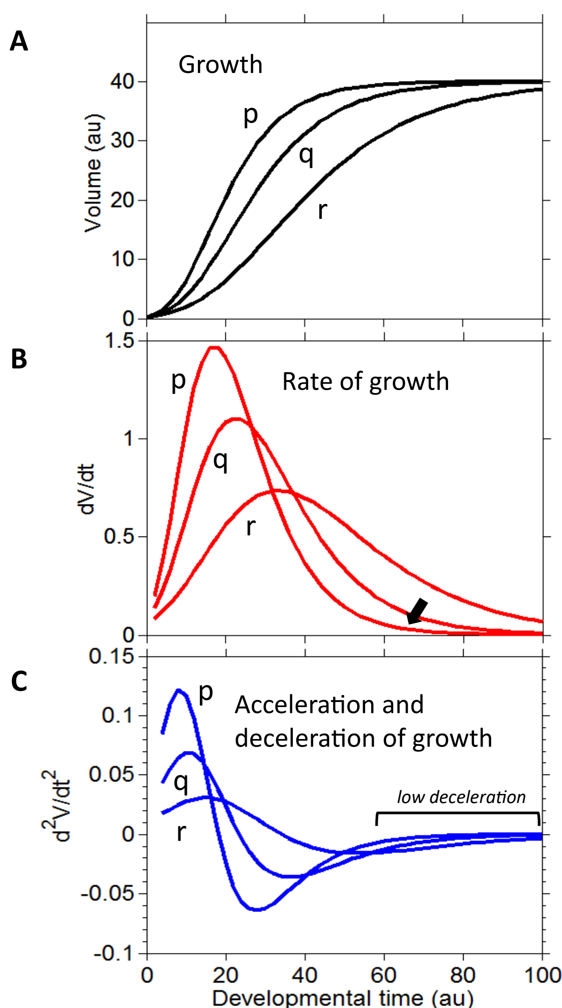

Fig. 5. Examining the dynamics of growth using the Gompertz equation as an example: *Hypothetical growth curves are plotted* **(A)** *using the non-linear Gompertz equation displaying volume against developmental time in arbitrary units (au, see text for equation), with a = 40, b = 5, c = 0.1 (p), 0.075 (q) and 0.05 (r). The derivatives of these curves are plotted in* **(B)** *and the second derivatives in* **(C).** *The block arrow in (B) indicates that growth rate is reaching zero in this curve. The black square bracket in (C) indicates the stage when deceleration is low in all three curves, p, q and r.*

Animal organ growth curves are generally sigmoid, with a phase of acceleration and a phase of deceleration. In Fig. 5A, the Gompertz non-linear equation ($Y= ae^{-be[-ct]}$) has been used to plot three hypothetical growth curves with arbitrary units (au) of volume plotted against au of developmental time. The Gompertz equation describes an asymmetrical form of a sigmoid curve, in which the point of inflection is during the first half of developmental time (see German and Meyers, 1989). The constant “a” defines the final target size, or the maximum size that is achieved. The constant “b” sets the displacement along the x axis (developmental time). If b is larger, it means that the lag time before growth starts to accelerate is longer. The constant “c” sets the growth rate. If c is larger, growth accelerates faster and reaches the peak growth rate at an earlier time of development. Gompertz curves have been used to describe tumour growth and the growth of bacterial cells in suspension (Laird, 1964; López *et al.,* 2004). These forms of growth display saturation often due to the depletion of resources or space. However, Gompertz curves have also been fitted to animal organ growth data although animal organ growth does not saturate due to depletion of resources or space (see Stewart and German, 1999; Reichling and German, 2000 for examples). They show slowing of growth due to organ intrinsic mechanisms as evident from the organ transplantation experiments (reviewed in Bryant and Simpson, 1984; discussed earlier in this paper).

The curves plotted in Fig. 5A are obtained by varying the constant “c” of the equation for each curve, but by retaining the same a and b values for all curves (see legend to Fig. 5A for details of the values used). Thus, the target size is the same and the time spent in the lag phase is the same, but the rate of growth varies between the curves. In all three curves the target size of 40 au is reached approximately between 70 and 100 au (developmental time). For curves p and q, this target size is reached earlier at about 75 au (developmental time). The derivatives below (Fig. 5B) show the time at which growth rate peaks for each curve, p, q and r. This time varies for each curve along with the length of time spent in slowing-down. Curve “p” which represents fast early growth, also slows rapidly (observe the steep fall in the red curve, p after reaching the peak) and reaches close to zero growth rate before the other curves do so (block arrow in Fig. 5B). Curve r represents slow early growth and takes much longer for its growth rate to reach a value close to zero (this time is beyond the developmental time shown here). The blue curves in Fig. 5C represent the second derivative, which tells us the rate of change of rate of growth (acceleration and deceleration). These blue curves, p, q and r also reveal clearly that a faster rate of acceleration correlates with a faster rate of deceleration. Notice the steep rise and fall of blue curve p when compared to the other blue curves, q and r. Importantly, one finds that all three blue curves reveal a similar length of time spent towards the end of the curve (between 60 and 100 au, developmental time), when changes in growth rate are at the slowest (indicated with a black bracket labelled ‘low

deceleration', Fig. 5C). I propose here that the target of the size control mechanism is to reach this phase of growth during which, deceleration is low (black bracket in Fig. 5C).

The curves shown in Fig. 5 depict organ growth. In principle, these dynamics could be applied to growth at a local level. So long as all cells followed the same mode of regulation, a slow-down would occur without the need to sense size. Such control of growth at a "local level" could involve rate-dependent negative feedback mediated by positive and/or negative growth regulators whose effects are experienced independently, within individual cells. These growth regulators might also act non-cell-autonomously at a short range. In this case, the term "local level" would imply a cell and its neighbours. Through such a mechanism growth would slow during regeneration without a need for input from patterning information. If target size is not sensed, and reaching this phase of low deceleration is instead, the target of the size control mechanism, we might have an explanation for why wildtype discs and compartments in *Minute* larvae continue to proliferate until the end of larval life (Martin and Morata, 2006). Despite having grown faster that the *Minute* discs and despite the greatly extended developmental time, they do not stop at a specific size because that size is not defined.

It is reasonable to consider that the final slow deceleration is brought about by a cumulative effect of the slowing down process that has started at the point of inflection, when growth rates reach their peak and then start to slow down. The point of inflection in wing imaginal disc growth curves has not been identified. Understanding size control would then need to shift its focus to understanding what causes the point of inflection. And how early rates of growth would influence the rates of slow down. According to this model, tissue or cells that grow faster earlier would slow down faster, resulting in a slower growth rate towards the end of the growth period, than that of the slow starters. In principle, the factors that determine the rates of ribosome biogenesis could play a role in bringing about such a cumulative effect on growth slow-down. It is known that levels of ribosomes are high in rapidly growing cells (Alberghina and Sturani, 1975; Daskal and Sinclair, 1975; Lempiaeinen and Shore, 2009). Ribosome assembly being a complex process depending on stoichiometry of the different components, a slight shift towards a slow-down in synthesis could lead to an increasing rate of slow-down in growth. Within a cell or group of cells, regulation of protein synthesis during the acceleration and subsequent deceleration phases can be expected to be complex, until the cells reach a phase when deceleration is low, steady and the cells are "cruising".

Such a mode of growth control is at the level of growth rates and does not sense changes in size. Thus, artificially altering size at the start of the growth period should alter final size. I am currently testing if this is true by using growth modulators to alter size only at the very start of the growth period. I have also begun to examine how early rates of growth influence the rate of slow down.

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