Ecdysone coordinates plastic growth with robust pattern in the developing wing

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Abstract

Animals develop in unpredictable, changing environments. To do this, they adjust their development according to environmental conditions to generate plastic variation in traits, while also buffering against environmental change to produce robust phenotypes. However, how organ development is coordinated to accommodate both plastic and robust developmental responses is poorly understood. Here, we propose that the steroid hormone ecdysone coordinates both plasticity in organ size and robustness of organ pattern in the developing wings of the fruit fly *Drosophila melanogaster*. Ablating the prothoracic glands (PGX), which synthesise and secrete ecdysone, resulted in wing discs that were reduced in size and delayed in the progression of Achaete and Senseless pattern. These effects were rescued by adding ecdysone to the food. Further, while wing growth was driven by both ecdysone and nutrition, ecdysone alone induced the progression of wing patterning. To further explore this difference in response, we quantified wing growth and patterning in PGX larvae that were either fed on standard diet or starved of yeast across a range of ecdysone concentrations. Disc growth rates increased in a graded, linear manner with ecdysone concentration in starved larvae. In contrast, Achaete and Senseless patterning rates showed threshold responses regardless of diet. This means that ecdysone confers robustness by turning on patterning once it exceeds threshold concentrations, while inducing graded responses for disc growth, tuning growth to environmental conditions. This potentially represents a generalizable mechanism through which hormones coordinate plastic growth with robust patterning in the face of environmental change.

Introduction

Developing animals respond to changes in their environment in a multitude of ways, for example, altering how long and how fast they grow, the time it takes them to mature, and their reproductive output [1, 2]. At the same time, however, other aspects of their phenotype must be unresponsive to environmental change to ensure that they function correctly regardless of environmental conditions. This presents a particular problem for morphological traits of developing animals. For any given trait, some aspects, such as final organ size, vary with changes in the environment, a phenomenon termed plasticity [3-7]. Other aspects, like establishing the cell types within an organ necessary for it to function, remain constant across environmental conditions, and are thus termed robust [6-9]. For many organs, growth and patterning occur at the same time during development, and may even be regulated by the same hormones [6]. How then do organs achieve plasticity in size while maintaining robustness of pattern?

If we want to extract general principles of how organisms regulate their development in variable environments, we need to understand how developmental processes unfold over time. Several recent studies that have applied systems approaches to development offer excellent examples, frequently employing methods to quantify how gene expression patterns change over time. These studies have used the dynamic changes in expression patterns to uncover the rules governing how insects build their segments [10, 11], how the gene regulatory network underlying segmentation evolves [12-17], how morphogen gradients scale across organs and

bodies [18-23], how sensory organs are positioned within epithelia [24], and how somites and digits form in vertebrates [25-27]. The power of these approaches is that they provide a framework for understanding how genes interact within a network to generate pattern that can be applied across a variety of contexts.

The success of these studies is, in part, due to the fact that the gene regulatory networks underlying each of these processes have been well described in their respective developmental contexts. In contrast, the gene regulatory networks governing growth and patterning at later stages of development, even at later stages of embryonic development, are not as well resolved. If we further complicate this by comparing development across environmental conditions and even across traits, approaches that rely on understanding the configuration of gene regulatory networks become much more difficult to implement.

Nevertheless, we can still use the principle of comparing the dynamics of developmental processes across environments to gain useful insights into the relationship between plasticity and robustness. Many types of environmental conditions impact organ development to induce changes in body and organ size. Malnutrition or starvation reduces growth rates in all animals, resulting in smaller body and organ sizes [28-30]. Similarly, changing temperature can alter animal growth. In insect species, rearing animals in warmer conditions results in smaller adult body sizes when compared to animals reared under cooler conditions [31-38]. Other factors like oxygen availability and the presence of toxic or noxious compounds also act to alter animal sizes [39-41]. Examining how organ growth and patterning progress across these environmental conditions helps us to understand how these two processes are coordinated.

We already have some understanding of the mechanisms that regulate growth and patterning in response to changing environmental conditions. The genetic mechanisms underlying plasticity in growth is best elucidated in insects. In insects, changes in available nutrition affect the synthesis and secretion of the conserved insulin-like peptides [42-44]. Insulin-like peptides bind to the Insulin Receptor in target tissues and activate the insulin signalling cascade, ultimately leading to increased growth [43, 45, 46]. Starvation reduces the concentration of insulin-like peptides in the bloodstream, and the resulting decrease in insulin signalling causes organs to grow more slowly [44, 47].

While changes in insulin signalling are known to affect organ size, they have little effect on organ pattern [48]. However, studies in the fruit fly *Drosophila melanogaster* have shown that, at least in this insect, insulin acts to control the synthesis of a second developmental hormone, the steroid hormone ecdysone [49-52]. Most of the body and organ growth in *D. melanogaster* occurs in the third, and final, larval instar, after which the animal initiates metamorphosis at pupariation. Either starving or reducing insulin signalling early in the third instar delays the timing of ecdysone synthesis, thereby prolonging the length of the third instar and the time it takes to metamorphose [49-53]. Changing the rearing temperature also alters the timing of ecdysone synthesis early in larval development [54].

In addition to its effects on developmental time, ecdysone controls the growth of the developing adult organs [55-58]. In *D. melanogaster* larvae, many of the adult organs form and grow inside the larvae as pouches of cells called imaginal discs. If ecdysone synthesis is reduced

or if the gland that produces ecdysone, the prothoracic gland (PG), is ablated, these imaginal discs grow at greatly reduced rates [56, 59].

Ecdysone signalling also regulates organ patterning. Reducing ecdysone signalling in either the wing imaginal disc or the developing ovary causes substantial delays in their patterning [56, 59-61]. In the wing disc, reducing ecdysone signalling stalls the progression of patterning of sensory bristles [56, 59]. Similarly, in the ovary terminal filament cell specification and the rate of terminal filament addition both require ecdysone to progress normally [60, 61]. Given its role in both the patterning and the growth of imaginal discs and ovaries, ecdysone is potentially a key coordinator of plastic growth and robust pattern.

Characterising organ growth rates is experimentally straight forward, requiring only accurate measurement of changes in organ size over time. To quantify the progression of organ patterning, however, requires developing a staging scheme. We previously developed such a scheme for the wing imaginal disc in *D. melanogaster*. This scheme makes use of the dynamic changes in expression from the moult to third instar to pupariation of up to seven patterning gene products in the developing wing [62]. Two of these patterning gene products, Achaete and Senseless, can be classed into seven different stages throughout third instar development ([62], Fig 1A), providing us with the ability to quantify the progression of wing disc pattern over a variety of conditions.

The ability to simultaneously quantify both organ growth and pattern allows us to generate, and test, hypotheses regarding how ecdysone coordinates plastic growth with robust pattern. One hypothesis is that growth and patterning occur at different times, with ecdysone driving growth first then pattern later, or vice versa [6]. If this were true, we would expect to identify an interval where ecdysone concentrations primarily affected growth and a second interval where they affected mostly pattern (Fig 1B). There is some precedence for this idea; most of the patterning in the wing discs and ovaries of *D. melanogaster* occurs 15 h after the moult to the third larval instar [60]. Similarly, wing discs are known to grow faster in the early part of the third instar and slow their growth in the mid-to-late third instar [63]. As a second hypothesis, ecdysone could coordinate plastic growth with robust pattern if the impacts of ecdysone on one of these processes depended on its effects on the other. For example, morphogens are known to regulate both growth and patterning of the wing. If ecdysone controlled the action of morphogens we would expect the progression of patterning would be tightly coupled to growth over time (Fig 1C). Finally, a third hypothesis is that ecdysone regulates growth and patterning of the wing discs independently, and that each process responds in a qualitatively different manner to ecdysone [6]. As an example of this, we might see that growth rates increase in a graded manner with increasing ecdysone while patterning shows threshold responses. If this were the case, we would expect that growth and the progression of pattern would be uncoupled over time (Fig 1D).

Here we test these hypotheses of whether and how ecdysone co-regulates plastic growth and robust pattern in wing imaginal discs in *D. melanogaster*. We blocked the production of ecdysone by either genetically ablating the PG [56], and quantified the effects on growth and patterning rates throughout the third instar. We then manipulated the rate of ecdysone synthesis, by up- or down-regulating the activity of the insulin-signalling pathway in

the PG [52, 64], to test how this alters the relationship between disc size and disc pattern. Finally, we tested our hypotheses about how a single steroid can regulate both plastic growth and robust patterning by conducting dose response experiments under two nutritional conditions. These studies provide a foundation for a broader understanding of how developmental hormones coordinate both plastic and robust responses across varying environmental conditions during animal development.

Results

Ecdysone is necessary for the progression of growth and patterning

To understand how ecdysone affects the dynamics of growth and patterning, we needed to be able to precisely manipulate ecdysone concentrations. For this reason, we made use of a technique we developed previously to genetically ablate the prothoracic glands (referred to as PGX) [56]. This technique pairs the temperature sensitive repressor of GAL4, GAL80^{ts}, with a prothoracic gland-specific GAL4 (*phm-GAL4*) to drive an apoptosis-inducing gene (*UAS-GRIM*). GAL80^{ts} is active at 17°C, where it represses GAL4 action, but inactive above 25°C, which allows *phm-GAL4* to drive expression of *UAS-GRIM* and ablate the PG [65, 66]. Because ecdysone is required at every moult, we reared larvae from egg to third instar at 17°C to repress GAL4, then shifted the larvae to 29°C at the moult to the third instar to generate PGX larvae. This allows us to examine how eliminating ecdysone affects disc size and pattern in third instar wing imaginal discs, and to manipulate ecdysone concentrations by adding it back in specific concentrations to the food [56]. For simplicity, all the data from the two control strains (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}), were pooled in all analyses.

Insect wing discs show damped exponential, or fast-then-slow, growth dynamics [63, 67], and have been modelled using a Gompertz function. The growth of wing discs from control and PGX larvae shows the same pattern, with a Gompertz function providing a significantly better fit to the relationship between log disc size and time than a linear function (ANOVA, linear v. Gompertz model, $n > 95$, $F > 65$, $P < 0.001$ for discs from both PGX and control larvae). Growth of the discs, however, followed a significantly different trajectory in PGX versus control larvae (Fig 2). In control larvae, discs continue to grow until 42h after ecdysis to the third instar (AEL3), when the larvae pupariate. In contrast, the wing imaginal discs of the PGX larvae stop growing at approximately 25h AEL3, and at a significantly smaller size. This is despite the fact that PGX larvae do not pupariate, and so disc growth is not truncated by metamorphosis.

We next explored how loss of ecdysone affected the progression of wing patterning. We used the staging scheme that we previously devised in [62] to quantify the progression of wing disc patterning in PGX and control larvae. We selected two genes from this scheme, Achaete and Senseless, as they each progress through seven stages throughout the third instar. Further we can stain for both antigens in the same discs, which allowed us to compare disc size, Achaete stage, and Senseless stage in the same sample.

The progression of Achaete patterning was best fit by a Gompertz function for discs from both PGX and control larvae (ANOVA, linear v. Gompertz model, $n > 50$, $F > 10.4$, $P < 0.002$) [62], and was significantly affected by loss of ecdysone. In control larvae, the wing discs progressed to Achaete stage 6 or 7 out of seven stages by 42h AEL3, while in PGX larvae, discs of the same age had not passed Achaete stage 3, and had not matured past Achaete stage 5 by 92h AEL3 (Fig 3A). The progression of Senseless patterning was best fit by a linear model (we could not fit a Gompertz function to the PGX data), but again was significantly affected by loss of ecdysone. In control larvae, most discs had progressed to Senseless stage 6 out of seven stages by 42h AEL3, while no disc progressed past Senseless stage 2 by 92h AEL3 (Fig 3B, Supplementary Table 3).

We find no evidence of temporal separation between wing disc growth and the progression of pattern (compare Figs 2 and 3). Both growth and patterning progress at steady rates throughout most the third instar in control larvae, slowing down only at the later stages of development. The hypothesis that ecdysone coordinates plastic growth with robust pattern by acting on each process at different times is therefore unlikely to be correct.

To confirm that the lack of ecdysone was responsible for delayed patterning, and not a systemic response to the death of the glands, we performed a second experiment where we added either the active form of ecdysone (20-hydroxyecdysone) or ethanol (the carrier) back to the food. PGX and control larvae were transferred onto either ecdysone or ethanol food and allowed to feed for 42 h, after which we dissected their wing discs and examined their size and pattern. On the control (ethanol) food, wing discs from PGX larvae were smaller (Fig 4A) and showed reduced patterning for both Achaete (Fig 4B) and Senseless (Fig 4C) when compared to control genotypes. Adding 1 µg/mg food of ecdysone fully restored disc size, and Achaete and Senseless pattern, such that they were indistinguishable from control genotypes fed on ecdysone-treated food.

Collectively, these data indicate that ecdysone is necessary for the normal progression of growth and patterning in wing imaginal discs. The loss of ecdysone has a more potent effect on patterning, however, which is effectively shut-down in PGX larvae, than on disc growth, which continues more-or-less at a normal rate for the first 24 hours of the third instar in PGX larvae.

Ecdysone rescues patterning and some growth in wing discs of yeast-starved larvae

The observation that ecdysone is necessary to drive both normal growth and patterning suggests that it may play a role in coordinating growth and patterning across environmental conditions. However, to do so it must lie downstream of the physiological mechanisms that sense and respond to environmental change. As discussed above, ecdysone synthesis is regulated by the activity of the insulin-signalling pathway, which is in turn regulated by nutrition. Starving larvae of yeast early in the third instar both suppresses insulin-signalling and inhibits growth and patterning of organs [59, 60]. We explored whether ecdysone was able to

rescue some of this inhibition by transferring larvae immediately after the moult to 1% sucrose food that contained either ecdysone or ethanol and comparing their growth and patterning after 24 hours to wing discs from larvae fed on normal food. Both the PGX and control genotype failed to grow and pattern on the 1% sucrose with ethanol (Fig 5A-C). Adding ecdysone to the 1% sucrose food rescued Achaete and Senseless patterning in both the control and the PGX larvae to levels seen in fed controls (Fig 5B, C). Ecdysone also partially rescued disc growth in PGX larvae, although not to the levels of the fed controls (Fig 5A). Collectively, these data suggest that the effect of nutrition on growth and patterning is at least partially mediated through ecdysone.

An important aspect of these data is that in PGX larvae, either supplementing the 1% sucrose food with ecdysone or feeding them on normal food both rescued wing disc growth (Fig 5A), albeit incompletely. This suggests that nutrition can drive growth through mechanisms independent of ecdysone, and vice versa. In contrast, nutrition alone did not promote Achaete and Senseless patterning in starved PGX larvae, while ecdysone alone completely restored patterning. Thus, the effect of nutrition on patterning appears to be wholly mediated by ecdysone, while the effect of nutrition on growth appears to be partially mediated by ecdysone and partially through another independent mechanism.

Ecdysone drives growth and patterning independently

The data above suggest a model of growth and patterning, where both ecdysone and nutrition can drive growth, but where patterning is driven by ecdysone. We next focused on exploring how ecdysone regulates both growth and patterning. Patterning genes, particularly morphogens, are known to regulate growth, so one hypothesis is that ecdysone promotes patterning by regulating morphogen signalling, which in turn promotes the ecdysone-driven component of disc growth. A second related hypothesis is that ecdysone-driven growth is necessary to promote patterning. Under either of these hypotheses, because the mechanisms regulating patterning and growth are inter-dependent, we would expect that changes in ecdysone levels would not change the relationship between disc size and disc pattern. An alternative hypothesis, therefore, is that ecdysone promotes growth and patterning through at least partially independent mechanisms. Under this hypothesis the relationship between size and patterning may change at different levels of ecdysone.

To distinguish between these two hypotheses, we increased or decreased the activity of the insulin-signalling pathway in the PG, which is known to increase or decrease the rate of ecdysone synthesis, respectively [49, 52, 64, 68]. We then looked at how these manipulations affected the relationship between disc size and disc pattern, again focusing on Achaete and Senseless patterning. We increased insulin signalling in the PG by overexpressing InR (*phm>InR*), and reduced insulin signalling by overexpressing the negative regulator of insulin signalling PTEN (*P0206>PTEN*).

We used a linear model to capture the relationship between disc size and Achaete stage when we either increased (*phm>InR*) or decreased (*P0206>PTEN*) ecdysone synthesis rates.

Changing ecdysone levels significantly changed the parameters of the linear model and altered the relationship between disc size and Achaete pattern. Specifically, increasing the rates of ecdysone synthesis shifted the relationship so that later stages of Achaete patterning occurred in smaller discs (Fig 6A, Supplementary Table 4).

The relationship between Senseless pattern and disc size is best fit using a four-parameter logistic (threshold) function, which provides a significantly better fit to the data for all genotypes than a linear function (ANOVA, linear v. four-parameter logistic model, $n > 30$, $F > 6.13$, $P < 0.007$). Changing ecdysone levels significantly changed the parameters of the logistic model and altered the relationship between disc size and Senseless pattern (Fig 6B, Supplementary Table 5). Again, increasing the rate of ecdysone synthesis shifted the relationship so that later stages of Senseless patterning occurred in smaller discs. Collectively, these data support the hypothesis that ecdysone acts on growth and patterning at least partially independently.

Ecdysone regulates disc growth and disc patterning through different mechanisms

The data above support a model whereby environmental signals act through ecdysone to co-regulate growth and patterning, generating organs of variable size but invariable pattern. Further, ecdysone appears to act at least partially independently on growth versus patterning, enabling similar progressions of pattern across discs of different sizes. Plasticity of growth with robustness of pattern may be achieved if each process differed in the way it responds to ecdysone.

The ecdysone titre fluctuates through a series of peaks throughout the third larval instar and the dynamics of these fluctuations are environmentally sensitive [69]. Two aspects of these fluctuations appear to be important with respect to growth and patterning. First, the timing of the ecdysone peaks set the pace of development, initiating key developmental transitions such as larval wandering and pupariation [52, 64, 69, 70]. Second, the basal levels of ecdysone appear to regulate the rate of growth, with an increase in basal level leading to a reduction in body growth [49, 56, 64, 68, 71]. Thus, one simple hypothesis is that patterning responds to ecdysone as a threshold response, being initiated as ecdysone concentrations rise toward a peak, while growth responds to ecdysone as a graded response, increasing or decreasing in response to basal levels of ecdysone.

To test our hypothesis, we reared PGX larvae on standard food supplemented with a range of ecdysone concentrations (0, 6.25, 12.5, 25, 50 and 100 ng of ecdysone/mg of food). However, as noted above, disc growth early in the third larval instar is only moderately affected by ablation of the PG, potentially obfuscating the effects of supplemental ecdysone. In contrast, discs from starved PGX larvae, however, show no growth or patterning without supplemental ecdysone. We therefore also reared PGX larvae on 20% sucrose medium (these larvae from here are referred to as 'starved') supplemented with a range of ecdysone concentrations. For both control genotypes and PGX larvae, increasing the concentration of ecdysone in the food

increases the hemolymph titres of ecdysone (Supplementary Fig 1). We dissected discs at 5 h intervals from immediately after the moult to the third instar (0 h AL3E) to 20 h AL3E. Because male and female larvae show differences in wing disc growth [72], we separated the sexes in this experiment. However, due to the size of this experiment, we focused our analysis on female wing discs.

As before, in both PGX and control larvae, wing disc growth was suppressed by starvation (Supplementary Fig 2, Supplementary Table 7). An increase in the concentration of supplemental ecdysone increased the rate of disc growth in starved PGX larvae such that there was a positive relationship between ecdysone concentration and the (linear) rate of disc growth (Fig 7, Supplementary Table 8 and 9). Thus, disc growth appears to show a graded response to ecdysone level in the absence of nutrition. In contrast, increasing ecdysone concentrations had no effect on disc growth rate in fed PGX larvae (Supplementary Fig 2, Supplementary Table 8). That is, the effect of nutrition on growth obfuscated the effect of ecdysone. This is not surprising since ablation of the PG has little effect on disc growth in the first 12 hours of the third instar in fed larvae (Fig 2), and again supports the hypothesis that disc growth early in the third instar is largely regulated by nutrition and not ecdysone [63].

The effect of ecdysone on Achaete patterning was qualitatively different than its effect on growth. As before, Achaete patterning did not progress in either starved or fed PGX larvae (Supplementary Fig 4A), or in starved controls (Supplementary Fig 4B). In contrast, Achaete patterning did progress in PGX larvae supplemented with ecdysone.

To explore how patterning progressed at different concentrations of ecdysone, we again modelled the progression of Achaete patterning using a Gompertz function, fitting a linear function when the Gompertz function failed to fit (when patterning did not progress sufficiently to fit a Gompertz function). In both fed and starved females, 6.25ng/ml ecdysone was insufficient to rescue patterning. Discs from larvae fed 12.5 ng/ml ecdysone showed intermediate levels of patterning, while levels of ecdysone above 25ng/ml all rescued Achaete patterning to the same extent (Fig 8, Supplementary Table 10 and 11). Thus, unlike growth, Achaete patterning appeared to show a threshold response to ecdysone level, not initiating unless ecdysone was above a certain level (12.5-25 ng/ml), but progressed at the same rate and to the same extent regardless of how high ecdysone was above this level. Comparing the timing of Achaete patterning in ecdysone-supplemented PGX larvae versus fed controls provides some indication of when in normal development this threshold is reached. Fed control larvae were at Achaete stage 4 by 20h AEL3 (Supplementary Fig 4B), while both fed and starved PGX larvae supplemented with >25 ng/ml of ecdysone were at stage 4 by 15h AEL3 (Fig 8). This suggests that in control larvae, ecdysone levels sufficient to initiate Achaete patterning are only reached toward the middle of the third larval instar.

Senseless patterning showed a similar threshold response to increases in ecdysone, although it did not progress as far as Achaete patterning, only achieving an average of stage 3 or stage 4 by 20h AEL3 in fed and starved larvae, respectively. We again fit a linear model between time and Senseless pattern. As before, Senseless patterning did not progress in either starved or fed PGX larvae (Supplementary Fig 5). In both fed and starved females, supplemental ecdysone at or below 12.5 ng/ml was insufficient to rescue patterning (Fig 9, Supplementary

Table 12 & 13). Supplemental ecdysone at or above 25 ng/ml rescued patterning to the same extent in both fed and starved PGX larvae (Fig 9A and B), with the exception that increasing ecdysone concentrations to 100 ng/ml increased patterning rate still further in starved PGX larvae alone (Fig 9B).

As for Achaete patterning, supplemental ecdysone initiated Senseless patterning in PGX larvae early versus fed controls. Fed control larvae were at Senseless stage 2 by 20h AEL3 (Supplementary Fig 5), while both fed and starved PGX larvae supplemented with >25 ng/ml of ecdysone were at stage 2 by 10h AEL3 (Fig 9).

Discussion

Organs are remarkably good at achieving correct pattern across a broad range of environmental conditions that generate variation in size. While this might seem a simple feat when growth and patterning occur at separate times or are regulated by different hormones, it is considerably less simple if both growth and patterning occur at the same time and are regulated by the same endocrine signal. In this work, we explored how the wing discs of developing *D. melanogaster* use the same hormonal signal to coordinate both plastic growth and robust progression of pattern. We found that ecdysone simultaneously regulates the growth and patterning of the wing disc through independent mechanisms. Further, there were qualitative differences in the way patterning and growth respond to this hormone. We propose that these differences in response represent a potentially general mechanism through which high levels of variation in one trait could be coordinated with low levels variation in another within an organ.

There is some evidence that our findings apply to patterning and growth of the wings in other insect species. Previous studies have explored the relationship between wing disc size and pattern in the buckeyed butterfly, *Junonia coenia*. In *J. coenia,* the patterning stage of wing discs can be quantified by the extent of tracheal invasion into the wing pouch [73]. In this species, wing vein patterning progressed independent of wing size in starved versus fed caterpillars (Miner et al 2000). In principle, these data would also support a model of independent regulation of wing disc growth and pattern.

Independent regulation of growth and pattern is likely to arise, at least in part, from the fact that growth in the wing disc relies not only on ecdysone, but also on insulin *D. melanogaster, Manduca sexta,* and *J. coenia* [7, 74-78]. In fact, early in the third instar the growth of wing discs and ovaries relies more on insulin signalling than ecdysone signalling in *D. melanogaster* [60, 63]. From the mid-third instar onward, ecdysone becomes important for driving growth in both organs [56, 60]. This likely explains why loss of ecdysone had only minimal effects on disc growth in the first 20 hours after the moult in fed PGX larvae. Further, relying on both insulin and ecdysone for growth might represent an important means through which growth and patterning can be uncoupled.

While ecdysone and insulin signalling provide systemic cues that tune organ growth to the environmental conditions, morphogens like Wingless and Decapentaplegic (Dpp) act to regulate growth in an organ autonomous manner. The extent to which morphogen gradients respond to these systemic cues is unclear, although the activity of morphogens is known to interact with those of systemic signals at the level of the target genes. For example, insulin/TOR signalling regulates the activity of Yorkie, a downstream effector of patterning morphogens, including Wingless and Dpp, that controls the rate of cell division [79]. Similarly, reducing ecdysone signalling in the wing reduces the expression of Wingless and reduces Dpp signalling, measured by the levels of phosphorylated Mothers against Dpp expression [56, 58, 59, 80]. Further, ecdysone acts to gate when cells of the wing can respond to these morphogens, limiting the extent of wing growth [80].Taken together, the signalling pathways that regulate organ growth in response to environmental conditions interact in complex ways with those that regulate organ-autonomous growth suggesting that these two growth regulating mechanisms are not as independent as previously thought [6].

Although the growth of the disc relies on insulin and ecdysone signalling, the progression of patterning for Achaete and Senseless in the wing disc appears to be driven by threshold responses to ecdysone. This is not to say that the progression of patterning does not depend on environmental conditions. Indeed, starvation early in the third instar impedes patterning in both the wing and ovary of *D. melanogaster* [59, 60]. However, rather than resulting from a direct effect of insulin signalling on patterning, the block in the progression of pattern occurs because insulin signalling controls the timing of the first ecdysone pulse in the third larval instar [52, 81]. Our results here confirm that patterning requires suprathreshold concentrations of ecdysone to be initiated. Further, the manner in which ecdysone regulates the progression of patterning ensures that it remains robust against further environmental perturbation. By switching on pattern above threshold ecdysone concentrations, the disc can continue to pattern across a range of environmental conditions, even while growth retains sensitivity to those conditions.

A similar threshold mechanism appears to regulate patterning in the wing discs of other insects. As for *Drosophila,* the earliest stages of patterning depend on nutrition in the butterfly *J. coenia*. If caterpillars are starved before the wing discs begin to pattern, then their discs remain small and their veins unpatterned [73]. In caterpillars starved at later stages after disc patterning has been initiated, however, the wing discs are small but reach the same vein patterning stage as those of fed control animals. Whether or not the initiation of patterning in *J. coenia* also depends on ecdysone has yet to be determined.

Our data suggest that Achaete and Senseless both show threshold responses to ecdysone, but that they differ in the threshold of ecdysone that initiates patterning. While Achaete patterns advance to stage four between 0 and 15 hours after the moult to the third instar, Senseless only progresses to stage two at 15 hours [62]. Given that Achaete initiates progression at 5 hours after the moult, while ecdysone titres are just beginning to increase [52], it seems likely that Achaete requires less ecdysone to initiate patterning progression. This hypothesis is supported by the observation that feeding larvae 12.5 ng/ml of ecdysone is more-or-less sufficient to initiate Achaete patterning (albeit incompletely in starved larvae), but insufficient to initiate Senseless patterning. This difference in threshold might also explain why

Achaete patterning is able to progress somewhat in PGX larvae. While genetically ablating the PG eliminates the ability of the animal to produce ecdysone [56], we expect that it takes some time for the gland to die after larvae are shifted to the permissive temperature. In this time, the gland might already produce sufficient ecdysone to induce some progression in Achaete patterning, but not enough to induce Senseless pattern.

What determines the rate of pattering once it has been initiated is unclear, but the temporal progression for both Achaete and Senseless was similar to how these patterning stages appear in control discs. In other words, we never observed discs progressing from stage 1 immediately to stage 7 within a single 5-hour time interval even at the highest ecdysone concentrations. This suggests that there are additional temporal factors that regulate how patterning progression unfolds. Almost certainly, interactions between the gene regulatory networks that regulate patterning control how patterning progresses across regions of the wing disc. We have very little understanding if/how the different regions of the wing communicate with each other to achieve this. In principle, differences between when cells turn on Achaete and Senseless across the disc could arise in response to other developmental signals, such as from the Dpp, Wingless, or Hedgehog morphogen gradients responsible for correctly scaling and patterning the wing.

Part of this temporal signature might arise from ecdysone itself. In this study we exposed animals to tonic concentrations of ecdysone. Developing larvae, however, secrete four pulses of ecdysone between the moult to third instar and pupariation [69]. We have little understanding of how developmental information is encoded within these pulses. In principle, individual pulses could either prime tissues to become responsive to hormones, or could alter their sensitivity - as the early ecdysone pulse does for wing disc growth and patterning [59, 60, 63]. Future studies comparing the difference between tonic and phasic exposure to hormone would provide further insights into the roles of the ecdysone pulses in directing development.

Temporal dynamics have been used extensively to understand how patterning remains robust to environmental conditions in other developmental contexts, particularly embryogenesis [82, 83]. The stages of embryogenesis in species of *D. melanogaster* are both spatially and temporally robust across thermal environments [82, 83]. Even though lowering the temperature increases the total time it takes for embryos to develop until hatching, the proportion of total development time that they spend to reach each stage is the same. If temperature is switched from cool-to-hot or hot-to-cool early in embryogenesis, embryos show an initial increase in variation in the timing between stages, after which timing returns to its normal trajectory [83]. This means that embryos that are developing too fast or too slow will adjust their developmental rates to achieve temporal robustness.

In contrast to the remarkable invariance of embryonic development to changes in temperature, the patterning of wing discs in *D. melanogaster* larvae shows considerable variation across thermal conditions [62]. In this case, rather than carefully adjusting rates of patterning with the increase in developmental time, wing discs ensure correct patterning by aligning patterning at developmental milestones [62]. Patterning stages show significant variation across thermal conditions throughout most of the third instar, but overlap completely at the moult to third instar and at pupariation [62]. The contrast between tight temporal

regulation in the embryo and loose temporal regulation with alignment at milestones in the wing discs of larvae highlights how developmental context matters, and might hint at differences in the mechanisms through which temporal regulation is achieved.

While our study has focussed on contrasting the robustness of patterning with plasticity of growth, depending on what is being measured there are instances where we expect patterning to also show plasticity [84]. For example, although the specification of cell types in the correct location within an organ may show little variation across environmental conditions, the number of structures specified can vary. The total number of abdominal and sternopleural bristles varies with temperature [85, 86], as does the number of terminal filament stacks that are specified in the ovary, which is also affected by nutrition [87-90]. Plasticity in the number of bristle cells or terminal filament stacks presumably occurs because the mechanisms that specify the number of each structure do not scale with tissues size. In other cases, the location of specific cell types may also be plastic. For example, there is an extensive literature exploring how the relative positions of veins in the wings of *D. melanogaster* and other insects is affected by environmental factors such as nutrition and temperature (e.g. [91-94]). Plasticity in wing shape is likely to be more complex, and may involve process that act at many different points during wing development [95, 96]. Future studies targeting how the mechanisms that establish the position of cell types differ from those that determine the number of cells of a given type would allow us to further define what makes traits either sensitive or robust towards changes in environmental conditions, and at what level.

Materials and Methods

Fly stocks and rearing conditions

We used a transgenic combination that allowed us to genetically ablate the prothoracic gland and eliminate native ecdysone synthesis specifically in the third larval instar. We crossed a *tub-GAL80^{ts}, phantom GAL4* strain with *UAS Grim* to generate *PGX* progeny [56]. GAL80^{ts} is a repressor of GAL4 active at temperatures lower than 22ºC [65]. Rearing PGX larvae at 17ºC allows GAL80^{ts} to remain active, thus the *phantom GAL4* cannot drive the expression of *UAS grim* to promote cell death. Under these conditions, larvae can moult, pupariate, and complete metamorphosis [56]. Changing the larval rearing temperature to 29ºC disables GAL80^{ts} activity, thus ablating the prothoracic gland [56]. The progeny of the isogenic control strain, *w1118*, crossed with one of two parental lines, either *phantom*-GAL4 (*PG* > +) or *UAS Grim* (+ > *Grim*), were used as controls for genetic background effects. The parental controls were reared under the same thermal conditions as PGX larvae.

We manipulated growth rates and developmental timing by altering the rates of ecdysone synthesis in developing *D. melanogaster* larvae. To accelerate the rates of ecdysone synthesis, we used the progeny from *w^{1118}*;*phantom*-GAL4, which is expressed in the prothoracic glands, crossed with yw *flp; UAS InR29.4* (*phm > InR*). We decreased rates of ecdysone synthesis by crossing *P0206-GAL4*, which drives expression throughout the ring gland, with *yw; UAS PTEN*

(*P0206 > PTEN*). Even though *P0206*-GAL4 is a weaker GAL4 driver for the prothoracic gland and also drives expression in the corpora allata, we chose to use it to drive *UAS PTEN* because *phm>PTEN* larvae die as first instar larvae [64]. The parental lines *yw flp; UAS InR29.4* (+ > *InR*) and *yw; UAS PTEN* (+ > *PTEN*) were used as controls for the *phm > InR* and *P0206 > PTEN* genotypes respectively.

Crosses, egg collections, and larval rearing were performed on either cornmeal/molasses medium (45 g of molasses, 75 g of sucrose, 70 g of cornmeal, 10 g of agar, 1100 ml of water, and 25 ml of a 10% Nipagin solution per liter) for the experiments in Figs 2-6 or, for the experiments in Figs 7-9, on Sugar-Yeast-Agar (SYA) medium (50g of autolysed Brewer's yeast powder (MP Biomedicals), 100 g of sugar, 10 g of agar, and 1200 ml of water). In addition, we added 3 ml of proprionic acid and 3 grams of nipagen to the SYA medium to prevent bacterial and fungal growth. Egg collections were performed for 2-6 hours at 25°C or overnight at 17℃ and larvae were reared at controlled densities of 200 eggs per food plate (60 x 15 mm Petri dish filled with either cornmeal/molasses or SYA medium) in a 12 h light/dark cycle at 70% humidity. The PGX larvae and their parental controls were reared at 17℃ until the moult to third instar. All other genotypes were reared at 25℃.

Animal staging and developmental time

Larvae were staged into 1-hour cohorts at ecdysis to the third larval instar as in [52, 60, 62, 64]. To do this, food plates were flooded with 20% sucrose and all second instar larvae were transferred to a new food plate. After one hour, the food plate was flooded once again with 20% sucrose and the newly moulted third instar larvae were collected and transferred to new food plates and left to grow until the desired time interval. Newly moulted larvae were transferred to new food plates and placed at either 29℃ for the PGX and parental controls genotypes, or at 25℃ for all other genotypes. Animals were staged and their wing discs dissected at defined intervals after the larval moult as in [62]. For the ecdysone dose response experiments in Figs 7-9, we separated female and male larvae by examining them for the presence of testes, which are significantly larger and more visible even in newly moulted males.

Exogenous ecdysone feeding treatments

To show that ecdysone could rescue patterning and growth in PGX larvae, we added either 0.15 mg of the active form of ecdysone (20-hydroxyecdysone, Cayman Chemical, Item No. 16145) dissolved in ethanol, or an equivalent volume of ethanol, to 1 ml of cornmeal/molasses food. Both the ethanol- and ecdysone-supplemented food were allowed to sit at room temperature for at least 4 hours to evaporate off excess ethanol before use. Twelve larvae were transferred to one of the two supplemented foods either at 0 h AL3E and left to feed for 42 h or at 42 h AL3E and left to feed for 24 h.

To determine the relative contributions of nutrition-dependent signalling or ecdysone to growth and patterning, we fed newly moulted PGX and control larvae 1 ml of starvation medium (1% sucrose with 1% agar) supplemented with either 0.15 mg of ecdysone dissolved in

ethanol, or an equivalent volume of ethanol (Fig Suppl 4). Supplemented food was left at room temperature for at least 4 hours to evaporate excess off ethanol before use. Larvae were collected at 24 h AL3E for tissue dissection.

For the ecdysone dose response experiments, we conducted an initial pilot that showed that supplementing the food with 100 ng of ecdysone/mg food could rescue most of the Achaete and Senseless patterning in PGX wing discs. We collected newly moulted third instar larvae, separated the sexes, and then transferred them to either sucrose food (20% sucrose, 1% agar; starved) or SYA food (fed) at 29°C. We fed these larvae on one of six ecdysone concentrations: 0, 6.25, 12.5, 25, 50, or 100 ng of ecdysone/ mg food. We added the same volume of ethanol to all treatments.

To quantify the relationship between the concentration of ecdysone administered and the concentration of ecdysone in the hemolymph, we allowed newly ecdysed larvae to feed on either 20% sucrose or SYA food that had been supplemented with one of the six concentrations of ecdysone for 20 h. We then transferred them onto either sucrose food or SYA food that did not contain ecdysone but was dyed blue. They were left to feed for 2 hours until their guts were filled with blue food. This extra step was taken so that we could be sure that our hemolymph ecdysone titres were not contaminated with ecdysone from the food. Thirty – forty larvae were then weighed as a group, and transferred to 5 times their weight in volume of ice cold methanol. Larvae were homogenized and ecdysone titres were determined using a 20-Hydroxyecdysone Enzyme ImmunoAssay Kit (Cayman Chemical, Item Number 501390) as per manufacturer's instructions.

Dissections and immunocytochemistry

For each sample, 10-20 larvae were dissected on ice cold phosphate-buffered saline (PBS), the anterior halves of the body were inverted and then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature or overnight at 4°C. After fixation, the tissue was washed four times (15 minutes per wash) with 0.3% Triton X-100 in PBS (PBT), then blocked for 30 minutes at room temperature in 2% heat-inactivated normal donkey serum in PBT. After blocking, the tissue was incubated in a primary antibody solution diluted with 2% heat-inactivated normal donkey serum in PBT overnight at 4°C. We used the guinea pig anti-Senseless ([97], 1:1000) and mouse anti-Achaete (supernatant, 1:10) primary antibodies. To compare signal across tissues, we stained for both antigens simultaneously. The washing and blocking procedures were repeated after primary antibody incubation and then the tissue was incubated in a secondary antibody solution (1:200 each of anti-guinea pig (546nm) and anti-mouse (488nm)) overnight at 4°C. The tissues were washed with PBT and rinsed with PBS and then the wing imaginal discs were mounted on poly-L-lysine-coated coverslips using Fluoromount-G (SouthernBiotech). Tissues were imaged using either a Leica LSM 510 or a Nikon C1 upright confocal microscope and processed using ImageJ (version 2.0) and Adobe Photoshop CC 2017.

Quantifications of wing imaginal disc size and Achaete and Senseless pattern

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

Achaete and Senseless stage was quantified using the staging scheme developed by [62], associating each of the wing imaginal discs to an Achaete or Senseless stage varying from 1 to 7.

Statistical analysis

All the analyses were conducted in R and the annotated R markdown scripts and data for the analyses are deposited on Figshare (10.26180/13393676).

For the relationship between time and disc size (log μm^2) or disc pattern (Achaete or Senseless), we fit either linear or Gompertz models and selected the model that best fit the data, using ANOVA. The Gompertz model was parameterized as $y = ae^{-b*c^x}$, where *y* is disc size/pattern, *x* is time, *a* is the asymptote of *y*, *b* controls where along the x-axis the curve is positioned and *c* is the scaling constant, such that $c = e^{-g}$ where *g* is the growth/patterning rate (thus, the higher *g* the lower *c*). To compare the parameters of linear models between treatments and genotypes we used ANOVA. To compare the parameters of Gompertz models between treatments and genotypes, we used ANOVA to compare the fit of models that assign the same constants across groups versus models that assigned group-specific constants.

For the relationship between disc size (log μm^2) and Senseless pattern we fit a four parameter logistic model $y = a + \frac{(b-a)}{1+e^{(c-x)/d}}$ where *y* is disc pattern, *a* is the minimum asymptote, *b* is the maximum asymptote, *c* is the inflection point and *d* is the scaling constant, such that $d = 1/k$, here *k* is the logistic growth rate. We again used ANOVA to compare the fit of models that assign the same parameters across groups versus models that assigned group-specific parameters. The relationship between disc size and Achaete pattern was fit using a linear model and compared across treatments using ANOVA.

We used ANOVA to compare disc size/patten at specific time points between treatments and genotypes, using a Tukey's HSD test to allow comparison among groups.

For all parametric tests, we checked for homoscedasticity and normality of errors.

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Author's Contributions

CKM and MMO conceived the study, MMO, ANA, TK, and CKM designed the experiments, MMO and ANA collected the data, CKM and AWS completed the analysis and wrote the manuscript. All authors contributed to revising the manuscript.

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Figures

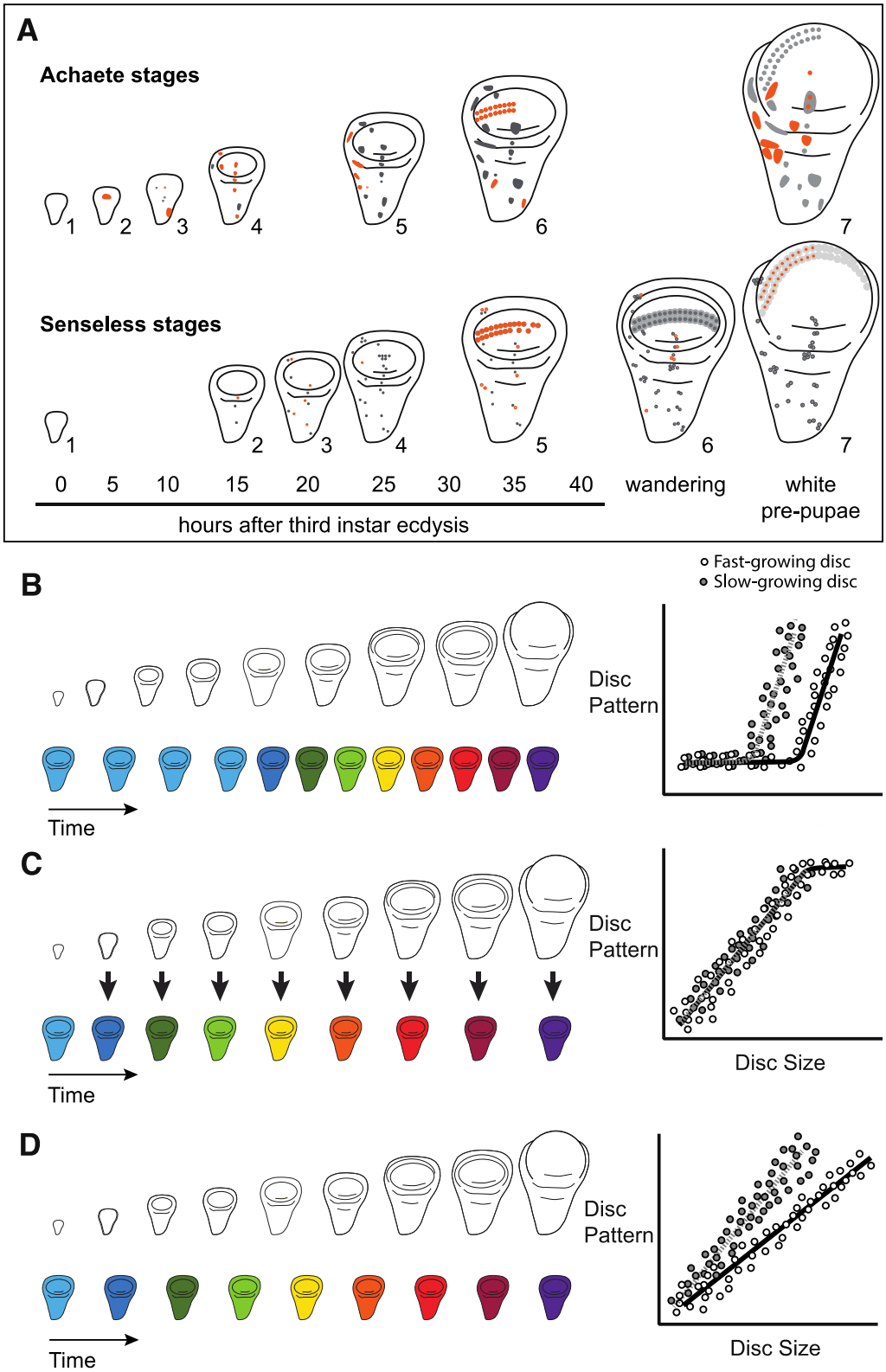
A
Achaete stages
1
2
3
4
5
6
7
Senseless stages
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
wandering
white pre-pupae
hours after third instar ecdysis
B
Fast-growing disc
Slow-growing disc
Disc Pattern
Time
C
Disc Pattern
Disc Size
Time
D
Disc Pattern
Disc Size
Time

Figure 1: Quantitative assessments of the progression of patterning allow us to test hypotheses about the relationship between the size and patterning stage of the developing wing. (A) The staging scheme developed by Oliveira *et al.* 2014 to quantify the progression of Achaete and Senseless pattern. The pattern elements shown in orange are diagnostic for each stage, which is indicated by the number beside the disc. (B) Hypothesis 1: the relationship between wing disc size and patterning stage if the discs were to grow first and then initiate pattern. (C) Hypothesis 2: If wing patterning depended on regulators of wing size (arrows) then the disc size and disc pattern would be tightly linked at these stages. (D) Hypothesis 3: If wing size and pattern were regulated independently, then we would not expect a tight relationship between them.

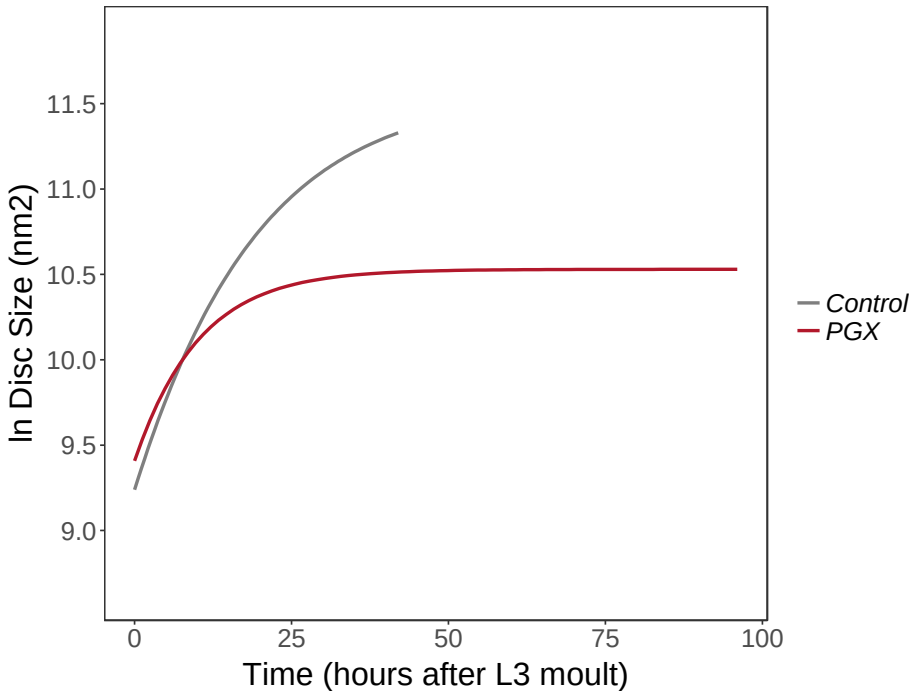

Figure 2: Growth rates of wing discs are reduced in larvae with genetically-ablated PGs (PGX) versus control larvae. Curves are Gompertz functions of disc size against time (hours after ecdysis to the third larval instar, AEL3). Parameters for the curves are significantly different between PGX and control (Supplementary Table 1). Control genotypes are the pooled results from both parental controls (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}).

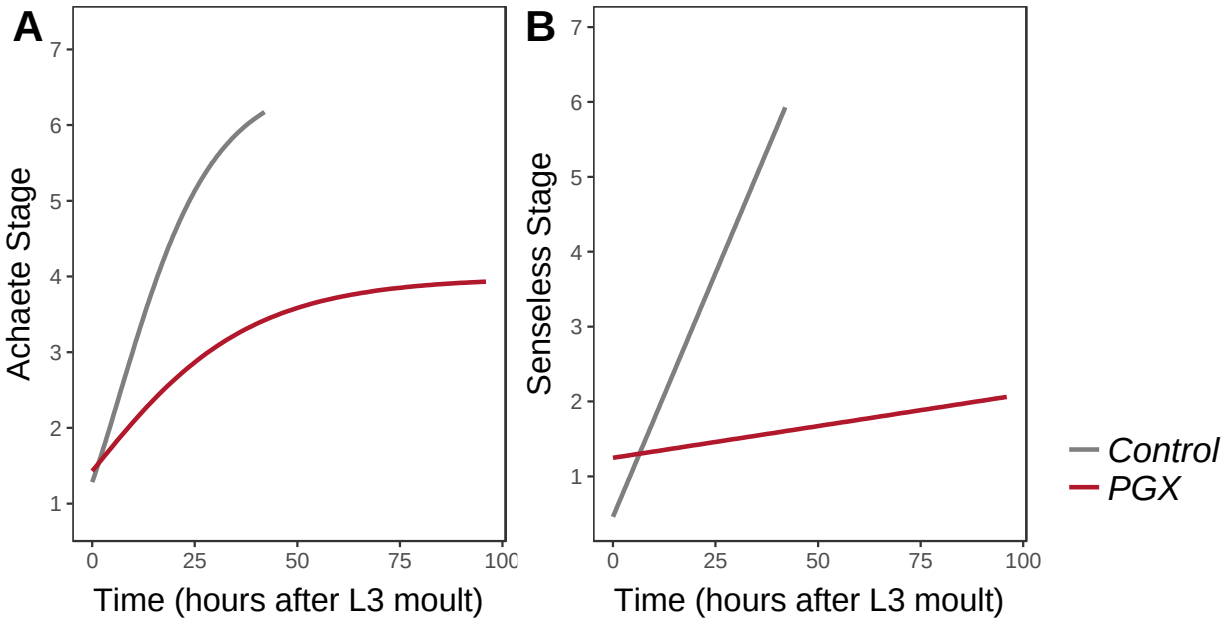

Figure 3: Achaete and Senseless patterning of wing discs is delayed in larvae with genetically-ablated PGs (PGX) versus control larvae. (A) Curves are Gompertz functions of Achaete stage against time (hours AEL3). Parameters for the curves are significantly different between PGX and control (Supplementary Table 2). (B) Lines are linear regression of Senseless stage against time (hours AEL3). Parameters for the lines are significantly different between PGX and control (Supplementary Table 3). Control genotypes are the pooled results from both parental controls (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}).

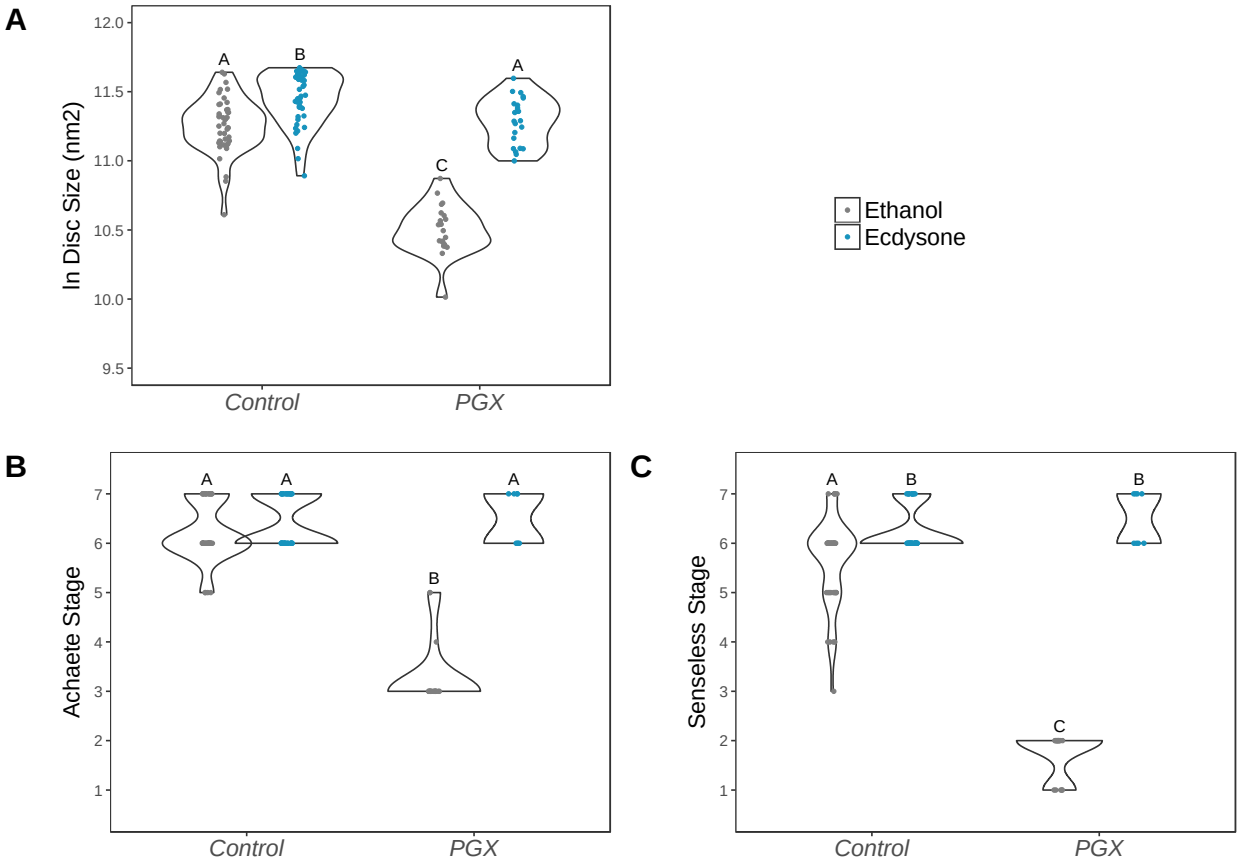

Figure 4: Supplementing PGX larvae with ecdysone rescues wing disc growth (A) and Achaete (B) and Senseless (C) patterning. Both the control and PGX larvae were exposed to ecdysone-treated food (0.15 mg of 20-hydroxyecdysone) or ethanol-treated food (which contains the same volume of ethanol) at 0 h AL3E. Wing discs were removed at 42 h AL3E. Control genotypes are the pooled results from both parental controls (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}).

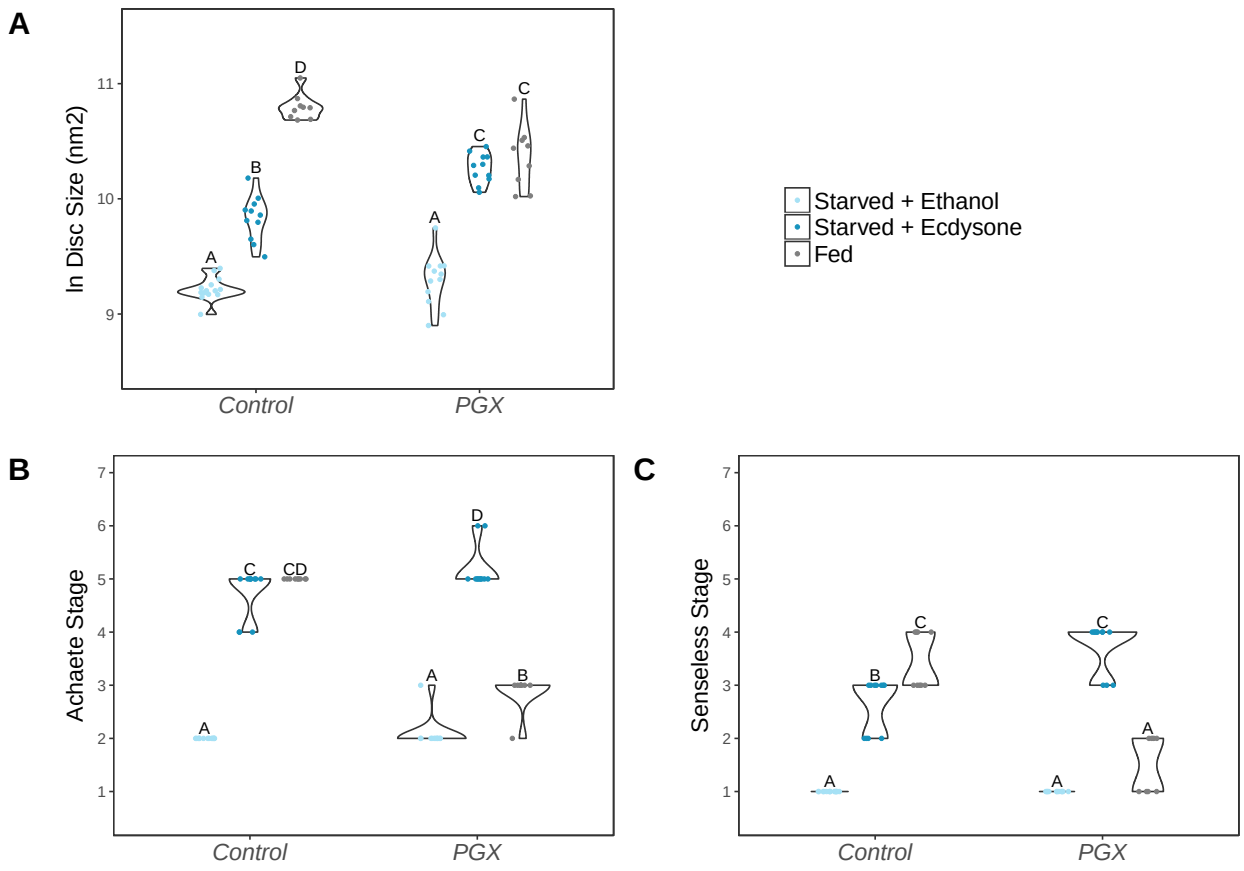

Figure 5: Supplementing PGX larvae with ecdysone is able to at least partially rescue the effect of yeast starvation on (A) wing discs growth, all fully rescue (B) Achaete, and (C) Senseless patterning. Both the control and PGX larvae were exposed from 0 h AL3E to one of three food types: 1) starved + ecdysone - starvation medium containing 1% sucrose and 1% agar supplemented with ecdysone (0.15 mg of 20-hydroxyecdysone), 2) starved + ethanol - starvation medium treated with the same volume of ethanol, or 3) fed - normal fly food. Wing discs were removed at 24 h AL3E. Control genotypes are the pooled results from both parental controls (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}). Treatments marked with different letters are significantly different (Tukey HSD, $P < 0.05$).

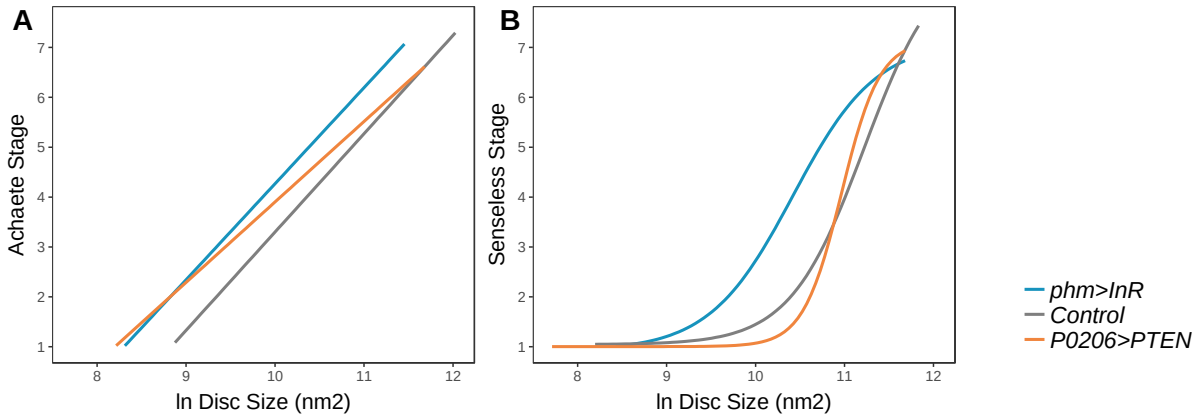

Figure 6: Changing level of ecdysteroidgenesis change the relationship between disc pattern and disc size. (A) The relationship between Achaete stage and disc size was fitted with a linear regression, the parameters of which are significantly different between genotypes (Supplementary Table 4). (B) The relationship between Senseless stage and disc size was fitted with a four-parameter logistic regression, the parameters of which are significantly different between genotypes (Supplementary Table 5). Control genotypes are the pooled results from both parental controls (either the *UAS-InR* or *UAS-PTEN* parental strain crossed to w^{1118}).

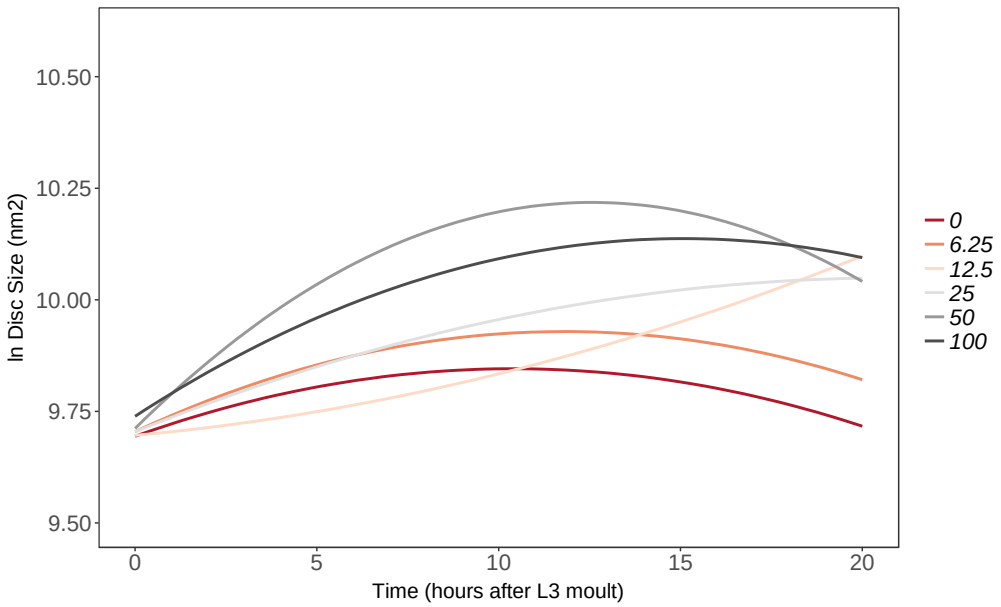

Figure 7: Effect of supplemental ecdysone dose on growth of the wing imaginal disc in starved PGX larvae. Growth was modelled as $S = E + T + T^2 + E*T + E*T^2$, where S = disc size, E = ecdysone concentration, and T = Time. There was a significant effect of E on the linear growth rate of the wing imaginal discs (Supplementary Table 8 and 9).

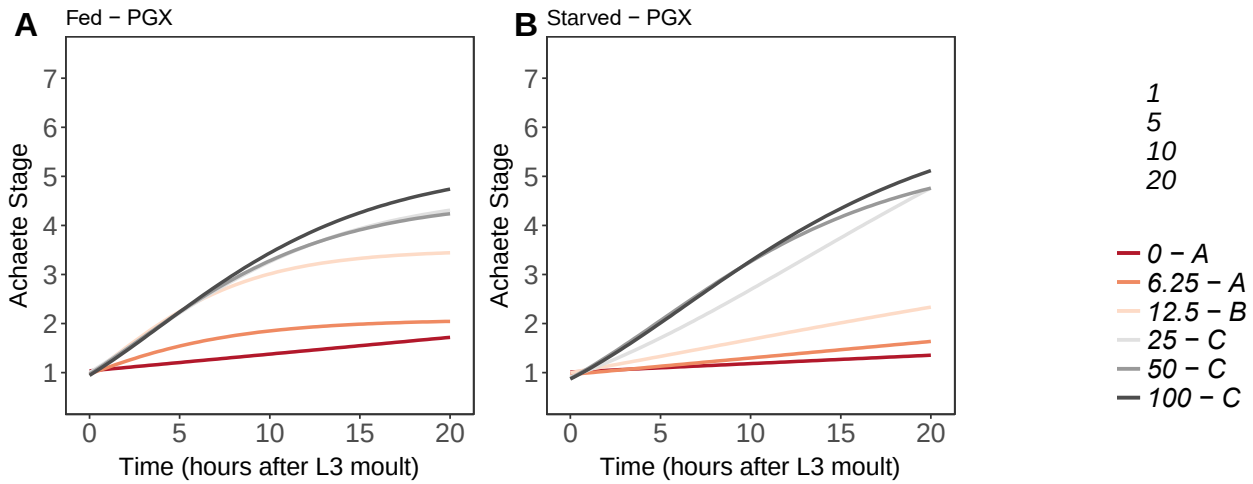

Figure 8: Effect of supplemental ecdysone dose on Achaete patterning of the wing imaginal disc in (A) fed and (B) starved PGX larvae. Larvae were fed on normal food, or starved of yeast on a 20% sucrose, 1% agar medium. Achaete stage is modelled either as a Gompertz or a linear function (where insufficient patterning occurred to fit a Gompertz function). Lines with different letters (see legend) are significantly different. See supplementary tables 9 and 10 for statistical details.

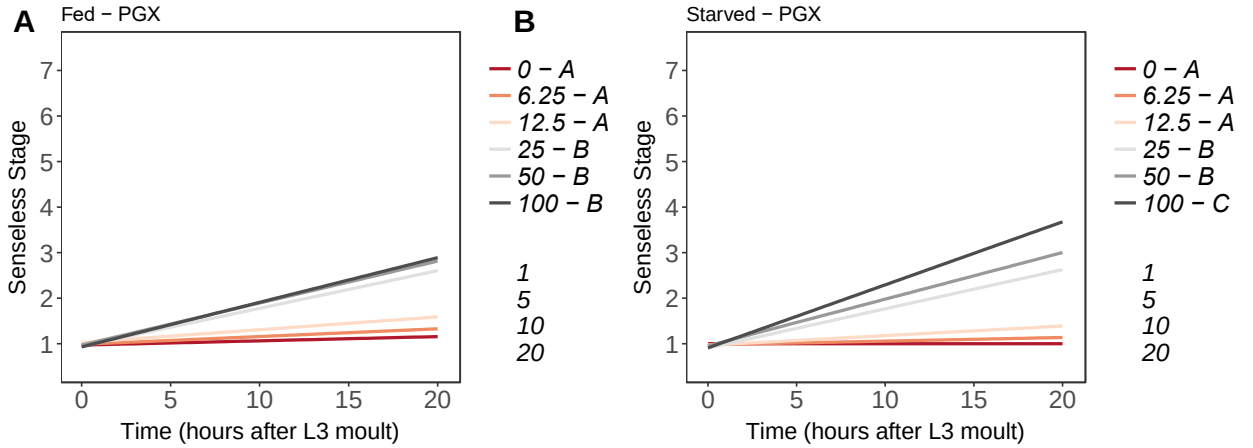

Figure 9: Effect of supplemental ecdysone dose on Senseless patterning of the wing imaginal disc in (A) fed and (B) starved PGX larvae. Growth is modelled as a linear function. Lines with different letters (see legend) are significantly different. See Supplementary Tables 12 and 13 for statistical details.