An Unbiased Analysis of Candidate Mechanisms for the Regulation of Drosophila Wing Disc Growth

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The control of organ size presents a fundamental open problem in biology. A declining growth rate is observed in all studied higher animals, and the growth limiting mechanism may therefore be evolutionarily conserved. Most studies of organ growth control have been carried out in Drosophila imaginal discs. We have previously shown that the area growth rate in the Drosophila eye primordium declines inversely proportional to the increase in its area, which is consistent with a dilution mechanism for growth control. Here, we show that a dilution mechanism cannot explain growth control in the Drosophila wing disc. We computationally evaluate a range of alternative candidate mechanisms and show that the experimental data can be best explained by a biphasic growth law. However, also logistic growth and an exponentially declining growth rate fit the data very well. The three growth laws correspond to fundamentally different growth mechanisms that we discuss. Since, as we show, a fit to the available experimental growth kinetics is insufficient to define the underlying mechanism of growth control, future experimental studies must focus on the molecular mechanisms to define the mechanism of growth control.

A fascinating aspect of embryonic development is how the growth of organ rudiments is globally coordinated such that all organs, tissues and the paired appendages grow to the correct (relative) size, even when final sizes differ between isogenic offspring because of external factors such as nutrition or temperature1. The mechanism by which growth is terminated has remained elusive.

Growth control has been studied extensively in Drosophila imaginal discs and can be separated into disc-intrinsic and disc-extrinsic mechanisms. Disc-extrinsic mechanisms ensure that body growth and organ growth are coupled in a way that correct size proportions are obtained, even when nutritional status and thus final size differs strongly during development, a phenomenon referred to as allometry2,3. Intriguingly however, similar final imaginal disc sizes are obtained even when imaginal discs are provided with additional developmental time4,5, when the frequency of cell divisions is perturbed6,7, after regeneration of lost tissue8,9, and when imaginal discs are cultured outside the larvae10,11. These results point to the existence of a disc autonomous mechanism of size control that ensures correct final sizes.

The morphogen Decapentaplegic (Dpp) affects growth in all fifteen imaginal discs in the Drosophila larvae, and models have been suggested how Dpp may result in a uniform declining growth rate12–18 in spite of its graded distribution in imaginal discs19–21. In the wing disc, in particular, Dpp forms a dynamically increasing gradient17 from the anterior-posterior boundary across the anterior and posterior sides12–21. In the eye disc, on the other hand, Dpp is secreted from the morphogenetic furrow (MF) that sweeps from the posterior to the anterior end of the eye disc22–24. Gonzalez-Gaitan and co-workers previously proposed that an exponential decline in the proliferation rate arises in both discs because cells sense the relative local change in the Dpp concentration and divide whenever they experience a fixed relative change of about 40%17,28. However, the area growth rate is unaltered in Mad-/Brk- clones that cannot sense Dpp28,29. Additionally, Dpp affects wing disc growth only in the first half of larval development30 and only in the medial part of the wing disc31. According to an alternative model, uniformly declining growth results from a combination of Dpp signaling and mechanical feedbacks12,15,32,33. Finally, we have recently shown that the growth rate of the apical area in the Drosophila eye imaginal disc declines inversely proportional to the total apical area, which is consistent with a dilution-based mechanism for growth control34.

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Here, we use published quantitative growth data to carry out an unbiased evaluation of alternative candidate growth mechanisms. Based on the quantitative analysis, we rule out growth control by dilution in the wing disc. Evaluating alternative candidate growth mechanisms, we find that biphasic exponential growth best fits the wing disc data. An exponential decline of the growth rate with developmental time and logistic growth are also consistent with the data. These three growth laws correspond to fundamentally different growth mechanisms that we discuss. Given the wide range of growth laws that are consistent with the data, we emphasize the need to explore a wider range of growth limiting mechanisms and to carefully check the consistency of any proposed mechanism with additional experimental data – the ability to recapitulate the growth kinetics alone provides insufficient support for any mechanism.

**Results and Discussion**

**Dilution of a cytokine cannot explain the Wing Disc Growth Kinetics.**  The growth of the apical area, $A$, of *Drosophila* imaginal discs over developmental time, $t$, can be described mathematically by

$$\frac{dA}{dt} = k(t)A.$$  

Here, $k$ refers to the area growth rate. We have previously shown that the declining area growth rate in the eye disc can be described by an area-dependent decline of the form

$$k(t) = k_0 A_0 / A.$$  

Here $A_0$ is the initial area and $k_0$ the maximal growth rate. We sought to test whether this mechanism would also apply to the wing disc. To this end, we used two independent, published datasets for wing disc area growth.
(Fig. 1A) to determine the area growth rate, $k$ (Fig. 1B), and to fit the model (Equations 1 and 2) to these data-sets (Fig. 1C,D). The poor fit to the data rejects this mechanism for growth control in the wing disc (Fig. 1C,D, Figs S1A–D and S2A–D).

Unbiased Analysis of Candidate Growth Laws for Wing Disc Growth. We next tested which other candidate growth laws would allow us to reproduce the wing disc data (Fig. 2). To this end, we simulated the growth model in Eq. (1) with the growth laws that we had previously considered as candidates for growth control in the Drosophila eye disc34. In addition, we considered biphasic growth, which had previously been shown to describe wing disc growth2, and logistic growth, which had previously been shown to describe the regeneration kinetics of newt limbs14. In summary, in the simplest model we assumed a constant growth rate (CST, Fig. 2A,B) = $kt_0$.

In biphasic growth (BPH), the value of $k_0$ changes once, thus giving rise to two phases of different exponential growth (biphasic, BPH, Fig. 2C,D). The switch point from phase I to II was defined as the one providing the lowest deviation from the data (Fig. S3). In addition, we considered models with a continuously declining growth rate, either as exponential decaying growth rate (EXP, Fig. 2E,F) of the form

$$k(t) = k_0 e^{-rt},$$

or by following a power law decay (POW, Fig. 2G,H) of the form
Finally, the growth rate could depend on the total wing disc area. Besides dilution (Equation 2, Fig. 1C,D), logistic growth (LOG, Fig. 2I,J) can result in an area-dependent growth rate

\[ k(t) = k_0 (A_{set} - A). \]  

We note that the models with a constant or an area-dependent growth rate have two parameters, the biphasic exponential model (BHP) has four parameters, while all other models (EXP, POW, LOG) have three independent parameters. A full list for the parameter values can be found in the supplementary information (Tables S1 & S2).

To fit the models to the data, we defined the residuals in two independent ways: either by normalizing with respect to the standard error (dashed lines in Figs 1C,D & 2A–J & Fig. S1) or by taking the logarithm of the data (solid lines in Figs 1C,D & 2A–J & Fig. S2) to correct for the different orders of magnitudes in the data and thus be able to fit the data well (Fig. 2A–J) (see Material & Methods for details). To compare the model fits, the sum of squared residuals (RSS, Fig. 2K, Table S3) and coefficient of determination \( R^2 \) (Table S4) were used. We note that fitting the logarithmic data provided the smaller deviation and better coefficient of determination \( R^2 \) in all cases (Tables S3 and S4). Using these measures, the biphasic model (BHP) performs best for almost all weightings and data sets (Fig. 2K). Interestingly, also the reported change in cell density\(^{17} \) correlates with the predicted switch (Tables S3 & S4).

Candidate Mechanisms for Growth Control in the Drosophila Wing Disc. While the biphasic growth law, logistic growth, and an exponentially declining growth rate all fit the data well, they point to very different underlying mechanisms. A biphasic growth law would require a sudden change in cell growth, potentially because of cell differentiation. It has been argued that larvae can monitor their size and trigger the switch to lower growth rates when reaching a ‘critical size’\(^{2} \). It remains unclear how wing discs would achieve this on the molecular level in a disc-autonomous way and how disc size could then vary in response to changes in external conditions (nutrients, temperature etc.).

\[ \frac{dk}{dt} = -\delta k. \]  

An exponential decline in the growth rate could be achieved by the Dpp-dependent mechanism proposed by Gonzalez-Gaitan and co-workers\(^{17,28} \), or if a growth factor to which the system responded linearly, was degraded at a constant rate \( \delta \), i.e.

An exponential decline in the growth rate would eventually be so close to zero that the expansion becomes negligible. Thus, the cell density increases until the inferred switch point (vertical lines) and subsequently stabilizes (Fig. 2L). Logarithmic growth (red) and to a slightly lesser extent an exponentially declining growth rate (yellow) also fit the data very well (Fig. 2K), and the difference to the biphasic model is only minor (Tables S3 & S4). The power law model fits the data slightly, but consistently, worse as judged by the \( R^2 \) value (Table S4) and the RSS (Table S3).

The models with a constant (CST) or an area-dependent growth rate both have only two free parameters, and thus at least one parameter less than the models discussed above (BHP, EXP, POW, LOG). The model with the area-dependent growth rate performs considerably worse than the model with a constant (CST) growth rate, and can thus be rejected (Fig. 2K, Tables S3 & S4). To compare the model with a constant (CST) growth rate to the other models, the different number of free parameters needs to be taken into account. Unlike other commonly used model selection criterions, such as the Bayesian information criterion (BIC) or the Akaike information criterion (AIC), the F-test provides a p-value on the null hypothesis that the better fit can be solely explained by the increased number of parameters. Its use, however, restricted to the case where the simpler model is nested within the more complex model, limiting it to the comparison of the biphasic (BHP) and single exponential model (CST). Conducting an F-test, we find that for both data sets and weightings, the biphasic exponential model fits the data significantly better than the model with a constant growth rate (Table S5).
In conclusion, we can rule out an area-dependent dilution mechanism for growth control in the wing disc. Rather, the responsible mechanism must give rise to biphasic growth, logistic growth, or an exponentially declining growth rate. Given that several growth laws match the measured growth curves, it needs to be stressed that the reproduction of the growth kinetics alone is insufficient evidence for any proposed mechanism and mechanistic proof needs to be provided. At the same time, there could be other growth laws that also reproduce the data and that we have not yet identified. It will be interesting whether the same mechanism limits growth in all organs and species or whether distinct mechanisms have evolved.

Materials and Methods

Software. All simulations and analysis were done using Matlab R2016A (The MathWorks, Natick, MA, USA) or the free software environment R in version 3.2.4.

Estimation of area growth rate. The area growth rate 
\[ k(t) = \frac{dA}{dt} \]  
(8)
in Eq. (1) can be determined by estimating the slope \( \frac{dA}{dt} \) at all time points and diving this slope by the measured area \( A \) at each timepoint. To estimate the slope \( \frac{dA}{dt} \) from the data without making any assumption about the (true) function underlying the growth dynamics, we used cubic smoothing splines as provided by the smooth.spline function in R. As the exact shape of the decline of the area growth rate \( k \) depends on the spline fits, we used two different fits for each data set with a varying smoothing parameter. The resulting spline fits are shown in Fig. 1A, and the estimated area growth rate \( k(t) \) in Fig. 1B.

Model Fitting. The two available independent studies do not provide the variance for all data points. We therefore quantified the deviation of the model from the data in form of the residual \( R \) at time point \( k \) as

\[ R_{1,k} = \log_{10}(D_k) - \log_{10}(f(x, t_k, p)) \]

or by weighting the difference by the given standard error (SE) as

\[ R_{2,k} = \frac{D_k - f(x, t_k, P)}{SE_k} \]

where \( D_k \) is the mean, \( SE_k \) its standard error, and \( f(x, t_k, p) \) refers to the model value at time point \( k \). We took the logarithm of the data for the first measure since the data is spread over several orders of magnitude and larger values would therefore be weighted much higher otherwise. In the data set from Wartlick et al., no SE was given for the time point at approximately 80 hours and for the last time point (SE = 0). We therefore excluded these two time points from the analysis with Eq. 10.

The model parameters were then determined by solving the minimization

\[ p^* = \min_p \sum_{k=1}^{N} R_k(p)^2 \]

where \( N \) is the number of distinct time points. This was done independently for both definitions of the residuals using the trust-region reflective algorithm as implemented in lsqnonlin (Matlab R2016A, MathWorks, Natick, MA, USA), resulting in two parameter sets for each model.

Comparison of model fits and statistical analysis. Models were compared based on the residual sum of squares (RSS) and their \( R^2 \) value. The \( R^2 \) value of model \( j \) was calculated as

\[ R_j^2 = 1 - \frac{\text{RSS}_j}{\text{SST}} \]

where RSS\(_j\) is the residual sum of squares of model \( j \) calculated as explained above (Equations 9 or 10) and SST is the total sum of squares.

To evaluate whether there is a statistical significance in the difference how the models fit the data, the F-test was used. The F-test can only be used to compare a simple model which is nested within a more complex one. Thus, here it can only be used to compare the biphasic exponential model to the single exponential model, as the latter one is nested within the first one. The F-statistic was calculated as

\[ F = \frac{\text{RSS}_j - \text{RSS}_k}{\text{RSS}_k / f_j} \]

where model \( j \) is nested within model \( k \), \( f_j \) and \( f_k \) are the degrees of freedom (number of data points minus number of parameters) for model \( j \) and \( k \), respectively, and \( \text{RSS} \) their residual sum of squares. The respective p-value can then be calculated from the F cumulative distribution function. The degrees of freedom to be used in the cumulative distribution function are \( (f_j - f_k, f_k) \).
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Author Contributions
D.I. conceived the study. J.V. performed the mathematical analysis of the different growth models. D.I., and J.V., wrote the manuscript.

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