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REVIEW ARTICLE

Measuring dimensions: the regulation of size and shape

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SUMMARY

Over many years evidence has accumulated that plants and animals can regulate growth with reference to overall size rather than cell number. Thus, organs and organisms grow until they reach their characteristic size and shape and then they stop – they can even compensate for experimental manipulations that change, over several fold, cell number or average cell size. If the cell size is altered, the organism responds with a change in cell number and vice versa. We look at the Drosophila wing in more detail: here, both extracellular and intracellular regulators have been identified that link cell growth, division and cell survival to final organ size. We discuss a hypothesis that the local steepness of a morphogen gradient is a measure of length in one axis, a measure that is used to determine whether there will be net growth or not.

Key words: Growth control, Angiosperm, Vertebrate, *Drosophila*

INTRODUCTION

Why are mice smaller than men? Why are pea pods smaller than pumpkins? Why are arms shorter than legs? What controls the progress of growth and, when the proper size is attained, what tells organs and organisms to stop growing? When looking for answers to these questions, the intuitive response is to search for mechanisms that count cell divisions or add up cell number. Yet there is persuasive evidence that size itself is measured and monitored – for example, despite manipulation of either cell proliferation or cell size, the resulting organs and/or organisms often attain the normal size. They may consist of fewer but larger cells, or more numerous but smaller cells. Thus it seems that, in at least some plants and animals, growth is regulated by correlates of absolute dimensions rather than correlates of cell number.

We summarise evidence from different animals and plants. We then discuss the *Drosophila* wing because it has been studied in greatest depth. We entertain the hypothesis that the regulation of size in the wing depends on the sensing of dimension (independently in different axes) and that some correlate of dimension is transferred to individual cells; this affects cell growth, the cell cycle and cell survival. We consider whether the dimension-sensing mechanism could be based on the gradients of morphogens* that pattern the wing.

*A morphogen is a molecule that usually spreads from a localised source; it forms a graded distribution, and the concentration (the scalar of the gradient) at a point or points some distance from the source determines the local differentiation of the cells. Morphogens may act directly on responding cells, and they may also initiate the production of secondary morphogens (see, for example, Lawrence and Struhl, 1996).

Further aspects of growth control in animals have been recently reviewed by others (Edgar and Lehner, 1996; Serrano and O'Farrell, 1997; Neufeld and Edgar, 1998; Conlon and Raff, 1999; Stern and Emlen, 1999; Milán and Cohen, 2000). The relationship between the cell and the organism during plant development has been discussed by Kaplan and Hagemann (1991) and Kaplan (1992).

EVIDENCE FOR THE REGULATION OF SIZE

(1) Animals

Variation in ploidy

The first evidence that animals can monitor dimension came from haploids and polyploids. For a given cell type, cell size is usually proportional to ploidy. Hence haploid cells are about half the volume of diploid cells, diploid cells are about half the volume of tetraploid cells, and so on.

The ploidy of newts and salamanders can be manipulated to produce animals with chromosome complements ranging from haploid to pentaploid (Frankhauser, 1945). In all cases, animals with unusual ploidy grow to the normal (diploid) size but contain very different numbers of cells. Thus mature tetraploid salamanders (*Amblystoma mexicanum*) look little different from diploid ones despite having half the number of cells. When plodding through mazes, tetraploid salamanders take about twice as many attempts to learn the route as diploids, perhaps because they have fewer neurones in the brain (Vernon and Butsch, 1957).

Mammals are not so robust; tetraploid mice usually die in

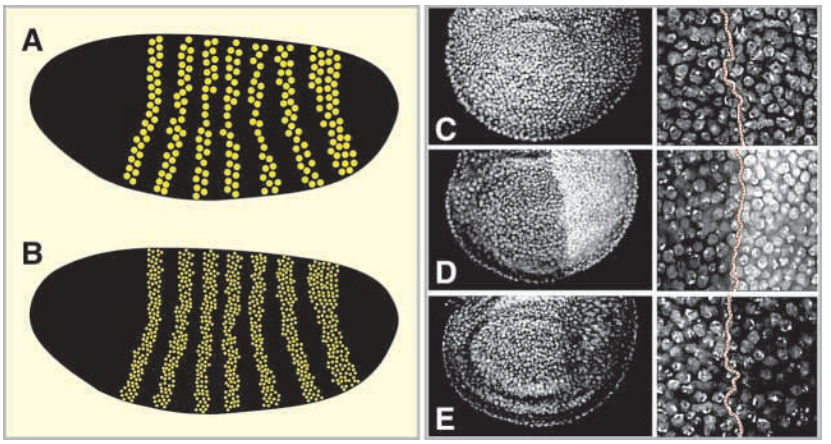

Fig. 1. Pattern can be conserved independently of cell size and number. (A,B) Two *Drosophila* embryos at the blastoderm stage. We see the 7 stripes of nuclei expressing the *fushi tarazu* gene. (A) The cell density is much reduced over the normal and average width of the stripes is only 1.7 cells (the wild-type stripes average 3.3 cells). (B) The cell density is much increased over the wild type (each stripe averages 5.7 cells). In both embryos, the dimensions and positions of the stripes are normal. (diagrams drawn from data and photographs of Sullivan, 1987). (C-E) Wing imaginal discs in which the cell density of the posterior compartments has been manipulated. There is no change in the shape and size of the compartments or the wing. The posterior compartments have been independently marked (not shown) and the interface between A and P cells accurately drawn with a dotted line. C is entirely wild type. In the P compartment, D has many more and E many fewer cells than normal. In the A compartment, D and E have normal cell densities (from Neufeld et al., 1998).

utero. However, they compensate for the larger size of their cells by a reduction in cell number. Tetraploid foetuses are about 85% the size of similar-stage diploid foetuses but have about 40% as many cells (Henery et al., 1992). But after birth, these mechanisms seem not to operate in mammals. Indeed there is some counter evidence: Mammalian $p27^{Kip1}$ (p27) is an inhibitor of the cyclin D- and cyclin E-associated kinases which are required for entry into S phase. Knockout mice that lack p27 are born normally sized, but subsequently grow considerably larger than littermates (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). The increase is a result of increased cell proliferation, presumably due to a reduction in the efficiency of the mechanisms that prevent the G_1-S transition. In these knockout mice, increased cell proliferation does *not* result in a compensatory decrease in cell size.

In *Drosophila* the growth and final size of diploid/haploid mosaics is about normal (Santamaria, 1983), the haploid regions of such flies containing more numerous but smaller cells. This kind of compensation can occur at various stages of development; for example, in young embryos there are seven stripes of pair-rule gene expression that are evenly spaced and of about the same width. If the cell sizes are varied, the width and spacing of the stripes are unchanged, as if 'painted' on the embryo according to position only (Sullivan, 1987; Fig. 1A,B).

The ploidy of *Drosophila* cells can be increased by loss of function of the cyclin-dependent kinase Cdc2. Cdc2 is required for mitosis in *Drosophila* and when the *Cdc2* gene is inactivated in cells of the wing imaginal disc, the cells switch from a mitotic cycle to cycles of endoreduplication without division (Weigmann et al., 1997). Inactivating this gene either specifically in the anterior compartment* of the wing disc, or in clones of wing cells, does not change the shape and size of the wing; even though the affected regions contain fewer but much larger, polyploid cells.

The *gigas* mutation provides an interesting contrast to the effects of reducing Cdc2 function. Loss of *gigas* gene function in clones leads to large, endopolyploid cells *and* to greatly increased growth (Ito and Rubin, 1999). Why is the response so different in the two mutations? One possibility is that *gigas* is required for a cell-size checkpoint so that *gigas* mutant cells are blind to signals that normally regulate disc size in the wild type (Ito and Rubin, 1999).

*Compartments are defined regions of the adult which were first identified in insects, but are also found in vertebrates (Lumsden, 1990). They are founded by small groups of cells, whose descendants form the whole compartment but do not contribute to neighbouring ones. The development of each compartment is specified by a unique set of 'selector genes'. Compartments are fundamental units of pattern formation and design in the fly (Garcia-Bellido et al., 1979; Lawrence, 1992; Lawrence and Struhl, 1996).

Measuring cell number, DNA or dimension?

The effects of polyploidy argue that, somehow, animals measure dimensions per se, rather than cell number. However, note that although pentaploid and haploid newts have different cell numbers they have the same total amount of DNA. Thus, if cell number were monitored indirectly, perhaps by measuring the number of copies of a particular gene, the above examples could be explained without recourse to a measurement of size. However, recent results with *Drosophila* contradict even this argument; there are two good cases where dimension is conserved in spite of large variations in DNA content.

(1) Neufeld et al. (1998) altered the expression of cell cycle genes either specifically in the posterior compartment of the wing disc or in clones of wing cells, producing either reduced or increased division rates without causing changes in ploidy. Despite a more than four-fold variation in cell number and DNA content in affected regions, compartments retained the normal size and shape. It seems that, locally in groups of cells, or more globally in the whole compartment, changes in cell number can be compensated for by changes in cell size (Fig. 1C-E).

(2) Johnston et al. (1999) have utilised loss or overexpression of the Myc transcription factor. Myc is a proto-oncogene and, in both vertebrates and *Drosophila*, Myc protein and mRNA are usually absent from quiescent cells but present in cycling cells. They found that hypomorphic *dm* mutants are smaller than wild type. The wings have substantially smaller cells, indicating that Myc is required for normal cell growth. Overexpression of *dm* increases cell growth rates and average cell size. Overexpression also drives cells through the G_1/S transition but not the G_2/M transition. Consequently, cells are larger with a far greater than normal proportion in G_2 compared to G_1. Yet, if *dm* is overexpressed specifically in the posterior compartment of the wing disc, there is no significant change

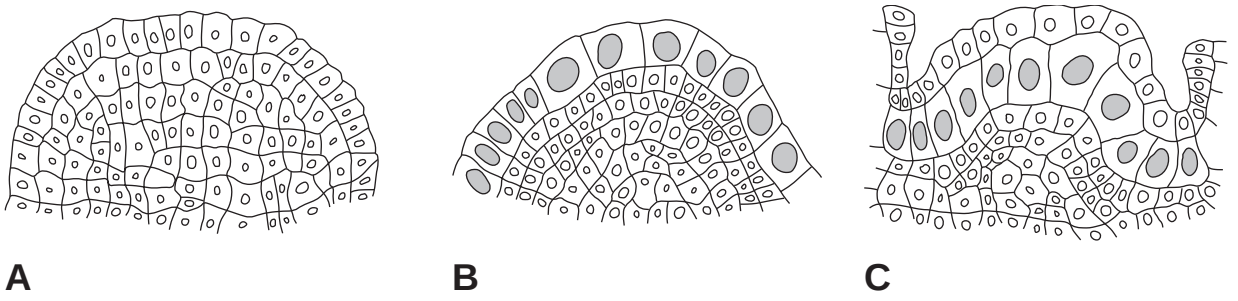

Fig. 2. Regulation of growth can be independent of cell number in plants. Sections through the shoot apex of chimeras in *Datura*. (A) All diploid; (B) outer layer octoploid, inner layers diploid; (C) outer layer diploid, second layer octoploid, inner layers diploid. The shoot apical meristem initiates all shoot tissues (after Satina et al., 1940).

in the size of the compartment or in the proportions of the wing as a whole – even though the posterior compartment now consists of larger, but fewer cells. It is thought that, in this case, any 'excess' cells, cells that would make the posterior compartment too large, are removed by apoptosis. This finding reminds us that net growth results from both cell division and cell death.

(2) Plants

In plants, growth occurs throughout life in conjunction with both determinate and indeterminate development. The leaf, for example, is a determinate organ whereas the shoot is often indeterminate – both along its longitudinal axis and (during secondary development in trees) along its radial axis. Although pattern formation in plants is poorly understood, laser ablation experiments (van den Berg et al., 1995) and clonal analysis (Poethig, 1987) indicate that patterning mechanisms are active throughout the growth of plant organs.

As in animal cells, the size of plant cells correlates with ploidy, but the effects of polyploidy in plants are more complex (Stebbins, 1950). Often, polyploid plants are simply larger than their diploid cousins. But in mosaic plants, we do see the same kind of compensation found in animals. For example, when the epidermis of an otherwise diploid thorn apple (*Datura*) is tetraploid, or even octoploid, growth and development occur normally despite greatly enlarged epidermal cells. In such plants, cell proliferation in the epidermis is markedly reduced so that the area of the epidermis matches that of the underlying cell layers. Similar compensation occurs if polyploid cells occur locally in other tissue layers (Satina et al., 1940; Satina and Blakeslee, 1941; Fig. 2).

In mosaic plants, the sensing of shape and size are unaffected by the growth rate of populations of cells within a leaf. For example, in *Pelargonium* leaves consisting of both wild-type cells that divide rapidly and mutant cells that divide slowly, the wild-type cells proliferate to occupy an excessive proportion of the leaf, yet the leaves are normal (Stewart et al., 1974).

Recent research has yielded more evidence of the control of size in plants.

(1) The Cdc2 protein (see above) is required for both DNA replication and mitosis in angiosperms (Hemerly et al., 1995; Mironov et al., 1999). Hemerly et al., (1995) made tobacco plants with reduced Cdc2 function; such plants have fewer cells than normal but there is no change in ploidy (Hemerly et al., 1995). Seedlings are at first smaller than wild type with oddly shaped cotyledons but, as they grow, they become more and more normal. Later leaves have an almost wild-type size and shape, despite being made of many fewer, but larger cells. In *Arabidopsis*, the *AINTEGUMENTA* (*ANT*) gene encodes a transcription factor with an AP2 domain. *ant* mutants have reduced proliferation leading to smaller leaves and floral organs. Nevertheless there is compensation with the cells being much larger than normal. Interestingly, overexpression of an *ANT* transgene increases the duration of proliferation, giving much larger leaves and flowers. But now there is no compensatory decrease in cell size (Mizukami and Fischer, 2000).

(2) Transforming tobacco plants with the *ABP1* gene (which encodes a receptor for the plant hormone, auxin) allows an inducible increase in leaf-cell expansion. Leaf cells can be increased to about twice the normal volume, yet affected leaves develop to the wild-type size and shape, compensating for this increase in cell size by reducing proliferation (Jones et al., 1998).

(3) There is convincing evidence that plants can measure the dimensions of specific axes. Since plant cells are immobile, it had been thought that normal cell shapes would be a necessary part of making wild-type organ shapes. The development of *tangled1* mutants of maize shows that this is not the case. The *tangled1* mutation results in frequent misorientation of the cell wall that separates daughter cells, giving highly irregular cell shapes. Nevertheless, although *tangled1* mutants are smaller than wild-type plants and grow more slowly, the leaves and other organs are normally proportioned (Smith et al., 1996; Cleary and Smith, 1998). This suggests a mechanism that regulates growth in accordance with the dimensions of each axis of the leaf and independently of the arrangement of leaf cells.

The *ANGUSTIFOLIA* and *ROTUNDIFOLIA3* genes of *Arabidopsis* may encode components of such a mechanism (Tsuge et al., 1996). Plants mutant at either locus have approximately the wild-type number of cells. However, cells in *angustifolia* mutants fail to elongate normally across the leaf, making narrow leaves; while *rotundifolia3* mutant cells do not stretch properly along the leaf, giving short leaves.

Measurement of specific axes is also suggested by plants in which proliferation has been experimentally increased. In the *Arabidopsis* root, longitudinal growth is indeterminate but radial growth is determinate. Overexpression of the mitotic cyclin gene *CYC1At* enhances cell proliferation in the roots (Doerner et al., 1996) and transformants develop a greatly

enlarged root system, with more and longer roots that contain cells of about normal size. However, the radial morphology of individual roots is unaffected. Thus the extra cells are exclusively deployed in making the roots longer and more branched – indicating that regulation of growth in the radial axis is separable from that of growth in the longitudinal axis.

THE BASIS FOR THE REGULATION OF ABSOLUTE SIZE: THE *DROSOPHILA* WING

The evidence discussed above indicates that, in both plants and animals, there are mechanisms for measuring the dimensions of organs. To consider these mechanisms, we now discuss the *Drosophila* wing, a system in which growth occurs in conjunction with patterning. Given the large proportion of homologous genes as well as the apparent similarities between the mechanisms that generate pattern in *Drosophila* and in the vertebrate embryo, we believe *Drosophila* is a model system for growth control in small-scale animal systems – there may be special mechanisms to monitor and determine the final size of organs in large systems such as postembryonic elephants.

We argue below that wing size is one outcome of patterning mechanisms intrinsic to the wing disc; in particular that gradient(s) of morphogen(s) in the wing may regulate cell growth, division and survival to fix wing size. For reviews of wing growth and patterning, see Bryant and Simpson (1984), Cohen (1993), Blair (1995), Edgar and Lehner (1996), Lawrence and Struhl (1996), Newman and Cohen (1997), and Serrano and O'Farrell (1997).

(1) Description of wing growth

The *Drosophila* wing is generated by the wing imaginal disc. The wing disc contains about 40 cells in the first instar larva of which about 30 will form the anterior (A) compartment and about 10 the posterior (P). In the larva, the cells first enlarge about sixfold and then divide steadily throughout subsequent larval life. Divisions initially reduce the cell size sharply, after which cell growth and cell division are roughly coordinated so that average cell size diminishes only slightly as the disc grows. There is a low level of apoptosis during the growth of the wild-type wing disc (Williams et al., 1993).

Until the mature larval stage, cell divisions occur all over the wing disc, the rate of growth being approximately uniform (Garcia-Bellido and Merriam, 1971; Gonzalez-Gaitan et al., 1994; Milán et al., 1996). Interestingly, cells in different phases of the cell cycle are present largely as small, synchronised clusters – but this is not because they descend from single cells dividing in a regular rhythm. Indeed, members of clusters are not clonally derived and the pattern of clusters is labile (Milán et al., 1996). In the final stages, there are regional differences in the patterns of cell growth and division, for example there is a temporary cessation of cell division at the future wing margin (O'Brochta and Bryant, 1985; Hartenstein and Posakony, 1989; Johnston and Edgar, 1998). The final number of cells in the wild-type wing disc at metamorphosis is about 50,000. The adult wing is produced by the eversion of the wing disc and its cells neither divide nor grow. The size and shape of the adult wing is therefore predetermined by the patterns of cell growth, division and death in the disc.

(2) Patterning of the wing disc

At the formation of the wing disc, the selector gene *engrailed* is already expressed in the P compartment and, amongst many other things, instructs all P cells to secrete the Hedgehog signalling protein. Hedgehog diffuses a short way into the A compartment where it induces A cells to produce another signalling protein: Decapentaplegic (Dpp) (Fig. 3A). Dpp acts as a morphogen and the gradient of Dpp concentration from the centre of the wing disc (the AP boundary) to the edges of the disc appears to regulate cell fate in both the A and P compartments. Thus the Spalt transcription factor is produced in a narrow band near the source of Dpp where the concentration is high, while the Omb transcription factor, whose production appears to be more sensitive to Dpp, is present in an overlapping but broader band (Lecuit et al., 1996; Nellen et al., 1996).

As it grows, the wing disc becomes further divided into a proximal compartment that will form the notum, and a distal compartment that will form the blade of the wing. The disc is then subdivided into a dorsal (D) compartment and a ventral (V) compartment. The DV compartment boundary runs along the edge of the wing. Interactions between dorsal and ventral wing cells lead to the production of another morphogen, the Wingless (Wg) protein, produced along the wing edge (Fig. 3B).

EXTRINSIC AND INTRINSIC REGULATION OF WING SIZE

We now turn to control of the size and shape of the wing. We will first discuss extrinsic regulation of growth and then turn to intrinsic mechanisms. It seems that, generally, extrinsic mechanisms are concerned with a link between growth and nutrition; they are not involved in proportion and pattern, but do affect the rate of growth and also the final size of the fly. Edgar and colleagues (Johnston et al., 1999) have argued that nutrition-based and pattern-based regulation of growth operate in distinct ways: nutrition regulates the cell cycle via cyclin E acting at the G_1/S checkpoint, and pattern acts through *cdc25*/*string* which intervenes at the G_2/M checkpoint. Our emphasis in this essay is on the pattern-based regulation of growth. Moreover we think pattern mechanisms intrinsic to the disc are the most important and *largely* determine wing size: as suggested by the capacity of discs to achieve the correct size when transplanted into an adult female host (Bryant and Simpson, 1984; Bryant and Levinson, 1985; Jursnich et al., 1990).

GROWTH, NUTRITION AND SIZE

Experiments on *Drosophila* and other insects show that the growth of the discs depends on extrinsic factors including hormones (reviewed by Stern and Emlen, 1999). There may be interactions between different imaginal discs; for example, removal of the hindwing discs in a caterpillar results in a butterfly with larger than normal forewings and forelegs (Nijhout and Emlen, 1998). Body and organ size are also related to nutrition: poorly fed larvae develop more slowly and can produce smaller flies. The wings of such flies are smaller

because they contain smaller and fewer cells. Apart from this reduction in size, wing pattern is unaffected (Robertson, 1963; Bryant and Simpson, 1984).

(1) The insulin pathway

Drosophila organ size may be influenced by factors produced in the fat body (Britton and Edgar, 1998). Candidates are a family of chitinase-related proteins that stimulate the proliferation of imaginal disc cells in culture (Imaginal Disc Growth Factors – IDGFs) (Kawamura et al., 1999). The effects of IDGFs are enhanced by insulin (Kawamura et al., 1999) and, indeed, an insulin-like peptide is present in the larval haemolymph (Seecof and Dewhurst, 1974; Meneses and De Los Angeles Ortiz, 1975), although its site of production is unknown. Furthermore, several recent papers have linked signalling through the *Drosophila* insulin receptor (InR) directly to size control (Chen et al., 1996; Leevers et al., 1996; Böhni et al., 1999; Montagne et al., 1999; Weinkove et al., 1999).

Strong loss-of-function mutations in the *InR* gene are lethal but flies with some loss of function survive and show a growth pattern similar to that induced by starvation: delayed development, smaller overall size and a reduction in both cell number and cell size (Chen et al., 1996; Böhni et al., 1999). The Flipper protein in *Drosophila* is homologous to the vertebrate insulin receptor substrate IRS1-4: an adapter for the insulin receptor. Mutations in the *flipper* gene cause a similar phenotype to a reduction in *InR* function. The effects of *flipper* mutations are cell autonomous (Böhni et al., 1999).

Studies on mammalian cells and *C. elegans* indicate that InR signalling is transduced via class I_A phosphoinositide 3-kinases (PI 3-kinases) and their adapter proteins, to a serine/threonine kinase cascade that includes PKB and p70S6 kinase (Weinkove et al., 1999 and references therein). Leevers and her colleagues (Leevers et al., 1996; Weinkove et al., 1999) have shown that inhibition of PI 3-kinase activity in *Drosophila* reduces both cell size and number. Overactivation of PI 3-kinase increases cell size, cell number and overall size. Interestingly, overactivation of PI 3-kinase has different effects on the cells in different stages of the cell cycle. In particular, it is more effective at driving cells through the G_1/S transition than the G_2/M transition (compare with the effects of overexpressing *dm* (Johnston et al., 1999, described above). All of these effects are cell autonomous.

Lastly, Montagne et al., (1999) have shown that loss-of-function mutations in the *Drosophila* S6 kinase gene cause a severe delay in development and reduction in size: although again without a change in pattern. Intriguingly, the smaller size of mutant flies is entirely due to a reduction in cell size, without a reduction in cell number. This mutation also acts cell autonomously.

Overall, the research suggests that the InR signalling pathway provides a cell autonomous mechanism through which the size of imaginal discs is regulated by an extrinsic insulin-like signal. Manipulation at different points of the pathway can affect both cell number and cell size (INR, Flipper, PI 3-kinase), or just cell size (S6 kinase). It is not clear how this pathway relates to other mechanisms that must control proportion, shape and size.

(2) Nitric oxide

The concentration of a diffusible growth inhibitor produced inside an organ could relate to organ size by a mechanism utilising the change in surface area to volume ratio of an organ as it grows. As size increases, the surface area to volume ratio decreases. Therefore, if all cells in a developing organ produced a growth inhibitor at a steady rate, then, as the organ grew, the internal concentration of the inhibitor would rise. At a critical level, the inhibitor could stop growth. This could give growth control based on total volume rather than cell number.

There is evidence that nitric oxide (NO) acts as such an intrinsic inhibitor (Kuzin et al., 1996). NO diffuses readily between cells and, among other functions, can suppress DNA synthesis and reduce cell proliferation (Garg and Hassid, 1989; Lepoivre et al., 1990; Kwon et al., 1991). NO synthase (Nos) activity in imaginal discs can be detected and increases from the third instar onwards. Overexpression of Nos in late larvae reduces final disc size, while inhibition of Nos increases it. The changes in size are associated with altered cell proliferation (Kuzin et al., 1996).

A mechanism for size control based on the surface area to volume ratio could work through NO but there are at least two complications. Firstly, the observations suggest that the rate of production of NO per cell is not constant but increases during the third instar. Secondly, it seems that the effects of Nos depend on which axis is considered. Inhibition of Nos increases leg size in the AP and DV axes but not in the proximodistal (PD) axis; whereas ectopic Nos expression decreases size in the PD axis but does not affect the AP and DV axes (Kuzin et al., 1996).

INTRINSIC MECHANISMS LINKED TO PATTERN FORMATION

Two lines of evidence suggest the important principle that, in the main, pattern determines growth rather than vice versa: firstly, the phenomenon of competition; and secondly the effects of changing the distribution of morphogens, or of manipulating the ability of cells to perceive them.

(1) Competition

More than 20 years ago a surprising and illuminating phenomenon was discovered in the wing disc (Morata and Ripoll, 1975; Simpson, 1976, 1979; Simpson and Morata, 1981). *Minutes* are a large class of mutations that, in one dose, reduce the capacity of protein synthesis (for example by cutting the number of complete ribosomes) and slow down development. Simpson and Morata made flies that contained both normal and *Minute* cells and found that cells compete with each other during growth in vivo. It is as if the final structure of the wing is mapped out in advance as a limited and shaped ‘space’ and, as cells proliferate to create and fill this space, there is a struggle to survive. Stronger cells, which grow and divide more rapidly, compete with weaker ones and the weaker ones die – they are ‘killed’ by the stronger ones (Fig. 4). Thus the wing becomes made entirely, or almost entirely by descendants of the strongest cells present. What is strong and weak is relative, so certain weak cells are eliminated if they are accompanied by stronger ones, yet the same type of weak cells will predominate and ‘kill’ if they are mixed with cells even weaker than they (Simpson and Morata, 1981). These observations establish the competitive nature of growth, they

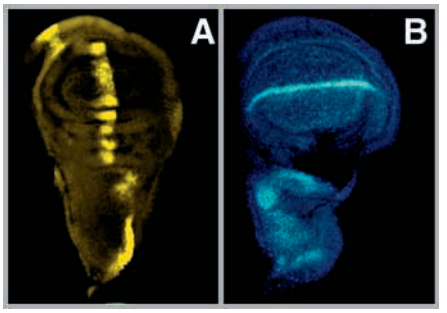

Fig. 3. Morphogens in the wing disc of *Drosophila.* (A) The expression pattern of the *Dpp* gene which is confined to a narrow strip of those A cells that are close to the P compartment. Dpp protein spreads outwards from this line source and forms gradients that cover both the A and P compartments, organising pattern and growth. (B) The expression pattern for the *wingless* gene which also sets up a morphogen gradient with its peak at the dorsoventral compartment border (sharp line in blue). There is also some *wingless* expression in parts of the notum (below). Images courtesy of Sean Carroll and Scott Weatherbee.

also suggest that apoptosis and cell division are integrated and that both are outcomes of this competition.

Competition is related to compartments. Simpson showed that, if compartments were founded by a mixture of weak and strong cells, then the weak ones were mostly eliminated; when there were a few surviving weak cells, they were found close to the compartment boundaries. However, if any compartment consisted entirely of weak cells these cells were protected from competition; for example from wild-type cells in the adjacent compartment. These and other findings showed that competition does not occur across compartment boundaries.

When most of the larva consisted of normal cells, compartments made entirely of a different kind of slow-growing cells were rushed into maturation and gave diminutive pieces of pattern, such as a tiny half-wing. However in other cases where these weaker cells constituted a larger proportion of the fly, development was delayed and all these slow-growing compartments had time to reach normal size and fit in perfectly with the rest of the fly. In these cases Simpson showed that the normal compartments first filled up with cells, stopped growing and then waited for the weak compartments to complete growth. This shows that growth is not strictly dependent on time, but continues until the preordained size is attained. We think these observations are important since, as pointed out by Simpson (1976), they show that pattern controls growth and not vice versa. Patterning mechanisms divide the wing into compartments, and these are the units in which size is controlled.

Competition experiments also show that net growth of a compartment (the outcome of cell division, cell growth and cell death) is under continuous global control. Since the decision whether to die, grow or divide must ultimately be made at the level of each cell, there should be a mechanism to convey information related to the size of the compartment to individual cells. Experiments on Dpp and Wg suggest that morphogen gradients provide a means to do this.

(2) The Dpp and Wg morphogens

Changes in the pattern or level of Dpp and Wg production can redesign the wing, suggesting that these morphogens not only pattern the organ but also determine dimensions.

Defective production of either Dpp (Spencer et al., 1982; Zecca et al., 1995) or Wg (Sharma and Chopra, 1976; Couso et al., 1994) in the wing primordium reduces the wing to a stump (Fig. 5B). Furthermore, downstream components (Omb, Spalt) needed for transduction of the Dpp (e.g. *tkv*, Burke and Basler, 1996, e.g. Spalt, de Celis et al., 1996) and Wg (e.g. *arm*, Peifer et al., 1991) signals are required cell autonomously throughout the wing to allow cell proliferation.

If extra Dpp (Capdevilla and Guerrero, 1994; Zecca et al., 1995) or Wg protein (Diaz-Benjumea and Cohen, 1995; Ng et al., 1996) is produced locally in clones, there is additional growth and the wing is substantially redesigned (Fig. 5). In a normal wing disc, Dpp and Wg are only produced together at the wing tip where the AP and the DV boundaries intersect. Notably, the growth effects of clones are most profound when a new site at which cells produce both Dpp and Wg is created; for example, when an ectopic Dpp-producing clone overlaps the DV boundary (the source of Wg). Ectopic sites at which both morphogens are produced can even organise the outgrowth of symmetrical winglets in which the spacing of pattern elements such as veins is normal (Zecca et al., 1995). These winglets may consist of elements of either the A or the P compartment, depending on where the clone originates. The ectopic Dpp/Wg-producing clones form a small strip including the tip of such winglets; the rest of the winglet is made by wild-type cells (Fig. 5A,C). In the eye, a DV border region near the equator organises growth, pattern and polarity at a distance; if an ectopic DV border is made by experiment, it induces ectopic eyelets (Cavodeassi et al., 1999). These observations illustrate that morphogen gradients specify pattern and scale.

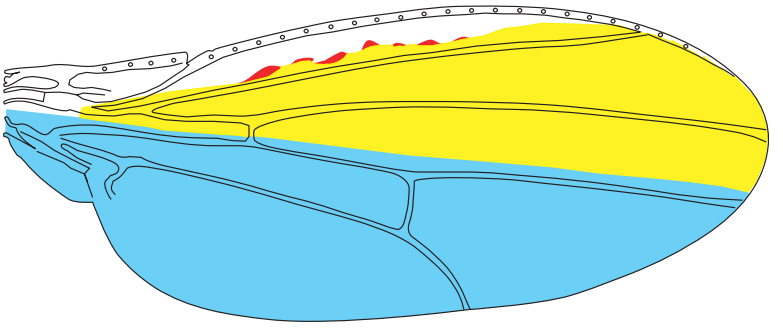

Fig. 4. Competition in the wing disc of *Drosophila*. If a large vigorous clone is initiated in a wing compartment made up of weaker cells, it grows at the expense of those weaker cells. The drawing shows a wing with the posterior compartment shaded in blue. There is a *Minute*$^+$ clone (yellow) in the anterior compartment of the wing; as it spreads in the disc, this clone has met with a different clone (red) that has the same genotype as the rest of the wing (*Minute*$^+$*/Minute*). The red clone of relatively weaker cells has been thinned and broken up by the more vigorous yellow clone (after Simpson and Morata, 1981).

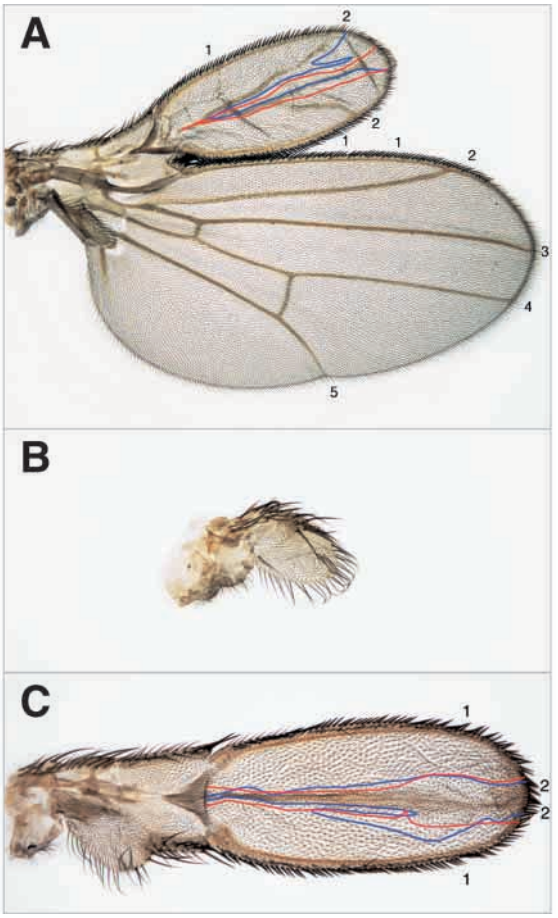

Fig. 5. Dpp gradients organise growth and pattern in the wing of *Drosophila*. (A) Small clones of *Dpp*-expressing cells can change the landscape of concentration of Dpp protein. The result is an outgrowth, in which the new peak in the concentration gradient leads to effects far beyond the clone (shown outlined by blue and red lines), the winglet formed being finely patterned in mirror symmetry. (B) A wing that lacks Dpp does not grow but forms a little stump. (C) If a clone of *Dpp*-expressing cells is induced in a stumpy wing, it now grows, the clone setting up gradients of Dpp protein which organise a symmetrical winglet. (from Zecca et al., 1995).

MODELS TO LINK WING DIMENSIONS WITH MORPHOGEN GRADIENTS

The above observations could lead and have led to the conclusion that Dpp and Wg are agents that resemble growth factors, like insulin. But our model is different, it places emphasis not on how the cells are kept cycling but on what *stops* growth when the wing has reached its final size. At this point, growth ceases in spite of the continued presence of Dpp and Wg proteins. We argue that local growth could depend on local reading of the steepness of morphogen concentration gradients. This information might be continually used to control cell division and cell death (our hypothesis is based on experiments by Bohn, 1967 elaborated in Lawrence, 1970, 1992; Lawrence and Struhl, 1996).

In its simplest form, the hypothesis suggests that the end points of a morphogen gradient – at the highest and lowest concentrations – are fixed. Individual cells, or local groups of cells, monitor the declivity of the gradient and grow and divide for as long as the gradient is sufficiently steep. Growth anywhere in the field of cells stretches the gradient and so reduces its rake. Eventually, in each region of the field, the local steepness falls below a threshold and cell proliferation ceases. If naturally, or in an experimental situation, the steepness of the gradient were to become too gentle, there would be no net growth and the steepness could be restored by an increase in the frequency of apoptosis.

How steepness relates to cell number would depend on the mechanism of morphogen spreading – if the morphogen spread unhindered through or around cells and cell membranes, and the concentration gradient took up a simple monotonic shape without steps, such as would occur if the morphogen diffused freely, like a gas, then the steepness could be largely dependent on distance itself rather than on cell number. If steepness itself could be measured, growth could respond to dimension per se. This feature of the gradient model is what makes it attractive, for the experimental results ask for such a property. This model has an additional advantage: it could explain why cell division occurs all across the disc, since the slope of a morphogen gradient would be read locally at every point in a field of cells.

There are different means by which cells could transduce the steepness of a concentration gradient into the control of cell division and survival. The polar coordinate model incorporates one such mechanism, devised in part to explain intercalary growth observed during regeneration of insect (Bohn, 1967; reviewed in Bryant and Simpson, 1984; Lawrence, 1992) and amphibian limbs (French et al., 1976; Bryant et al., 1981) after surgical juxtaposition of proximal to distal tissue. This intercalation mechanism depends on cells acquiring a ‘positional value’ (Wolpert, 1969) according to the local concentration of a morphogen. Neighbouring cells then compare positional values. If the comparison reveals that the cells are too different to be nearest neighbours, intercalary cell proliferation is stimulated. Cell division and growth ‘stretches’ and reduces the steepness of the morphogen gradient. The newly produced cells adopt intervening positional values. The process reiterates until the complete range of positional values is created (Bryant and Simpson, 1984) with the morphogen gradient now being sufficiently gentle to give neighbouring cells neighbouring positional values.

Although this intercalation mechanism is an attractive model for regeneration, in its simplest form it cannot explain the ability of organs to regulate dimension independent of cell size. The model assumes that each cell has a unique positional value and if so it will only generate an axis of normal length if cells are the normal size.

If cells or groups of cells could measure the steepness of the morphogen gradient per se, size regulation could become independent of variation in cell size and number. These matters have been studied in *Dictyostelium* and leucocytes and it seems that such cells can compare the local concentration perceived at (a minimum of) two sites on the cell surface that are a fixed distance apart. They can detect differences in concentration of as little as 2% from one end of the cell to another, even in widely varying ambient concentrations (Zigmund, 1981; Parent et al., 1998; Jin et al, 2000; Servant et al, 2000; reviewed in Parent and Devreotes, 1999). This capacity should allow, in principle, for both the direction and the steepness of the gradient to be measured by individual epithelial cells. If such

measurements were made on a basis of distance itself, the mechanisms could be independent of cell size.

Tests of gradient models for size control

We have seen that morphogen gradients originate from compartment borders; our model therefore predicts that these borders should be crucial for growth. In the milkweed bug *Oncopeltus*, the abdominal segments grow at a fixed rate so that at every instar they enlarge by a certain proportion. In young larvae, two segments can be fused by excising the border region between them: the fused segment now continues to grow but only as a single segment, even though it contains cells from two (Wright and Lawrence, 1981). The segments of the adult abdomen of *Drosophila* are most probably homologous to those in *Oncopeltus* – in the fly, it is known that morphogens do emanate from borders between A and P compartments; here too they would be expected to determine growth of the segment as a whole (Struhl et al., 1997). Indeed, in some mutant lines of *Drosophila*, adjacent segments of the abdomen (sometimes only on one side) seem to lose the separating borders, become fused and, if so, grow only as much as one unit, causing distortion of the abdomen (Sobels, 1952). Also, in the wing of *Drosophila*, a combination of two mutations makes the wing enormous and deformed. Clones of mutant cells in the middle of the wing had no effects on wing size or shape, but the same mutant cells at the DV border changed the shape of the wing and caused much extra growth, extending far beyond the clone itself (Lawrence and Morata, 1976). All these above findings point to the borders as localised regions that can organise the growth of the whole unit.

Relevant experiments on the effects of morphogen gradients on size have also been done in the *Drosophila* embryo and larva, although the circumstances are somewhat different to the wing disc. If the body pattern is disturbed so that segments are created with too many cells, there is increased cell death in these segments and their normal size is restored. This again suggests some measure of size or cell counting, with the segments or compartments acting as units in the control of growth. However, if segments are made with too few cells, the embryo does not regulate, there is no extra cell division— but this may be because development of the *Drosophila* embryo is so rapid it does not have time to undertake extra cell divisions (Busturia and Lawrence, 1994; Namba et al., 1997; Li et al., 1999).

If mutant embryos are made that lack all maternally provided morphogens responsible for the AP axis, they are unpatterned and unsegmented in that axis, even though their cells differentiate and secrete cuticle. In such embryos the length of the AP axis is reduced to about 30% by cell death, yet the length of the DV axis is reasonably normal. The outcome is a tiny spherical larva (Struhl et al., 1992). Later in development, the zygotic morphogen thought to be largely responsible for patterning the AP axes of the A compartments is Wingless. Embryos that lack both *engrailed* and *wingless* genes lack compartment boundaries and also become nearly spherical. If Wg is provided uniformly at a high level to such embryos, little growth is restored. But, if a few stripes of Wg are provided, the embryos gain much more in length (Fig. 6). These results suggest that it is not the presence or absence of Wg that is important for growth, but rather its distribution. If it is uneven in distribution the cells survive, if it is either uniform, or missing altogether, the cells tend to die (Lawrence et al., 1996).

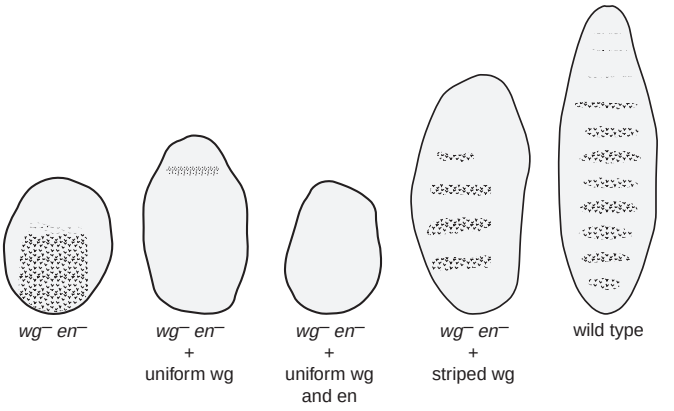

Fig. 6. Size may depend on the slope of a morphogen gradient. The wild-type larva of *Drosophila* hatches from the egg as shown on the right, but if it lacks compartments and is all made by A cells (*wg⁻ en⁻)* it is small and spherical. If Wingless protein is added uniformly to such embryos they elongate slightly but if Wingless is added in a few stripes the growth is greater (we like to think that if Wingless were added in more stripes the growth would be greater still). Drawings to same scale (after Lawrence et al., 1996).

Counterevidence

Although it is appealing to extrapolate these models to the wing and conclude that the slopes of the Dpp and Wg gradients control the size of the wing, there is no real confirmation of this. On the contrary, there are at least two pieces of evidence against the rake of Dpp and Wg gradients being directly responsible for growth.

(1) Ubiquitous expression of Dpp in the disc leads to greatly increased growth along the AP axis (Nellen et al., 1996). Yet, under the model, generalised production of Dpp, if it is strong enough, should abolish the Dpp concentration gradient by creating a uniformly high concentration. The gradient model predicts that a flat field of Dpp concentration might produce cells of one sort, with no growth and increased apoptosis.

(2) Ubiquitous activation of the Wg pathway in the wing disc via the expression of constitutively active Armadillo, causes no change in the size of the wing itself (Nagaraj et al., 1999). Again, the gradient model might predict that ubiquitous activation of the Wg pathway could prevent cells from perceiving the Wg gradient and reduce or abolish growth.

It is hard to weigh the significance of these observations and decide how much they argue against the gradient model because there are even more complications that need to be considered:

Complications

We list four kinds of complications.

(1) Cell affinity: consider the effects of blocking receipt of the Dpp signal in a cell that is located in the middle of the wing, near the source of Dpp. Failure to ‘see’ Dpp will change the positional information received by that cell, it will now differentiate as if it were at a site remote from the source of Dpp, that is at the edge of the wing. It, and its descendants, will acquire the affinities of cells at that remote location, and the clone will round up as it tends to sort out from its now different neighbours, its cells dying not because they cannot grow, but because they become crowded, and/or because they

cleave from the epithelium to form a separate vesicle (Wigglesworth, 1940; Steinberg, 1963; Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). There is some evidence that this may happen in the wing where *tkv*$^{-}$ clones (which lack the receptor for Dpp) survive if they are far from the source of Dpp. However, if these same clones are induced near to the source of Dpp they tend to disappear (see Burke and Basler, 1996, but note these authors offer different explanations for their observations).

(2) Changes of mechanism that occur during development: for example, the D/V boundary of the wing is the source of the Wg morphogen gradient, and yet it is not formed until the second instar. Thus growth of the young disc cannot depend on a gradient of Wg, at least not in the same way as later on. The response to Wg also varies depending on the location and stage: we have seen that the Wg morphogen gradient may promote and regulate growth in much of the wing disc, yet Wg is specifically required in the third instar for the *cessation* of cell proliferation at the wing margin (O'Brochta and Bryant, 1985; Phillips and Whittle, 1993; Johnston and Edgar, 1998).

(3) Receptors: Wg and Dpp can influence the amount and distribution of their receptors (Cadigan et al., 1998; Lecuit and Cohen, 1998). Other feedback loops of this kind can affect the shapes and behaviour of morphogen gradients; see for example studies of the *brinker* gene (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999).

(4) Secondary morphogens: there is another possibility that perhaps wing size is not controlled directly by the gradients of Wg and Dpp, but instead by concentration gradient(s) of some secondary morphogen(s) downstream of these agents. This would be a blatant violation of Occam's razor, but his razor has not proved too useful a standby in the increasingly baroque world of developmental genetics. In any case, there is some evidence for secondary morphogens in *Drosophila*: The orientation of hairs and bristles in the fly epidermis is thought to depend on the local direction of slope – or vector – of a morphogen gradient (Lawrence, 1966; Stumpf, 1966; Usui et al., 1999). In the adult abdomen, it has been shown that polarity is dependent on the Hedgehog (Hh) primary morphogen, but is not determined directly by it. Instead it appears that a secondary morphogen with a longer range is induced by Hh and it is the vector of this secondary gradient that determines polarity (Struhl et al., 1997; Lawrence et al., 1999).

This hypothesis of a secondary gradient raises an important question about our model: if growth is to depend on local measurement of steepness of a morphogen gradient, we need to know which gradient; is it the primary gradient that gives positional information, or is it a gradient of a secondary morphogen that might also be responsible for polarity? Some results on the adult abdomen argue that the primary (Hh) gradient may not be directly responsible for growth: clones of *smoothened*$^{-}$ cells cannot receive Hh, and therefore differentiate the type of cuticle usually found at the centre of the A compartment, regardless of their actual position. If such clones are located in the centre of the A compartment, remote from the sources of Hh, they grow normally and are normally polarised (Struhl et al., 1997). In this region at least, growth and polarity are apparently independent of Hh; one explanation is that they might both depend on the same secondary gradient.

To conclude – we have argued that shape and size in both animals and plants is controlled in part by mechanisms that read absolute dimensions rather than cell number. We have discussed and advocated models that utilise gradients of morphogens but freely admit the evidence is equivocal.

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