Ecdysone coordinates plastic growth with robust pattern in the developing wing

André Nogueira Alves[1,2*], Marisa Mateus Oliveira[1*], Takashi Koyama[1,3], Alexander Shingleton[4Ψ], Christen Mirth[1,2Ψ]

1 Instituto Gulbenkian de Ciência, Oeiras, Portugal

2 School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

3 Department of Biology, University of Copenhagen, Copenhagen, Denmark

4 Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

* These authors contributed equally to this work.

Ψ Co-senior authors.

Keywords: phenotypic plasticity, developmental robustness, growth rates, patterning rates, Achaete, Senseless, wing disc, *Drosophila melanogaster*

Running title: Plastic Growth & Robust Pattern

Abstract

Animals develop in unpredictable, variable environments. In response to environmental change some aspects of development adjust to generate plastic phenotypes. Other aspects of development, however, are buffered against environmental change to produce robust phenotypes. How organ development is coordinated to accommodate both plastic and robust developmental responses is poorly understood. Here, we demonstrate that the steroid hormone ecdysone coordinates both plasticity of organ size and robustness of organ pattern in the developing wings of the fruit fly *Drosophila melanogaster*. Using fed and starved larvae that lack prothoracic glands, which synthesise ecdysone, we show that nutrition regulates growth both via ecdysone and via an ecdysone-independent mechanism, while nutrition regulates patterning only via ecdysone. We then demonstrate that growth shows a graded response to ecdysone concentration, while patterning shows a threshold response. Collectively, these data support a model where nutritionally-regulated ecdysone fluctuations confer plasticity by regulating disc growth in response to basal ecdysone levels, and confers robustness by initiating patterning only once ecdysone peaks exceeds a threshold concentration. This could represent 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]. Other aspects of their phenotype, however, 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 patterning 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.

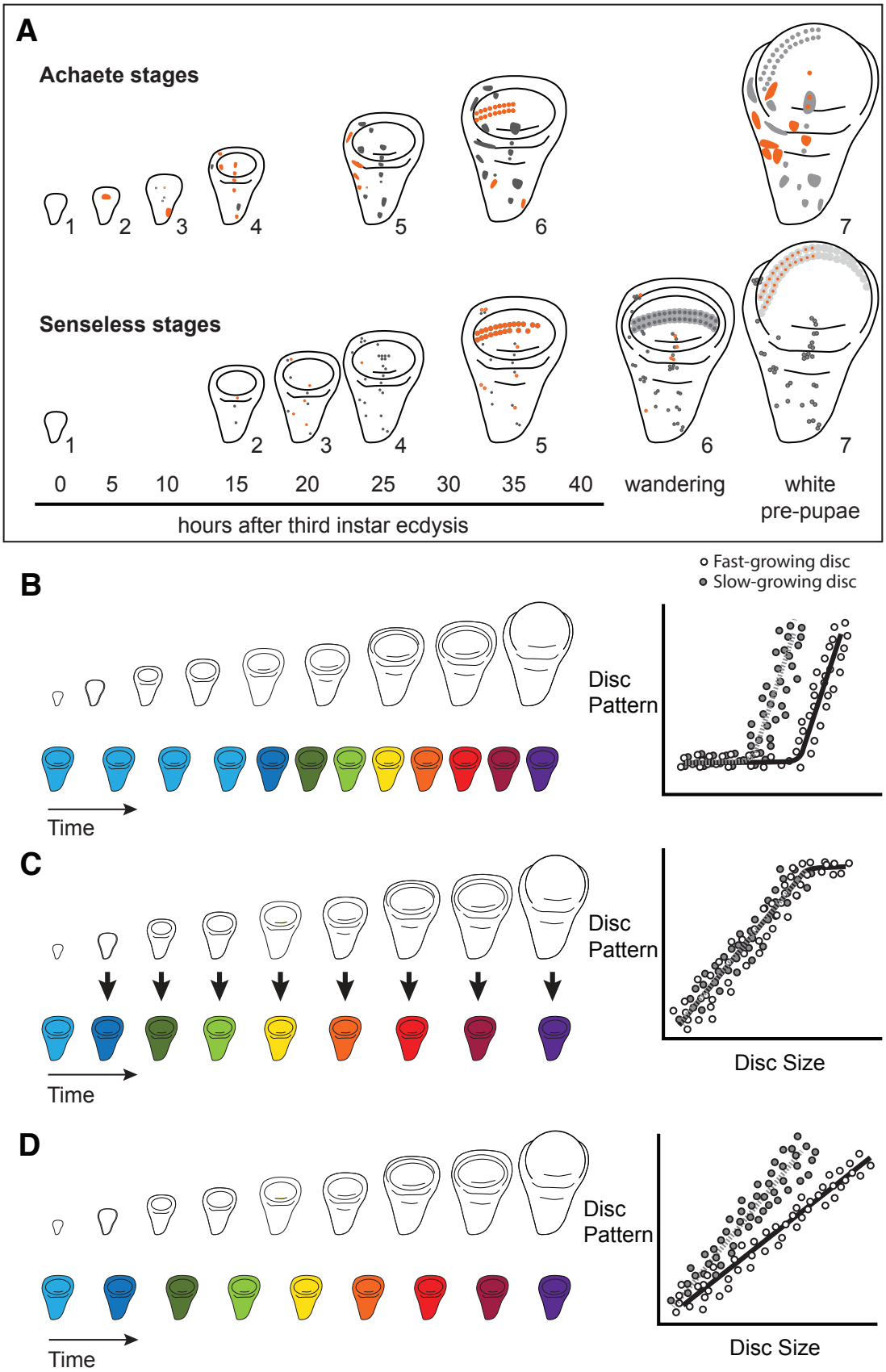

A
Achaete stages
1
2
3
4
5
6
7
Senseless stages
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
wandering
white pre-pupae
hours after third instar ecdysis
B
Fast-growing disc
Slow-growing disc
Disc Pattern
Time
C
Disc Pattern
Disc Size
Time
D
Disc Pattern
Disc Size
Time

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 [42] 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-D) The relationship between wing disc size and patterning stage (represented as wing discs progressing through a series of colours) if; (B) Hypothesis 1: Wing discs grow first and then initiate pattern; (C) Hypothesis 2: Wing disc patterning is regulated by wing disc size (arrows); (D) Hypothesis 3: Wing disc pattern and growth are regulated at least partially independently.

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 [43-45]. Insulin-like peptides bind to the Insulin Receptor in target tissues and activate the insulin signalling cascade, ultimately leading to increased growth [44, 46, 47]. Starvation reduces the concentration of insulin-like peptides in the bloodstream, and the resulting decrease in insulin signalling causes organs to grow more slowly [45, 48].

While changes in insulin signalling are known to affect organ size, they have little effect on organ pattern [49]. 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 [50-53]. 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 [50-54].

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 glands that produce ecdysone, the prothoracic glands (PG), are 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 [42]. Two of these patterning gene products, Achaete and Senseless, can be classed into seven different stages throughout third instar development ([42], Figure 1A), providing us with the ability to quantify the progression of wing disc pattern over a variety of conditions. In short, by describing patterning on a near continuous scale, our scheme not only allows us to determine under what conditions patterning is initiated, but also the rate at which it progresses.

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 (Figure 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 [62]. 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, with different aspects of patterning being initiated at different disc sizes (Figure 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 and quantitively different manner to ecdysone [6]. As an example of this, we might see that growth rates increase in a graded response to increasing ecdysone while patterning shows threshold responses, or vice versa. If this were the case, we would

159 expect that growth and the progression of pattern would be uncoupled over time
160 (Figure 1D).

161 Here we test these hypotheses of whether and how ecdysone co-regulates
162 plastic growth and robust pattern in wing imaginal discs in *D. melanogaster*. We
163 blocked the production of ecdysone by genetically ablating the PG [56], and
164 quantified the effects on growth and patterning rates throughout the third instar. We
165 then manipulated the rate of ecdysone synthesis, by up- or down-regulating the
166 activity of the insulin-signalling pathway in the PG [52, 53], to test how this alters the
167 relationship between disc size and disc pattern. Finally, we tested our hypotheses
168 about how a single steroid can regulate both plastic growth and robust patterning by
169 conducting dose response experiments under two nutritional conditions. These
170 studies provide a foundation for a broader understanding of how developmental
171 hormones coordinate both plastic and robust responses across varying
172 environmental conditions during animal development.

173

174 Results

175 Ecdysone is necessary for the progression of growth and 176 patterning

177 To understand how ecdysone affects the dynamics of growth and patterning,
178 we needed to be able to precisely manipulate ecdysone concentrations. For this
179 reason, we made use of a technique we developed previously to genetically ablate
180 the prothoracic glands (referred to as PGX) [56]. This technique pairs the temperature
181 sensitive repressor of GAL4, GAL80^{ts}, with a prothoracic gland-specific GAL4 (*phm-*
182 *GAL4*) to drive an apoptosis-inducing gene (*UAS-GRIM*). GAL80^{ts} is active at 17°C,
183 where it represses GAL4 action, but inactive above 25°C, which allows *phm-GAL4* to
184 drive expression of *UAS-GRIM* and ablate the PG [63, 64]. Because ecdysone is
185 required at every moult, we reared larvae from egg to third instar at 17°C to repress
186 GAL4, then shifted the larvae to 29°C at the moult to the third instar to generate PGX
187 larvae.

188 PGX larvae had significantly reduced ecdysteroid titres than control genotypes
189 (Figure 2 Supplement 1). This method of reducing ecdysteroid concentration in the
190 larvae allows us to examine how reducing ecdysone titres affects disc size and pattern
191 in third instar wing imaginal discs, and to manipulate ecdysone concentrations by
192 adding it back in specific concentrations to the food [56]. For simplicity, all the data

193 from the two control strains (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental
194 strain crossed to w^{1118}), were pooled in all analyses.

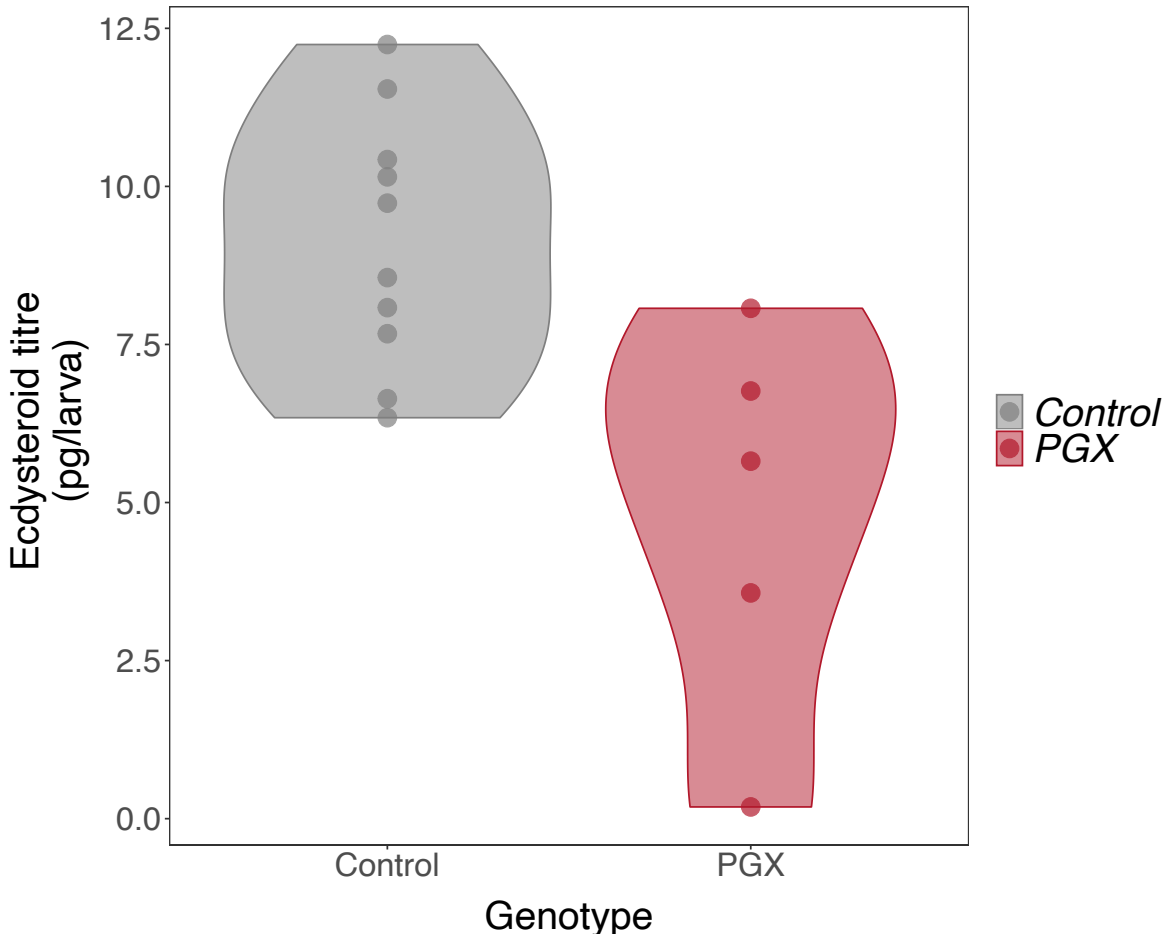


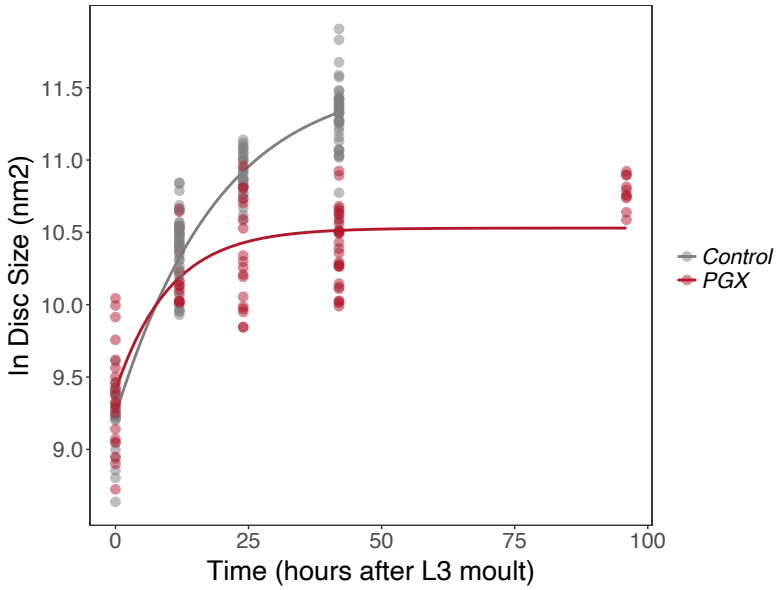
Figure 2 Supplement 1: Ecdysteroid titres in PGX and control larvae. Newly ecdysed larvae were placed on sucrose/yeast diets. Ecdysteroid titres in control (phm > + and + > grim) are significantly higher ecdysteroid titres than PGX larvae ,as determined by linear models and pairwise comparisons of the means (F $value_{1, 13}$ = 10.75, p value = 0.006, $N_{control}$ = 10, N_{PGX} = 5). Data is plotted with violin plots and the individual replicates (5 per genotype) are included as points overlaying the violin plots.

195

196 Insect wing discs show damped exponential, or fast-then-slow, growth
197 dynamics [62, 65]. These types of growth dynamics have frequently been modelled
198 using a Gompertz function, which assumes that exponential growth rates slow down
199 with time. The growth of wing discs from control and PGX larvae shows the same
200 pattern, with a Gompertz function providing a significantly better fit to the
201 relationship between log disc size and time than a linear function (ANOVA, linear vs.
202 Gompertz model, n> 93, F>65, P<0.001 for discs from both PGX and control larvae).

203 Growth of the discs, however, followed a significantly different trajectory in PGX
204 versus control larvae (Figure 2, Supplementary Table 1). In control larvae, discs
205 continue to grow until 42h after ecdysis to the third instar (AEL3), when the larvae
206 pupariate. In contrast, the wing imaginal discs of the PGX larvae grow at slower rates
207 between 0 and 25h AEL3 (Supplementary Table 1) and stop growing at
208 approximately 25h AEL3 at a significantly smaller size. This is despite the fact that
209 PGX larvae do not pupariate, and so disc growth is not truncated by metamorphosis.

210



211

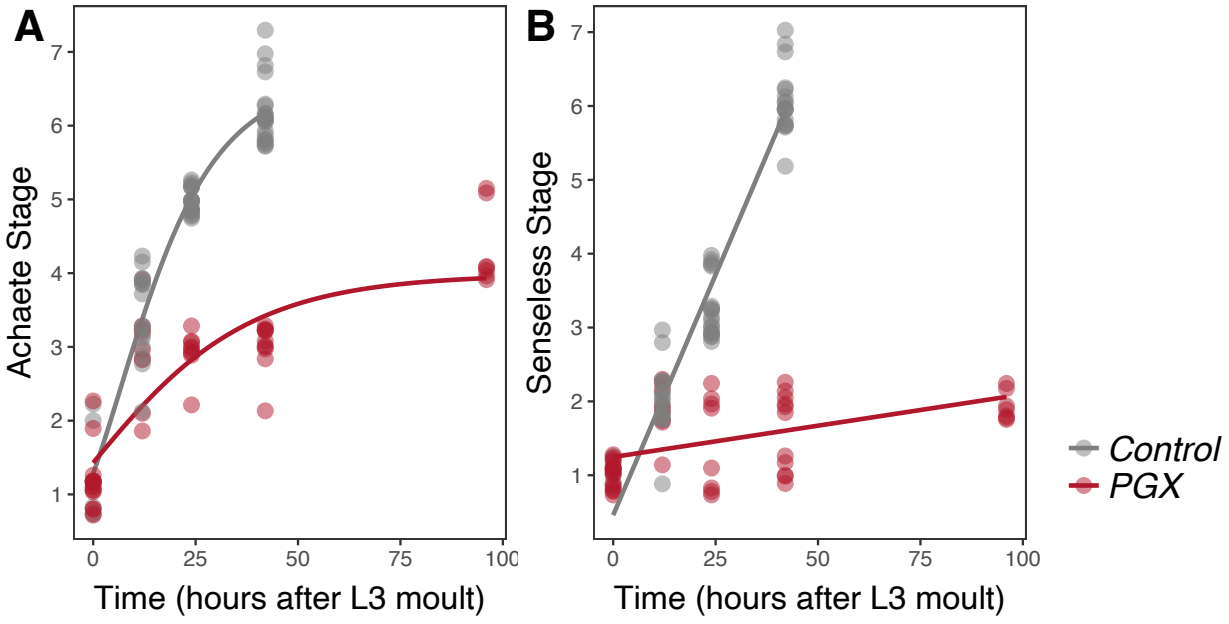
212 **Figure 2:** Growth rates of wing discs are reduced in larvae with genetically-ablated PG
213 (PGX) versus control larvae. Curves are Gompertz functions of disc size against disc age
214 (hours after ecdysis to the third larval instar, AEL3). Parameters for the curves are significantly
215 different between PGX and control (Supplementary Table 1). Control genotypes are the
216 pooled results from both parental controls (either the ***phm-GAL4; GAL80ts*** or ***UAS-GRIM***
217 parental strain crossed to w^{1118}). Each point represents the size of an individual wing disc.
218 N_{PGX} = 95, $N_{Control}$ = 125 across all time points.

219

220 We next explored how loss of ecdysone affected the progression of wing
221 patterning. We used the staging scheme that we previously devised in [42] to
222 quantify the progression of wing disc patterning in PGX and control larvae. We
223 selected two genes from this scheme, Achaete and Senseless, as they each progress
224 through seven stages throughout the third instar. Further we can stain for both
225 antigens in the same discs, which allowed us to compare disc size, Achaete stage,
226 and Senseless stage in the same sample.

227 The progression of Achaete patterning was best fit by a Gompertz function for
228 discs from both PGX and control larvae (ANOVA, linear versus Gompertz model, $n>$
229 48, $F>10.4$, $P=0.002$) [42], and was significantly affected by reduced ecdysone titres
230 in PGX larvae. In control larvae, the wing discs progressed to Achaete stage 6 or 7 out
231 of 7 stages by 42h AEL3, while in PGX larvae, discs of the same age had not passed
232 Achaete stage 3, and had not matured past Achaete stage 5 by 92h AEL3 (Figure 3A,
233 Supplementary Table 2). The progression of Senseless patterning was best fit by a
234 linear model, but again was significantly affected by reduced ecdysone titres. In
235 control larvae, most discs had progressed to Senseless stage 6 out of seven stages by
236 42h AEL3, while no disc progressed past Senseless stage 2 by 92h AEL3 (Figure 3B,
237 Supplementary Table 3).

238 We find no evidence of temporal separation between wing disc growth and
239 the progression of pattern (compare Figures 2 and 3). Both growth and patterning
240 progress at steady rates throughout most of the third instar in control larvae, slowing
241 down only at the later stages of development. Thus, the hypothesis that ecdysone
242 coordinates plastic growth with robust pattern by acting on each process at different
243 times (Figure 1C; Hypothesis 1) is not correct.



244

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

crossed to w^{1118}). For Achaete: N_{PGX} = 50, $N_{Control}$ = 61, for Senseless: N_{PGX} = 52, $N_{Control}$ = 54 across all time points.

To confirm that reduced ecdysone titres were 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 (20E), or ethanol (the carrier) back to the food. PGX and control larvae were transferred onto either 20E 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 (Figure 4A) and showed reduced patterning for both Achaete (Figure 4B) and Senseless (Figure 4C) when compared to control genotypes. Adding 0.15 mg of 20E/mg food fully restored disc size, and Achaete and Senseless pattern, such that they were indistinguishable from control genotypes fed on ecdysone-treated food.

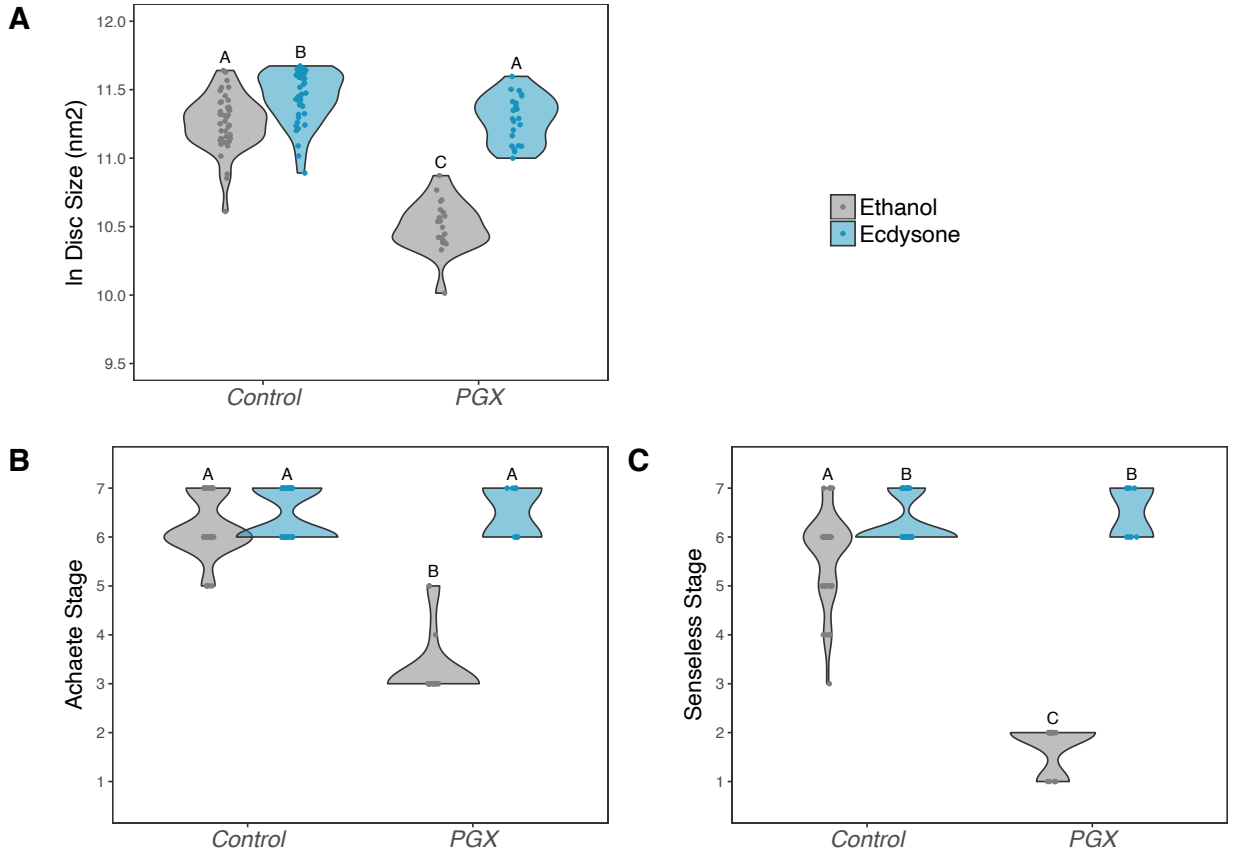


Figure 4: Supplementing PGX larvae with 20-hydroxyecdysone (20E) rescues wing disc growth (A), and Achaete (B) and Senseless (C) patterning. Both the control and PGX larvae were exposed to 20E-treated food (0.15 mg/mg of food) or ethanol-treated food (which contains the same volume of ethanol) at 0 h AL3E. Wing discs were removed at 42 h

271 AL3E. Control genotypes are the pooled results from both parental controls (either the ***phm-***
272 ***GAL4; GAL80ts*** or ***UAS-GRIM*** parental strain crossed to w^{1118}). Treatments marked with
273 different letters are significantly different (Tukey HSD, $P < 0.05$, for ANOVA see
274 Supplementary Table 4). Data were plotted using violin plots with individual wing discs
275 displayed over the plots. $N_{PGX + ethanol} = 21$, $N_{PGX + 20E} = 23$, $N_{Control + ethanol} = 43$, $N_{Control + 20E} = 42$.

276

277 Collectively, these data indicate that ecdysone is necessary for the normal
278 progression of growth and patterning in wing imaginal discs. The loss of ecdysone
279 has a more potent effect on patterning, however, which is effectively shut-down in
280 PGX larvae, than on disc growth, which continues, albeit at a slower rate, for the first
281 24 hours of the third instar in PGX larvae.

282 Ecdysone rescues patterning and some growth in wing discs of 283 yeast-starved larvae

284 The observation that ecdysone is necessary to drive both normal growth and
285 patterning suggests that it may play a role in coordinating growth and patterning
286 across environmental conditions. However, to do so it must lie downstream of the
287 physiological mechanisms that sense and respond to environmental change. As
288 discussed above, ecdysone synthesis is regulated by the activity of the insulin-
289 signalling pathway, which is in turn regulated by nutrition. Starving larvae of yeast
290 early in the third instar both suppresses insulin-signalling and inhibits growth and
291 patterning of organs [59, 60]. We explored whether ecdysone was able to rescue
292 some of this inhibition by transferring larvae immediately after the moult to 1%
293 sucrose food that contained either 20E or ethanol and comparing their growth and
294 patterning after 24 hours to wing discs from larvae fed on normal food. Both the PGX
295 and control genotype failed to grow and pattern on the 1% sucrose with ethanol
296 (Figure 5A-C). Adding 20E to the 1% sucrose food rescued Achaete and Senseless
297 patterning in both the control and the PGX larvae to levels seen in fed controls
298 (Figure 5B, C). 20E also partially rescued disc growth in PGX larvae, although not to
299 the levels of the fed controls (Figure 5A). Collectively, these data suggest that the
300 effect of nutrition on growth and patterning is at least partially mediated through
301 ecdysone.

302 An important aspect of these data is that in PGX larvae, either supplementing
303 the 1% sucrose food with 20E or feeding them on normal food both rescued wing
304 disc growth (Figure 5A), albeit incompletely. This suggests that nutrition can drive
305 growth through mechanisms independent of ecdysone, and vice versa. In contrast,

306 nutrition alone only marginally promoted Achaete and Senseless patterning in
307 starved PGX larvae, while 20E alone completely restored patterning. Further, even
308 early patterning does not progress in PGX larvae (Figure 3). Thus, the effect of
309 nutrition on patterning appears to be wholly mediated by ecdysone, while the effect
310 of nutrition on growth appears to be partially mediated by ecdysone and partially
311 through another independent mechanism. Ecdysone-independent growth appears to
312 occur early in the third larval instar, however, since disc growth in PGX and control
313 larvae is more-or-less the same in the first 12 hours after ecdysis to L3 (Figure 2).

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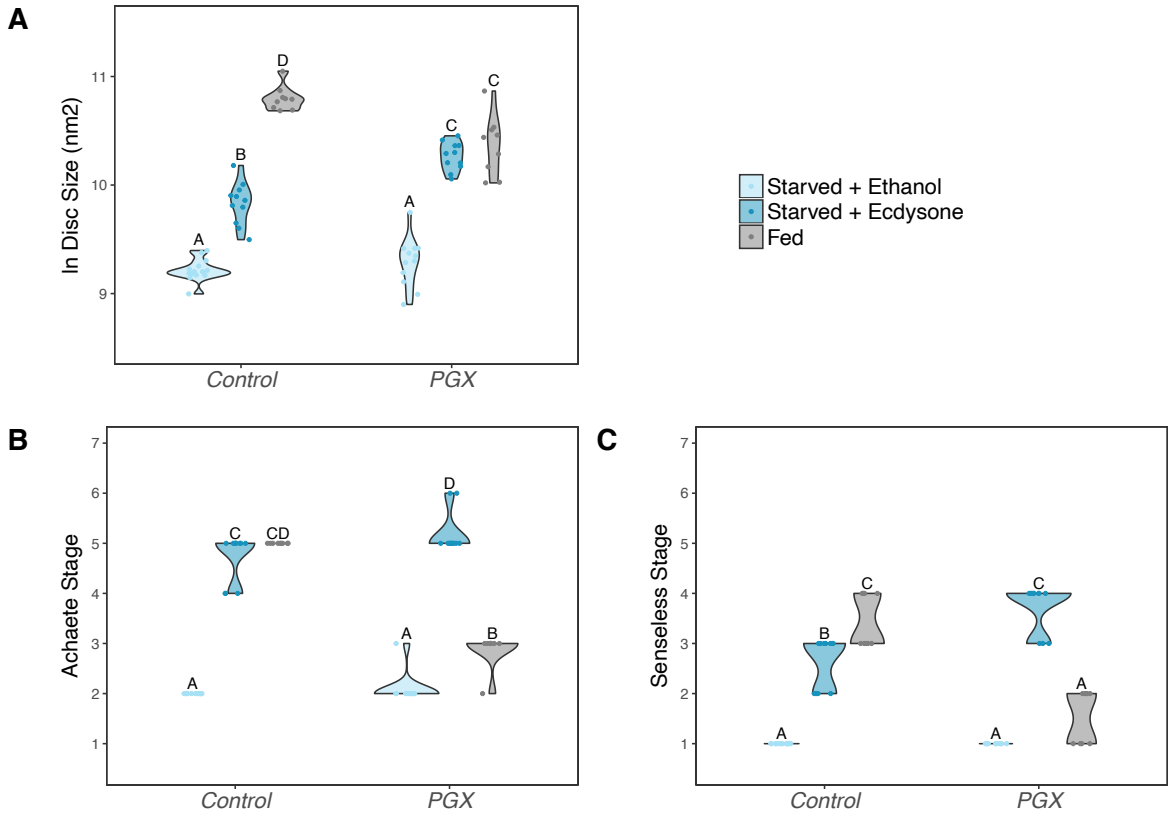


315

316 **Figure 5:** Supplementing PGX larvae with 20-hydroxyecdysone (20E) is able to
317 partially rescue the effect of yeast starvation on (A) wing discs growth, and fully rescue (B)
318 Achaete, and (C) Senseless patterning. Both the control and PGX larvae were exposed from 0
319 h AL3E to one of three food types: 1) starved + 20E - starvation medium containing 1%
320 sucrose and 1% agar laced with 20E (0.15 mg/mg of food), 2) starved + ethanol - starvation
321 medium treated with the same volume of ethanol, or 3) fed - normal fly food. Wing discs were
322 removed at 24 h AL3E. Control genotypes are the pooled results from both parental controls
323 (the *UAS-GRIM* parental strain crossed to w^{1118}). Treatments marked with different letters are
324 significantly different (Tukey HSD, $P < 0.05$, for ANOVA see Supplementary Table 5). Data

were plotted using violin plots with individual wing discs displayed over the plots. $N_{PGX + starved - ethanol} = 23$, $N_{PGX + starved - 20E} = 22$, $N_{PGX + fed} = 26$, $N_{Control + starved-ethanol} = 28$, $N_{Control + starved-20E} = 22$, $N_{Control + fed} = 27$.

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, 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 level of circulating ecdysone, respectively [50, 53, 66, 67]. 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 found that a linear model is sufficient to capture the relationship between disc size and Achaete stage when we either increase (*phm>InR*: $AIC_{linear} - AIC_{logistic} = 22$, ANOVA, $F_{(25,27)} = 1.71$, $P = 0.2018$) or decrease ecdysone synthesis rates (*P0206>PTEN*). Changing ecdysone levels, however, significantly changed the parameters of the linear model and altered the relationship between disc size and Achaete pattern. Specifically, increasing ecdysone level shifted the relationship so that later stages of Achaete patterning occurred in smaller discs (Figure 6A, Supplementary Table 6).

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 than a linear function ($AIC_{linear} - AIC_{logistic} = 32.2$; ANOVA, $F_{(44,46)} = 25.8$, $P < 0.001$). Changing ecdysone levels significantly changed the parameters of the logistic model and altered the relationship between disc size and Senseless pattern (Figure 6B, Supplementary Table 7). Again, increasing ecdysone level 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, and that patterning is not regulated by wing disc size (Figure 1D; Hypothesis 2).

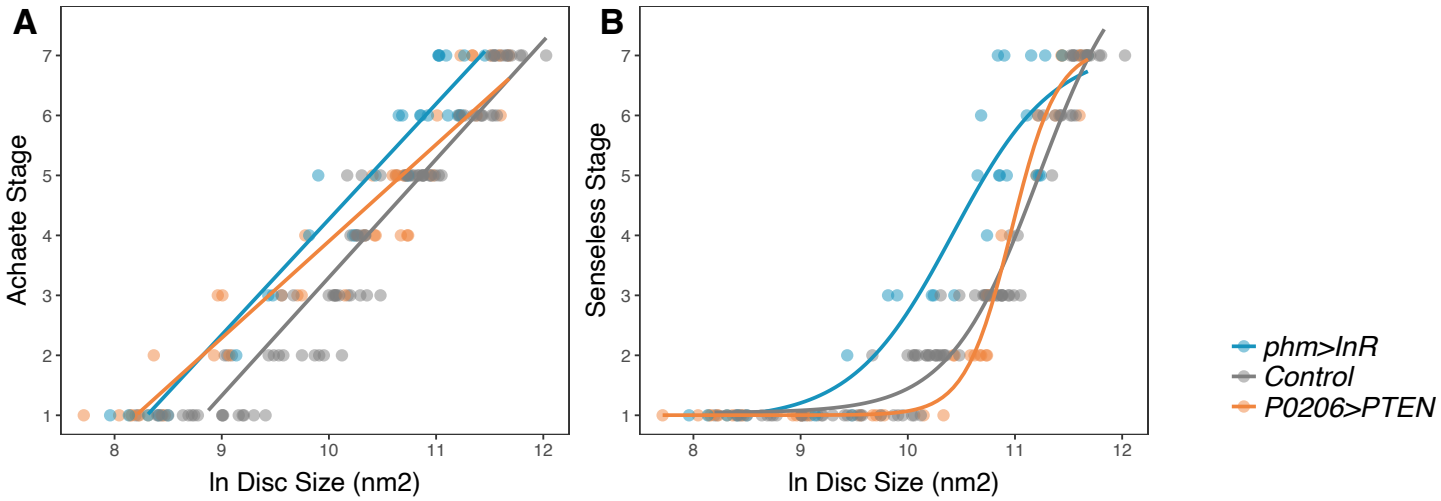


Figure 6: Changing level of ecdysteroidgenesis change the relationship between disc pattern and disc size throughout the third instar larval stage. (A) The relationship between Achaete stage and disc size was fitted with a linear regression, the parameters of which are significantly different between genotypes (Supplementary Table 6). (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 7). We staged third instar larvae from the onset of the moult to the formation of white prepupae. The length of this developmental interval varied across genotypes. For control genotypes, we sampled larvae at 0, 10, 20, 30, 48, and 51 (InR control)/53 (PTEN control) h AL3E. For phm>InR, we sampled larvae at 0, 10, 20, 29, 30, and 36 h AL3E. For P0206>PTEN, we sampled larvae at 0, 10, 20, 30, 40, 60, 73, and 80 h AL3E. The number of discs sampled for each genotype and patterning gene was: for Acheate: $N_{phm>InR} = 29$, $N_{Control} = 99$, $N_{P0206>PTEN} = 40$, for Senseless: $N_{phm>InR} = 29$, $N_{Control} = 99$, $N_{P0206>PTEN} = 41$.

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, growth is also regulated by an ecdysone independent mechanism, enabling similar progressions of pattern across discs of different sizes. An added nuance, however, is that ecdysone levels are not constant throughout development. Rather, the ecdysone titre fluctuates through a series of peaks throughout the third larval instar and the dynamics of these fluctuations are environmentally sensitive [68]. To gain further insight into how ecdysone co-regulates plasticity and robustness, we therefore explored which aspects of ecdysone dynamics regulate growth and patterning.

Two characteristics of ecdysone 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 [53, 67-69]. Second, the basal levels of ecdysone appear to regulate the rate of body growth, with an increase in basal level leading to a reduction in body growth [50, 56, 66, 67, 70]. While several studies, including this one, have established that disc growth is positively regulated by ecdysone [56, 58, 71], whether disc growth is driven by basal levels or peaks of ecdysone is unknown.

There are a number of hypotheses as to how ecdysone levels may drive patterning and growth. One hypothesis is that patterning and ecdysone-regulated disc growth show threshold responses, which are initiated once ecdysone rises above a certain level. This would manifest as low patterning and growth rates when ecdysone titres were sub-threshold, and a sharp, switch-like increase in patterning and growth rates after threshold ecdysone concentrations were reached. Alternatively, both may show a graded response, with patterning and growth rates increasing continuously with increasing ecdysone titres. Finally, disc patterning may show one type of response to ecdysone, while disc growth may show another. Separating these hypotheses requires the ability to titrate levels of ecdysone.

413 To do this, we reared PGX larvae on standard food supplemented with a range of 20E
414 concentrations (0, 6.25, 12.5, 25, 50 and 100 ng of ecdysone/mg of food). However,
415 as noted above, disc growth early in the third larval instar is only moderately affected
416 by ablation of the PG, potentially obfuscating the effects of supplemental 20E. In
417 contrast, discs from starved PGX larvae show no growth or patterning without
418 supplemental 20E. We therefore also reared PGX larvae on either on standard food

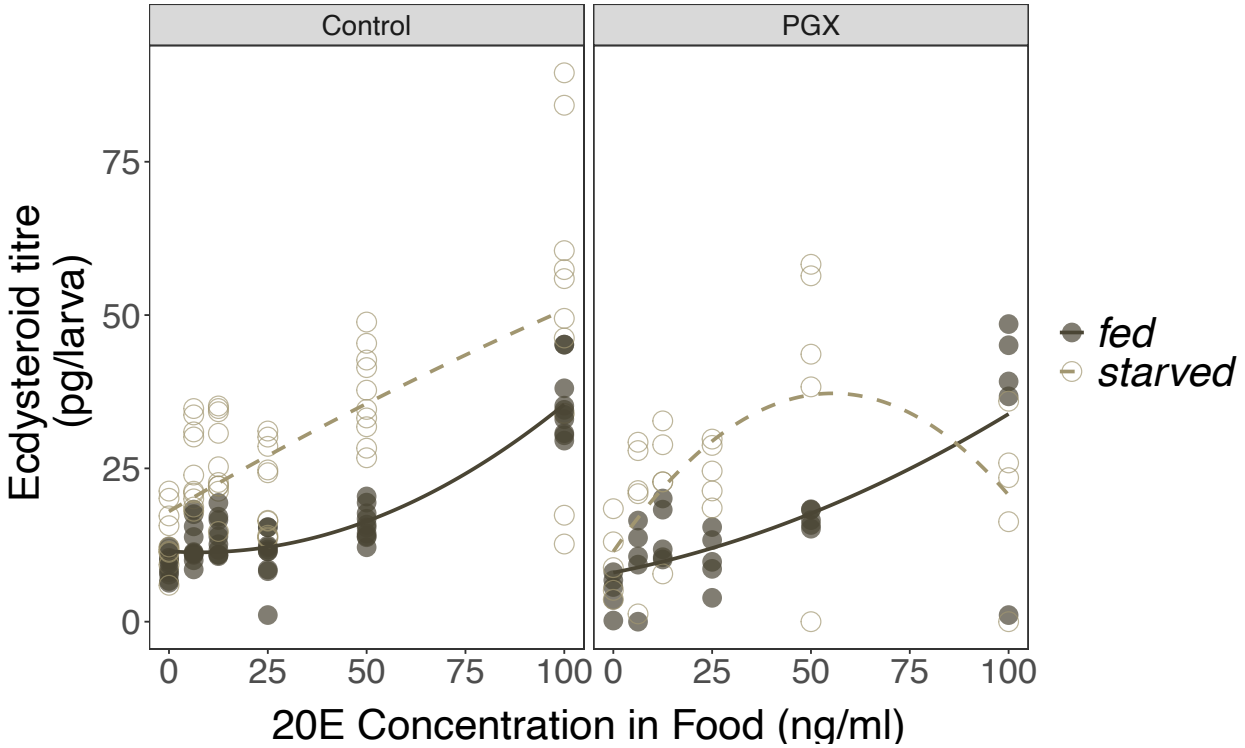


Figure 7 Supplement 1: The effects of 20E concentration in the food on ecdysteroid titres in control and PGX larvae. Newly ecdysed larvae were placed on sucrose/yeast (fed)or 20% sucrose/1% agar (starved) diets supplemented with a range of 20E concentrations for 20h. They were then transferred to the same diet without 20E and dyed with blue food colouring for 2 h, to eliminate residual 20E in the gut. Ecdysteroid titres in fed and starved control (phm > + and + > grim) and PGX larvae fed a range of 20E concentrations. There is a significant positive relationship between 20E concentration in the food and the concentration of ecdysteroids in the larvae, as indicated by a significant 20E term (Supplementary Table 8). Furthermore, starved larvae had higher ecdysone titres than fed larvae. Open and closed points represent the biological replicates, and solid and dashed lines are linear regressions. $N_{control - starved}$ = 60, $N_{control - fed}$ = 60, $N_{PGX - starved}$ = 30, $N_{PGX - fed}$ = 30 across all 20E concentrations.

419 or on 20% sucrose / 1% agar medium (from here on referred to as 'starved' larvae)
420 supplemented with a range of 20E concentrations. For both control genotypes and

421 PGX larvae, increasing the concentration of 20E in the food increased ecdysteroids
422 titres in the larvae (Figure 7 Supplement 1, Supplementary Table 6).

423 To quantify the effects of 20E concentration in the on wing disc growth, we
424 dissected discs at 5 h intervals starting immediately after the moult to the third instar
425 (0 h AL3E) to 20 h AL3E. Because male and female larvae show differences in wing
426 disc growth [72], we separated the sexes in this experiment and focused our analysis
427 on female wing discs.

428 As before, in both PGX and control larvae, wing disc growth was suppressed by
429 starvation (Figure 7 Supplement 2, Supplementary Table 9). To explore how disc size
430 changed over time with increasing 20E concentration, we modelled the data using a
431 second order polynomial regression for time after third instar ecdysis. Increasing the
432 concentration of supplemental 20E increased the disc growth rate in starved PGX
433 larvae (Figure 7A, Supplementary Table 10). In contrast, increasing 20E
434 concentrations had no effect on disc growth rate in fed PGX larvae (Figure 7
435 Supplement 3, Supplementary Table 10). This confirms that the effect of nutrition on
436 growth hides the effect of 20E early in the third larval instar, and supports that
437 hypothesis that disc growth during this period is primarily regulated by nutrition and
438 only moderately regulated by ecdysone [62].

439 To test whether wing disc growth rates show either a graded or threshold
440 response to 20E concentration in starved PGX larvae, we extracted the linear growth
441 rate coefficients from our models. We then modelled the relationship between
442 growth rate and 20E concentration with three non-linear functions: a graded
443 Michaelis Menten function, and with two threshold functions, three and four
444 parameter log-logistic functions. Finally, we tested which model best fit the data
445 using Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) for
446 model selection. The model with the lowest AIC and BIC values best fits the data.
447 When wing disc growth rate was modelled with the graded Michaelis Menten
448 function, both the AIC and BIC values were lower than when it was modelled with
449 either threshold function (Supplementary Table 11). This supports the hypothesis that
450 growth rate increases continuously with increasing 20E concentration, with growth
451 rate plateauing after 20E concentrations reach 25 ng/ml (Figure 7B). Thus, disc
452 growth rate appears to show a graded response to 20E level in the absence of
453 nutrition. This is in line with recent findings from the Teleman lab, that show that
454 proliferation and growth in the wing discs increase with increasing 20E concentration
455 in the diet [73].

456

457

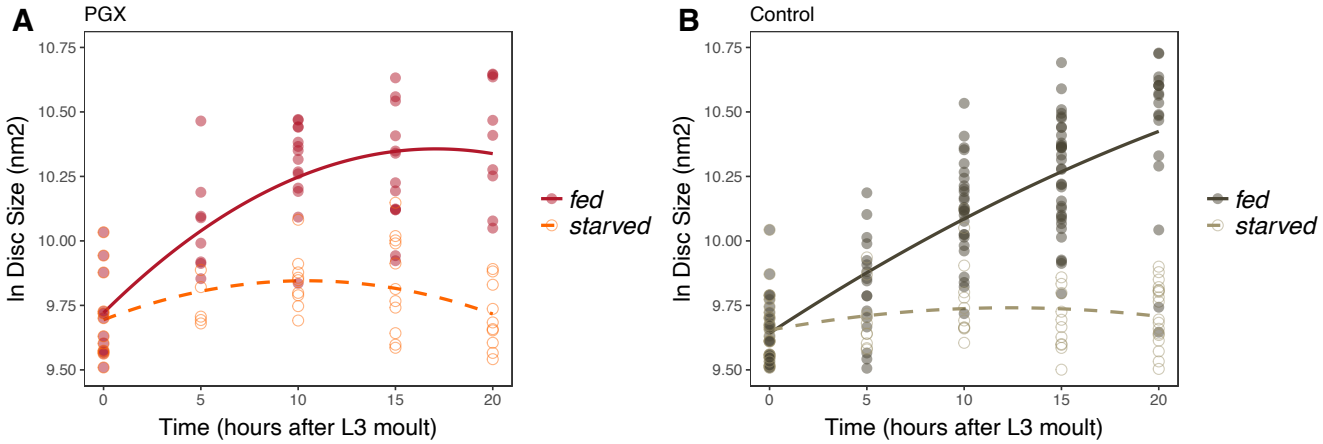


Figure 7 Supplement 2: Wing imaginal disc growth is suppressed in fed PGX larvae relative to controls, and in starved larvae of both genotypes. Wing disc growth was modelled as a quadratic, and there was a significant interaction between genotype (PGX v. Control) and nutrition (fed v. starved) on growth (Supplementary Table 9). Solid line/closed point = fed larvae, broken line/open point = starved larvae. Each point corresponds to a wing disc, $N_{PGX\text{ - }starved}$ = 67, $N_{PGX\text{ - }fed}$ = 74, $N_{control\text{ - }starved}$ = 118, $N_{control\text{ - }fed}$ = 151 across all time points.

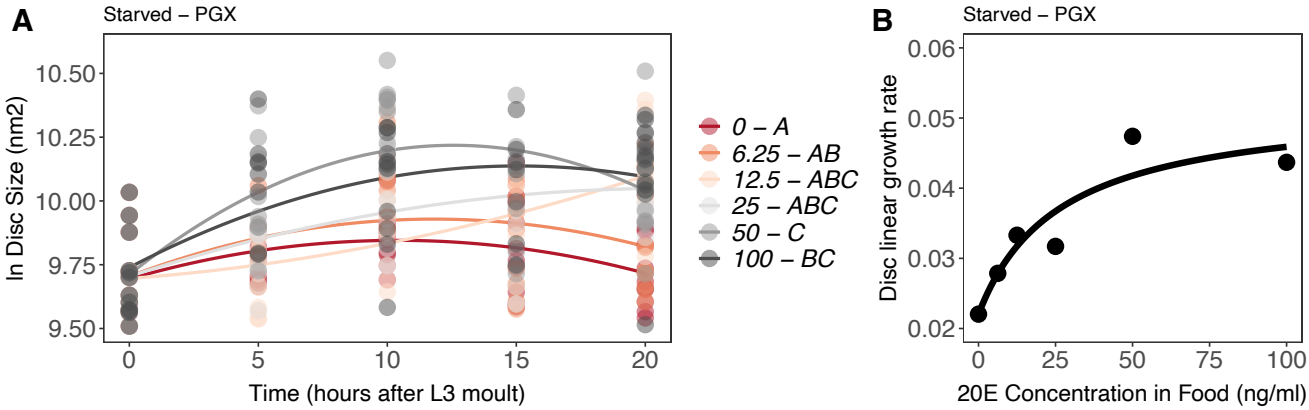
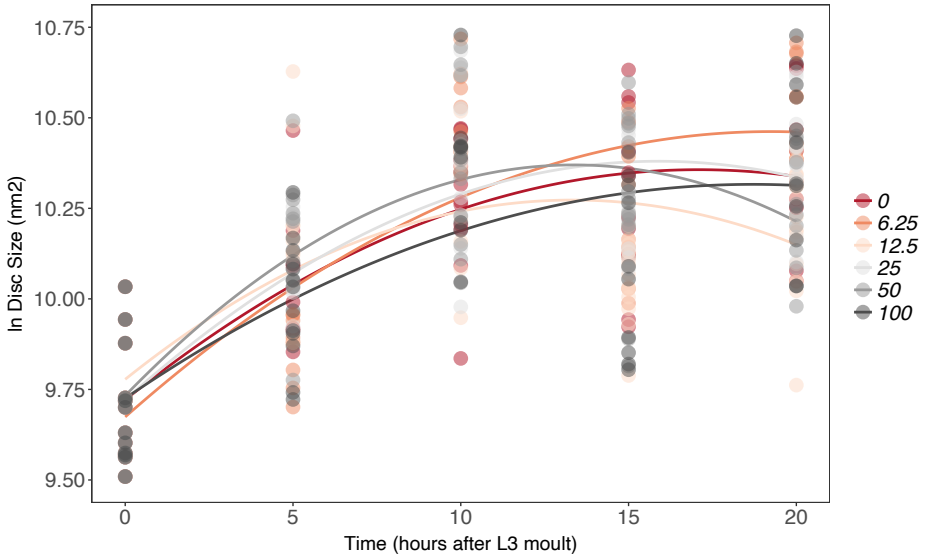


Figure 7: Effect of supplemental 20E on growth of the wing imaginal disc in starved PGX larvae. Growth was modelled as $S = E + T + T^2 + E*T + E*T^2$, where S = disc size, E = 20E concentration, and T = disc age. A) There was a significant effect of E on the linear growth rate of the wing imaginal discs (Supplementary Table 8 and 9). Each point corresponds to a wing disc, $N_{PGX\text{ - }starved}$ = 409 (63-72 discs were sampled per treatment across all time points). 20E treatments that do not share a letter (see legend) are significantly different in patterning rates as determined by post-hoc test on the slopes (Supplementary Table 10). B) The linear growth rate of the wing disc was extracted from the growth model for each concentration of 20E, and modelled using a three parameter Michaelis Menten equation: $y = c + (d-c)/(1+(b/x))$, where c is y at x=0, $d = y[max]$, and b is x where y is halfway between c and d. Linear growth

477 rate increases steadily with ecdysone concentration in the food up until 25 ng of 20E/ml of
478 food, after which growth rate increases more slowly with increasing 20E concentration.

479



480

481 **Figure 7 Supplement 3:** There is no effect of supplemental 20E on growth of the
482 wing imaginal disc in fed PGX larvae. Growth was modelled as $S = E + T + T^2 + E*T + E*T^2$,
483 where S = disc size, E = 20E concentration, and T = disc age. There was no significant effect
484 of E on the linear or quadratic growth rate of the wing imaginal discs (Supplementary Table
485 10). Each point corresponds to a wing disc, $N_{PGX - fed}$ = 459 (73-86 discs were sampled per
486 treatment across all time points).

487

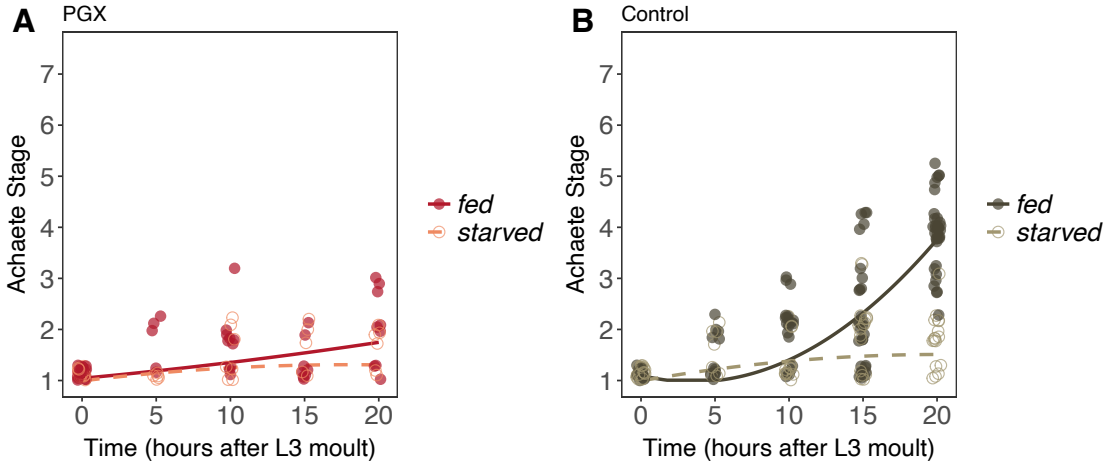
488 The effect of 20E concentration on Achaete patterning was qualitatively different
489 to its effect on growth. As before, Achaete patterning did not progress in either
490 starved or fed PGX larvae (Supplementary Figure 4A). In contrast, Achaete patterning
491 did progress in PGX larvae supplemented with 20E. Patterning rates for Achaete did
492 not differ significantly between 0-6.25 ng/ml (fed) and 0-12.5 ng/ml (starved) of 20E
493 (Figure 8 A&C). Above 25 ng/ml of 20E, Achaete patterning occurred at the same
494 rapid rate in both fed and starved PGX larvae (Figure 8 A&C).

495 To compare the progression of Achaete at different levels of 20E with the
496 progression of disc growth, we modelled the relationship between Achaete pattern,
497 time after third instar ecdysisis, and 20E concentration as second order polynomial
498 regression for fed and starved PGX larvae. As for disc growth, we then extracted the
499 linear coefficients from this model at each level of 20E and modelled the relationship
500 between patterning rate and 20E using a Michaelis Menten function and a three and
501 four parameter log-logistic function. Both the AIC and BIC indicated that that a
502 threshold four parameter log-logistic function fit the data better than a graded

503 Michaelis Menten function (Supplementary Table 9). Thus, unlike growth, Achaete
504 patterning appears to show a threshold response to 20E concentration. Specifically,
505 Achaete patterning is not initiated unless 20E is above a certain level (12.5-25ng/ml),
506 but progresses at the same rate and to the same extent regardless of how high 20E is
507 above this level.

508 Comparing the timing of Achaete patterning in 20E-supplemented PGX larvae
509 versus fed controls provides some indication of when in normal development the
510 threshold level of 20E necessary to initiate Achaete patterning is reached. Discs from
511 fed control larvae began to reach Achaete stage 4 by 15h AEL3 (Figure 8 Supplement
512 1B), while discs from both fed and starved PGX larvae supplemented with >25ng/ml
513 of 20E began to reach stage 4 by 10h AEL3 (Figure 8A & C). This suggests that in
514 control larvae, ecdysone levels sufficient to initiate Achaete patterning are only
515 reached 15 h after the mount to the third larval instar.

516



517

518 **Figure 8 Supplement 1:** Achaete patterning in wing discs from fed and starved PGX
519 and control larvae. (A) Patterning does not progress in either fed or starved PGX larvae. (B)
520 Patterning does not progress in starved control larvae but does in fed control larvae. There is
521 a significant interaction between the effects of disc age and food on Achaete patterning in
522 control larvae (orthogonal polynomial regression: $F_{food*disc\ age^2}$=67.98, P < 0.001), but not in
523 PGX larvae (orthogonal polynomial regression: $F_{food*disc\ age^2}$=1.81, P = 0.163). Each point
524 corresponds to a wing disc, $N_{PGX\ -\ starved}$ = 67, $N_{PGX\ -\ fed}$ = 74, $N_{control\ -\ starved}$ = 118, $N_{control\ -\ fed}$ =
525 151 across all time points.

526

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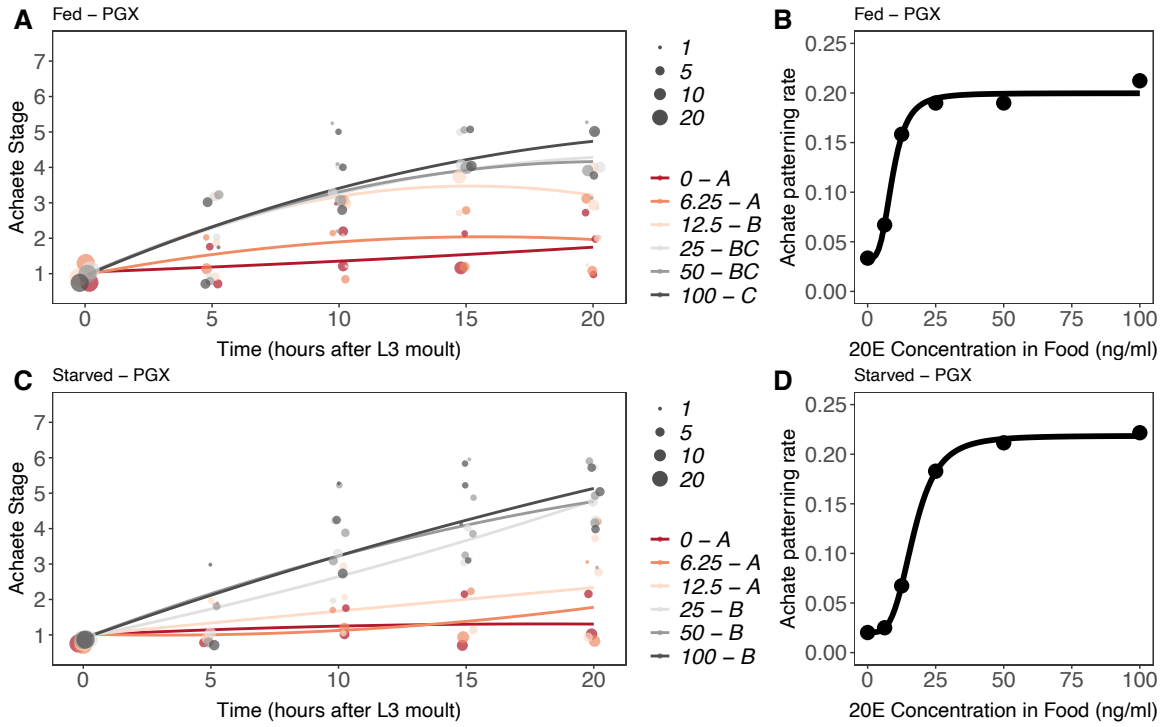


Figure 8: Effect of supplemental 20E on Achaete patterning of the wing imaginal disc in (A-B) fed and (C-D) starved PGX larvae. In A and C, patterning stage was modelled as $A = E + T + T^2 + E*T + E*T^2$, where A = Achaete stage, E = 20E concentration, and T = disc age. The size of each point corresponds to the number of wing disc in each category. 20E treatments that do not share a letter (see legend) are significantly different in patterning rates as determined by post-hoc test on the slopes (for ANOVA see Supplementary Table 12). In B and D, we extracted the linear patterning rate from fed (B) or starved (D) PGX larvae. We then modelled the relationship between patterning rate and 20E concentration using a four parameter log-logistic equation: $y = c + (d-c)/(1+e^{(b(\log(x)-\log(a))})$, where c is the lower asymptote, d is the upper asymptote, b is the rate of increase, and a is the inflection point. $N_{PGX\text{ - fed}} = 459$, $N_{PGX\text{ - starved}} = 409$, 63-86 discs were sampled per treatment across all time points.

Senseless patterning did not progress as far as Achaete patterning, only achieving an average of stage 3 in fed and stage 4 in starved larvae at 20 h AL3E when supplemented with 20E. In both fed and starved larvae, supplemental 20E at or below 12.5 ng/ml was insufficient to rescue Senseless patterning, while supplemental 20E at or above 25 ng/ml rescued patterning to approximately the same extent (Figure 9 A&C). As for Achaete patterning, supplemental 20E also initiated Senseless patterning in PGX larvae early when compared to fed controls. Discs from fed control larvae began to reach Senseless stage 3 at 20h AEL3 (Figure 9 Supplement 1), while discs from both fed and starved PGX larvae supplemented with ≥25ng/ml of 20E were at stage 3 by 15h AEL3 (Figure 9A & C).

We again used a second order polynomial regression to model the relationship between Senseless pattern, time after third instar ecdysis, and 20E concentration. The relationship between the linear coefficient from our model for Senseless patterning and 20E were best fit with a log-logistic rather than a Michaelis Menten function. For fed PGX larvae, the four parameter logistic function provided the best fit to the data (Figure 9B, Supplementary Table 11), whereas for starved PGX larvae, the three parameter logistic function was the best fit (Figure 9D, Supplementary Table 11. Thus, like Achaete patterning rate, Senseless patterning rate shows threshold responses to 20E concentration.

Collectively, these data support a model of disc growth and patterning where ecdysone regulates disc growth as a graded response to basal levels of ecdysone, while ecdysone regulates disc patterning as a single threshold response.

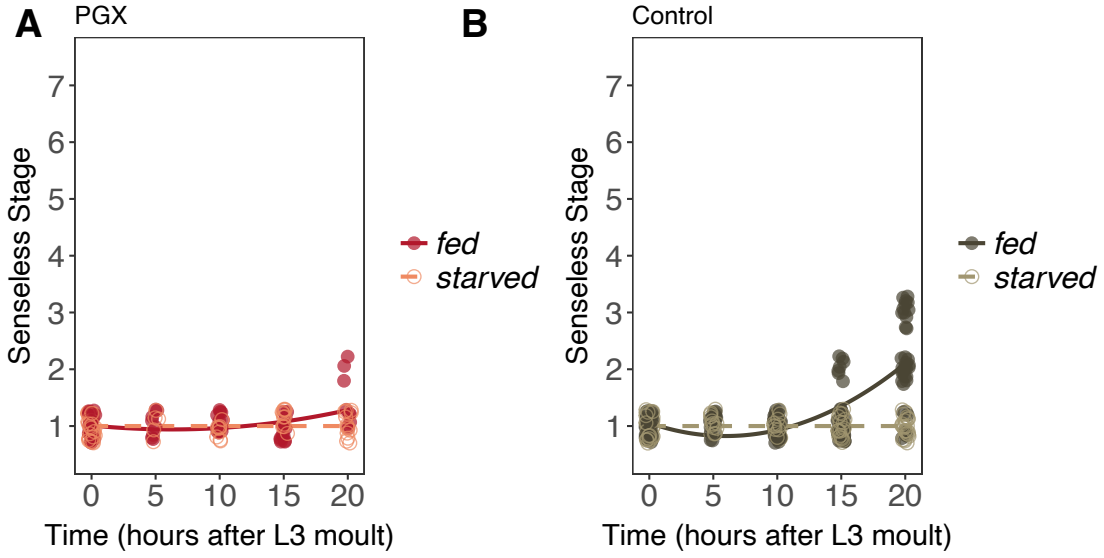


Figure 9 Supplement 1: Senseless patterning in wing discs from fed and starved PGX and control larvae. (A) Patterning does not progress in either fed or starved PGX larvae. (B) Patterning does not progress in starved control larvae but does in fed control larvae. There is a significant interaction between the effects of time and food on Achaete patterning in control larvae (linear regression: $F_{\mathrm{food*time}}$=67.98, $P < 0.001$). In PGX larvae Senseless patterning does not progress at all in starved larvae (linear regression: F_{time}=0.057, P =0.82), but does in fed larvae (linear regression: F_{time}=9.76, $P < 0.01$). Control genotypes are the pooled results from both parental controls (either the *phm-GAL4; GAL80ts* or *UAS-GRIM* parental strain crossed to w^{1118}). Each point corresponds to a wing disc, $N_{\mathrm{PGX\ -\ starved}}$ = 67, $N_{\mathrm{PGX\ -\ fed}}$ = 74, $N_{\mathrm{control\ -\ starved}}$ = 118, $N_{\mathrm{control\ -\ fed}}$ = 151 across all time points.

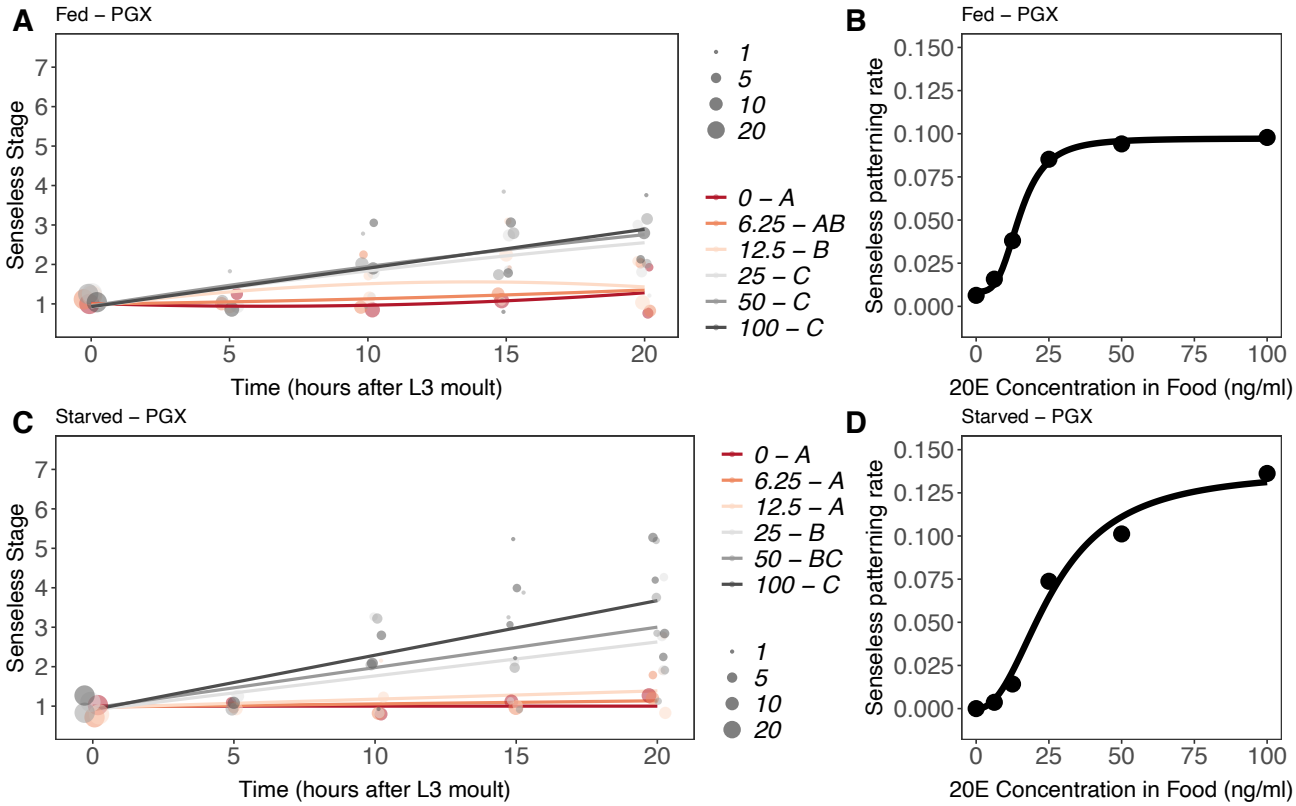


Figure 9: Effect of supplemental 20E on Senseless patterning of the wing imaginal disc in (A-B) fed and (C-D) starved PGX larvae. In A and C, patterning stage was modelled as $S = E + T + T^2 + E*T + E*T^2$, where S = Senseless stage, E = 20E concentration, and T = disc age. The size of each point corresponds to the number of wing disc in each category. 20E treatments that do not share a letter (see legend) are significantly different in patterning rates as determined by post-hoc test on the slopes (for ANOVA see Supplementary Table 13). In B and D, we extracted the linear patterning rate in fed (B) or starved (D) larvae. We then modelled the relationship between patterning rate and 20E concentration in B using a four parameter log-logistic equation: $y = c + (d-c)/(1+e^{(b(\log(x)-\log(a))})$, where c is the lower asymptote, d is the upper asymptote, b is the rate of increase, and a is the inflection point. In D, we used a three parameter log-logistic equation: $y = d/(1+e^{(b(\log(x)-\log(a))})$, where d is the upper asymptote, b is the rate of increase, and a is the inflection point. $N_{PGX\text{ - fed}} = 459$, $N_{PGX\text{ - starved}} = 409$, 63-86 discs were sampled per treatment across all time points.

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

592 simple feat when growth and patterning occur at separate times or are regulated by
593 different hormones, it is considerably less simple if both growth and patterning occur
594 at the same time and are regulated by the same endocrine signal. In this work, we
595 explored how the wing discs of developing *D. melanogaster* use the same hormonal
596 signal to coordinate both their growth and their progression of pattern. We found
597 that ecdysone simultaneously regulates the plastic growth and robust patterning of
598 the wing disc through independent mechanisms: plastic growth responds to
599 ecdysone with a graded response, while robust patterning responds with a single
600 threshold response. We propose that these differences in response represent a
601 potentially general mechanism through which high levels of variation in one organ
602 characteristic, for example size, could be coordinated with low levels of variation in
603 another characteristic of the same organ, for example pattern.

604 These data make an important contribution to our understanding of how
605 environmental factors, specifically nutrition, affect growth and patterning in
606 developing organs in *Drosophila*. During normal development, circulating ecdysone
607 levels are low during the first 8 hours of the third larval instar, until attainment of a
608 critical size initiates a hormonal cascade that causes ecdysone to fluctuate through a
609 series of characteristic peaks. Each of these peaks is associated with key
610 developmental transitions and prepares the larva for metamorphosis. Low nutrition
611 delays attainment of critical size and the initiation of these peaks, but also appears to
612 raise basal levels of circulating ecdysone between these peaks [74], which slows the
613 growth of the body [74]. At the same time, low nutrition also lowers levels of
614 circulating insulin-like peptides, further slowing growth of the body. While low levels
615 of insulin signalling will suppress imaginal disc growth, the increase in ecdysone
616 concentrations resulting from starvation opposes some of these effects [56, 58, 71] by
617 promoting imaginal disc growth.

618 Our data suggest that these opposing effects are critical to robust patterning
619 of the wing under different nutritional conditions. At low nutritional conditions, low
620 insulin-signalling at the beginning of the third larval instar slows growth of the body
621 and of the imaginal discs. At this stage, growth of the wing imaginal discs is less
622 dependent on ecdysone [62], evident from the more moderate effects on growth of
623 the wing discs during this period in fed PGX larvae. In the middle of the third larval
624 instar, however, low nutritional conditions elevate basal levels of ecdysone [74]. This
625 drives disc growth independent of insulin-signalling to ensure the discs are of
626 sufficient size to generate viable adult appendages, even as elevated ecdysone
627 suppresses growth of the body as a whole [50, 66, 67]. At the same time, changes in
628 the tempo of the ecdysone fluctuations may ensure that patterning is initiated at the
629 appropriate developmental time, when discs are a sufficient size to generate a viable

630 adult appendage. Three factors therefore appear necessary to achieve variable size
631 but robust patterning under a range of nutritional conditions: (1) a graded growth
632 response to ecdysone; (2) nutritionally-sensitive growth that is independent of
633 ecdysone; and (3) a threshold patterning response to ecdysone.

634 There is some evidence that our findings apply to patterning and growth of the
635 wings in other insect species. In the tobacco hornworm *Manduca sexta* and the buck-
636 eyed butterfly *Junonia coenia*, wing disc growth is regulated by both ecdysone and
637 insulin (73,76). In the butterfly *J. coenia*, the patterning stage of wing discs can be
638 quantified by the extent of tracheal invasion resulting in wing vein patterning [75]. In
639 this species, wing vein patterning progresses independently of wing size in starved
640 versus fed caterpillars [75]. Thus, the independent regulation of growth and
641 patterning, with growth regulated by both insulin and ecdysone signalling, may be a
642 general mechanism to achieve robust patterning across a range of wing sizes.

643 While ecdysone and insulin signalling provide systemic cues that tune organ
644 growth to the environmental conditions, morphogens like Wingless and
645 Decapentaplegic (Dpp) act to regulate growth in an organ autonomous manner. The
646 extent to which morphogen gradients respond to these systemic cues is unclear,
647 although the activity of morphogens is known to interact with those of systemic
648 signals at the level of the target genes. For example, insulin/TOR-signalling regulates
649 the activity of Yorkie, a downstream effector of patterning morphogens, including
650 Wingless and Dpp, which controls the rate of cell division [76]. Similarly, reducing
651 ecdysone signalling in the wing reduces the expression of Wingless and reduces Dpp
652 signalling, measured by the levels of phosphorylated Mothers against Dpp
653 expression [56, 58, 59]. Taken together, the signalling pathways that regulate organ
654 growth in response to environmental conditions interact in complex ways with those
655 that regulate organ-autonomous growth suggesting that these two growth regulating
656 mechanisms are not as independent as previously thought [6].

657 Although the growth of the disc relies on insulin and ecdysone signalling, the
658 progression of patterning for Achaete and Senseless in the wing disc appears to be
659 driven by threshold responses to ecdysone. This is not to say that the progression of
660 patterning does not depend on environmental conditions. Indeed, starvation early in
661 the third instar impedes patterning in both the wing and ovary of *D. melanogaster*
662 [59, 60]. However, rather than resulting from a direct effect of insulin signalling on
663 patterning, the block in the progression of pattern occurs because insulin signalling
664 controls the timing of the first ecdysone pulse in the third larval instar [53, 77]. Our
665 results here confirm that patterning requires suprathreshold concentrations of
666 ecdysone to be initiated. Further, the manner in which ecdysone regulates the
667 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 wing patterning depend on nutrition in *J. coenia*. If caterpillars are starved before the wing discs begin to pattern, then their discs remain small and their veins unpatterned [75]. In caterpillars starved at later stages after disc patterning has been initiated, 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.

At first glance, the observation that patterning shows a threshold response to ecdysone may not be surprising. In any given cell, patterning is inherently regulated by threshold responses because the expression of the patterning gene product is either on or off in that cell. However, our patterning scheme considers the progression of patterning across the entire field of cells that make up the wing disc. Cells across the wing disc turn on Achaete and Senseless expression at different times, resulting in a continuous progression of pattern with time [42]. Furthermore, like growth, the progression of pattern can vary in rate depending on environmental and hormonal conditions [42]. Consequently, the progression of patterning could, in principle, also show a graded response to ecdysone levels. The observation that once ecdysone concentrations are above threshold, the rate of patterning for Achaete and Senseless is independent of ecdysone provides evidence that the rate of patterning across an entire organ can also show a threshold response: an assumption that, hitherto, has not been tested.

What determines how progression of pattering unfolds through time is unclear. We did not observe discs progressing from stage 1 immediately to stage 7 within a single 5-hour time interval even at the highest 20E concentrations. This suggests that there are additional temporal factors that regulate the order of patterning progression. 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,

706 secrete four pulses of ecdysteroids between the moult to third instar and pupariation
707 [68]. We have little understanding of how developmental information is encoded
708 within these pulses. In principle, individual pulses could either prime tissues to
709 become responsive to hormones, or could alter their sensitivity – as the early
710 ecdysone pulse does for wing disc growth and patterning [59, 60, 62]. Future studies
711 comparing the difference between tonic and phasic exposure to hormone would
712 help clarify the roles of the ecdysone pulses.

713 While our study has focussed on contrasting the robustness of patterning with
714 plasticity of growth, depending on what is being measured there are instances where
715 we expect patterning to also show plasticity [78]. For example, although the
716 specification of cell types in the correct location within an organ may show little
717 variation across environmental conditions, the number of structures specified can
718 vary. The total number of abdominal and sternopleural bristles varies with
719 temperature [79, 80], as does the number of terminal filament stacks that are
720 specified in the ovary, which is also affected by nutrition [81-84]. Plasticity in the
721 number of bristle cells or terminal filament stacks presumably occurs because the
722 mechanisms that specify the number of each structure do not scale with organ size. In
723 other cases, the location of specific cell types may also be plastic. For example, there
724 is an extensive literature exploring how the relative positions of veins in the wings of
725 *D. melanogaster* and other insects is affected by environmental factors such as
726 nutrition and temperature (e.g. [85-88]). Plasticity in wing shape is likely to be more
727 complex, and may involve process that act at many different points during wing
728 development [89, 90]. Future studies targeting how the mechanisms that establish
729 the position of cell types differ from those that determine the number of cells of a
730 given type would allow us to further define what makes traits either sensitive or
731 robust towards changes in environmental conditions, and at what level.

732 Materials and Methods

733 Fly stocks and rearing conditions

734 We initially manipulated growth rates and developmental timing by either
735 altering the rearing temperature or the rates of ecdysone synthesis in developing *D.*
736 *melanogaster* larvae. We used the isogenic strain, Samarkand (SAM), which also bears
737 the w^{1118} mutation, when rearing larvae under one of three thermal conditions: 18˚C,
738 25 ˚C, and 29˚C. To accelerate the rates of ecdysone synthesis, we used the progeny
739 from w^{1118};*phantom*-GAL4, which is expressed in the prothoracic glands, crossed with
740 yw *flp; UAS InR29.4* (*phm > InR*). We decreased rates of ecdysone synthesis by

741 crossing *P0206-GAL4*, which drives expression throughout the ring gland, with *yw;*
742 *UAS PTEN* (*P0206 > PTEN*). Even though *P0206*-GAL4 is a weaker GAL4 driver for
743 the prothoracic gland and also drives expression in the corpora allata, we chose to
744 use it to drive *UAS PTEN* because *phm>PTEN* larvae die as first instar larvae [52]. The
745 parental lines *yw flp; UAS InR29.4* (+ > *InR*) and *yw; UAS PTEN* (+ > *PTEN*) were used
746 as a reference for the *phm > InR* and *P0206 > PTEN* genotypes respectively.

747 Flies of the above genotypes were raised from timed egg collections (2-6 hours)
748 on cornmeal/molasses medium containing 45 g of molasses, 75 g of sucrose, 70 g of
749 cornmeal, 10 g of agar, 1100 ml of water, and 25 ml of a 10% Nipagin solution per
750 liter. Larvae were reared at low density (200 eggs per 60 x 15 mm Petri dish) in a 12 h
751 light-dark cycle with 70% humidity, and maintained at 25°C unless stated otherwise.

752 We used a transgenic combination that allowed us to genetically ablate the
753 prothoracic gland and eliminate native ecdysone synthesis specifically in the third
754 larval instar. We crossed a *tub-GAL80^{ts}, phantom GAL4* strain with *UAS Grim* to
755 generate *PGX* progeny [56]. GAL80^{ts} is a repressor of GAL4 active at temperatures
756 lower than 22°C [63]. Rearing PGX larvae at 17°C allows GAL80^{ts} to remain active,
757 thus the *phantom GAL4* cannot drive the expression of *UAS grim* to promote cell
758 death. Under these conditions, larvae can moult, pupariate, and complete
759 metamorphosis [56]. Changing the larval rearing temperature to 29°C disables
760 GAL80^{ts} activity, thus ablating the prothoracic gland [56]. The progeny of the isogenic
761 control strain, *w1118*, crossed with one of two parental lines, either *phantom*-GAL4
762 (*PG* > +) or *UAS Grim* (+ > *Grim*), were used as controls for genetic background
763 effects. The parental controls were reared under the same thermal conditions as PGX
764 larvae.

765 Crosses, egg collections, and larval rearing were done on the cornmeal/molasses
766 medium (above) for the experiments in Figures 2-6 or, for the experiments in Figures
767 7-9, on Sugar-Yeast-Agar (SYA) medium: 50g of autolysed Brewer's yeast powder (MP
768 Biomedicals), 100 g of sugar, 10 g of agar, and 1200 ml of water. In addition, we
769 added 3 ml of proprionic acid and 3 grams of nipagen to the SYA medium to prevent
770 bacterial and fungal growth. Egg collections were performed on SYA medium for 4
771 hours at 25°C or overnight at 17°C and larvae were reared at controlled densities of
772 200 eggs per food plate (60 x 15 mm Petri dish filled with SYA medium) at 17°C, as
773 described previously [56].

774

775 Animal staging and developmental time

776 To measure the effects of larval rearing temperature or changes in the rates in
777 ecdysone synthesis on wing disc growth and wing disc patterning, larvae were staged

778 into 1-hour cohorts at ecdysis to the third larval instar as in [52, 59]. To do this, food
779 plates were flooded with 20% sucrose and all second instar larvae were transferred to
780 a new food plate. After one hour, the food plate was flooded once again with 20%
781 sucrose and the newly moulted third instar larvae were collected and transferred to
782 new food plates and left to grow until the desired time interval. Animals were staged
783 and their wing discs dissected at defined intervals after the larval moult as in [42].

784 For the experiments in Figures 7-9, PGX, *phm>*, and *>Grim* genotypes, larvae
785 were raised from egg to second instar at 17°C. Larvae were staged into 2-hour
786 cohorts at ecdysis to the third larval instar using the methods described above. We
787 separated female and male larvae by examining them for the presence of testes,
788 which are significantly larger and more visible even in newly moulted males.

789

790 Exogenous ecdysone feeding treatments

791 To show that ecdysone could rescue patterning and growth in PGX larvae (Fig
792 4C&D), we added either 0.15 mg of 20E (Cayman Chemical, Item No. 16145)
793 dissolved in ethanol, or an equivalent volume of ethanol, to 1 ml of standard food.
794 Both the ethanol- and ecdysone-supplemented food were allowed to sit at room
795 temperature for at least 4 hours to evaporate off excess ethanol before use. Twelve
796 larvae were transferred to one of the two supplemented foods either at 0 h AL3E and
797 left to feed for 42 h or at 42 h AL3E and left to feed for 24 h.

798 To determine the relative contributions of nutrition-dependent signalling or
799 ecdysone to growth and patterning, we fed newly moulted PGX and control larvae 1
800 ml of starvation medium (1% sucrose with 1% agar) supplemented with either 0.15
801 mg of 20E dissolved in ethanol, or an equivalent volume of ethanol (Fig Suppl 4).
802 Supplemented food was left at room temperature for at least 4 hours to evaporate
803 excess off ethanol before use. Larvae were collected at 24 h AL3E for tissue
804 dissection.

805 For the 20E dose response experiments, we conducted an initial pilot that
806 showed that supplementing the food with 100 ng of ecdysone/mg food could rescue
807 most of the Achaete and Senseless patterning in PGX wing discs. We collected newly
808 moulted third instar larvae, separated the sexes, and then transferred 10-20 larvae to
809 either sucrose food (20% sucrose, 1% agar; starved) or SYA food (fed) at 29°C. We
810 fed these larvae on one of six ecdysone concentrations: 0, 6.25, 12.5, 25, 50, or 100
811 ng of 20E/ mg food. We added the same volume of ethanol to all treatments.

812 To quantify the relationship between the concentration of 20E administered and
813 the concentration of ecdysteroids in the hemolymph, we allowed newly-ecdysed

814 larvae to feed on either sucrose or SYA food that had been supplemented with one of
815 the six concentrations of ecdysone for 20h at 29°C. We then transferred them onto
816 either sucrose food or SYA food that did not contain ecdysone but was dyed blue.
817 They were left to feed for 2 hours until their guts were filled with blue food. This extra
818 step was taken so that we could be sure that our hemolymph ecdysone titres were
819 not contaminated with ecdysone from the food. Thirty – forty larvae were then
820 weighed as a group, and transferred to 5 times their weight in volume of ice cold
821 methanol. Larvae were homogenized and ecdysone titres were determined using a
822 20-Hydroxyecdysone Enzyme ImmunoAssay Kit (Cayman Chemical, Item Number
823 501390) as per manufacturer's instructions.

824

825 Dissections and immunocytochemistry

826 For each sample, 10-20 larvae were dissected on ice cold phosphate-buffered
827 saline (PBS) and fixed in 4% formaldehyde in PBS overnight at 4°C. After fixation, the
828 tissue was washed four times (15 minutes per wash) with 0.3% Triton X-100 in PBS
829 (PBT), then blocked for 30 minutes at room temperature in 2% heat-inactivated
830 normal donkey serum in PBT. After blocking, the tissue was incubated in a primary
831 antibody solution diluted with 2% heat-inactivated normal donkey serum in PBT
832 overnight at 4°C. We used the guinea pig anti-Senseless (Nolo et al., 2000, 1:1000)
833 and mouse anti-Achaete (supernatant, 1:10) primary antibodies. To compare signal
834 across tissues, we stained for both antigens simultaneously. The washing and
835 blocking procedure was repeated after primary antibody incubation and then the
836 tissue was incubated in a secondary antibody (1:200 each of anti-guinea pig (546nm)
837 and anti-mouse (488nm)) overnight at 4°C. The tissues were washed with PBT and
838 rinsed with PBS and then the wing imaginal discs were mounted on poly-L-lysine-
839 coated coverslips using Fluoromount-G (SouthernBiotech). Tissues were imaged
840 using either a Leica LSM 510 or a Nikon C1 upright confocal microscope and
841 processed using ImageJ (version 2.0) and Adobe Photoshop CC 2017.

842

843 Quantifications of wing imaginal disc size and Achaete and
844 Senseless pattern

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

Achaete and Senseless stage was quantified using the staging scheme developed by [42], 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 (DOI: 10.26180/13393676).

For the relationship between time after third instar ecdysis 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 and AIC. 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 parametrized as $y = c + \frac{(d-c)}{1+e^{(b-x)/a}}$ where y is disc pattern, x is disc size, c is the minimum asymptote, d is the maximum asymptote, b is the inflection point and a is the scaling constant, such that $a = 1/k$, where 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.

Finally, to compare the effects of 20E supplementation in the diet on the progression of wing disc growth, Achaete patterning, and Senseless patterning, we fit a second-order orthogonal polynomial regression using disc size/patterning stage as our dependent variable, and 20E concentration and linear and quadratic terms for time as fixed effects. Fitting a single model to the data allowed us to compare the same model parameters for growth and patterning. We then extracted the linear rate of change at each 20E concentration using the *emtrends* function of the *emmeans*

package in *R* [91]. The changes in growth/patterning rate with 20E concentration were modelled using three non-linear functions: (1) a continuous Michaelis Menten function: $y = c + \frac{(d-c)}{1+b/x}$, where *c* is *y* at *x*=0, *d* is the maximum asymptote, and *b* is *x* where *y* is halfway between *c* and *d*, (2) a threshold three-parameter log-logistic function: $y = \frac{d}{1+e^{b(\log x - \log a)}}$ where *d* is the maximum asymptote, *b* is the rate of increase, and *a* is the inflection point, and (3) a threshold four-parameter log-logistic function: $y = c + \frac{(d-c)}{1+e^{b(\log x - \log a)}}$ where *c* is the minimum asymptote, *d* is the maximum asymptote, *b* is the rate of increase, and *a* is the inflection point. For each model we calculated the Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) to allow model selection. The model that produces the lowest AIC and BIC value best fits the data.

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

Acknowledgements

We would like to thank members of the Mirth and Shingleton labs, past and present, for their useful discussions relating to this project. This research was supported by NSF grants IOS-0919855, IOS-1557638 and IOS-1952385 to AWS and an ARC Future Fellowship (FT170100259) to CKM.

Competing Interests

The authors declare that they have no competing interests.

References

1. Nylin S, Gotthard K. Plasticity in life-history traits. Annual Review of Entomology 1998, 43:63-83.
2. West-Eberhard MJ. Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics 1989, 20:249–278.
3. Beldade P, Mateus ARA, Keller RA. Evolution and molecular mechanisms of adaptive developmental plasticity. Molecular Ecology 2011, 20:1347-1363.
4. Koyama T, Mendes CC, Mirth CK. Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. Frontiers in Physiology 2013, 4:1-12.
5. Shingleton AW. The regulation of organ size in *Drosophila*: physiology, plasticity, patterning and physical force. Organogenesis 2010, 6:76-87.
6. Mirth CK, Shingleton AW. Coordinating Development: How Do Animals Integrate Plastic and Robust Developmental Processes? Frontiers in Cell and Developmental Biology 2019, 7:8.
7. Nijhout HF, Sadre-Marandi F, Best J, Reed MC. Systems biology of phenotypic robustness and plasticity. Integrative and Comparative Biology 2017, 57:171-184.
8. Félix MA, Barkoulas M. Pervasive robustness in biological systems. Nature Reviews Genetics 2015, 16:483-496.
9. Félix MA, Wagner A. Robustness and evolution: concepts, insights and challenges from a developmental model system. Heredity 2008, 100:132-140.
10. Manu SS, Spirov AV, Gursky VV, Janssens H, Kim AR, Radulescu O, Vanario-Alonso CE, Sharp DH, Samsonova M, Reinitz J. Canalization of gene expression and domain shifts in the Drosophila blastoderm by dynamical attractors. PLoS Computational Biology 2009, 5:e1000303.
11. Manu SS, Spirov AV, V.V. G, Janssens H, Kim AR, Radulescu O, Vanario-Alonso CE, Sharp DH, Samsonova M, J. R. Canalization of gene expression in the Drosophila blastoderm by gap gene cross regulation. PLoS Biology 2009, 3:e1000049.
12. Clark E. Dynamic patterning by the Drosophila pair-rule network reconciles long-germ and short-germ segmentation. PLoS Biology 2017, 15(9):e2002439.
13. Clark E, Akam M. Odd-paired controls frequency doubling in Drosophila segmentation by altering the pair-rule gene regulatory network. eLife 2016, 5:e18215.
14. Clark E, Peel AD. Evidence for the temporal regulation of insect segmentation by a conserved sequence of transcription factors. Development 2018, 145(10):dev155580.

943 15. Crombach A, Wotton KR, Jiménez-Guri E, Jaeger J. Gap gene regulatory
944 dynamics evolve along a genotype network. Molecular Biology and Evolution
945 2016, 33:1293-1307.

946 16. Verd B, Clark E, Wotton KR, Janssens H, Jiménez-Guri E, Crombach A, Jaeger
947 J. A damped oscillator imposes temporal order on posterior gap gene expression
948 in Drosophila. PLoS Biology 2018, 16(2):e2003174.

949 17. Wotton KR, Jiménez-Guri E, Crombach A, Janssens H, Alcaine-Colet A, Lemke
950 S, Schmidt-Ott U, Jaeger J. Quantitative system drift compensates for altered
951 maternal inputs to the gap gene network of the scuttle fly *Megaselia abdita*. eLife
952 2015, 4:e04785.

953 18. Zhou S, Lo W-C, Suhalim JL, Digman MA, Gratton E, Nie Q, Lander AD. Free
954 extracellular diffusion creates the Dpp morphogen gradient of the *Drosophila*
955 wing disc. Current Biology 2012, 22:668-675.

956 19. Almuedo-Castillo M, Bläßle A, Mörsdorf D, Marcon L, Soh GH, Rogers KW,
957 Schier AF, Müller P. Scale-invariant patterning by size-dependent inhibition of
958 Nodal signalling. Nature Cell Biology 2018, 20(9):1032-1042.

959 20. Zhu Y, Qiu Y, Chen W, Nie Q, Lander AD. Scaling a Dpp Morphogen Gradient
960 through Feedback Control of Receptors and Co-receptors. Developmental Cell
961 2020, 53(6):724-739.e714.

962 21. Schwank G, Tauriello G, Yagi R, Kranz E, Koumoutsakos P, Basler K.
963 Antagonistic growth regulation by Dpp and Fat drives uniform cell proliferation.
964 Developmental Cell 2011, 20:123-130.

965 22. Wartlick O, Mumcu P, Kicheva A, Bittig T, Seum C, Jülicher F, González-Gaitán
966 M. Dynamics of Dpp signaling and proliferation control. Science 2011, 331:1154-
967 1159.

968 23. Hamaratoglu F, de Lachapelle AM, Pyrowolakis G, Bergmann S, Affolter M. Dpp
969 Signaling Activity Requires Pentagone to Scale with Tissue Size in the Growing
970 Drosophila Wing Imaginal Disc. PLoS Biology 2011, 9(10):e1001182.

971 24. Corson F, Couturier L, Rouault H, Mazouni K, Schweisguth F. Self-organized
972 Notch dynamics generate stereotyped sensory organ patterns in *Drosophila*.
973 Science 2017, 356:eaai7407.

974 25. Raspopovic J, Marcon L, Russo L, Sharpe J. Modeling digits. Digit patterning is
975 controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen
976 gradients. Science 2014, 345:566-570.

977 26. Dubrulle J, McGrew MJ, Pourquié O. FGF signalling controls somite boundary
978 position and regulates segmentation clock control of spatiotem- poral Hox gene
979 activation. Cell 2001, 106:219–232.

980 27. Bakera RE, Schnella S, Maini PK. A clock and wavefront mechanism for somite
981 formation Develomental Biology 2006, 293:116-126.

982 28. Nijhout HF. The control of body size in insects. Developmental Biology 2003,
983 261:1-9.

984 29. Nijhout HJ, Riddiford LM, Mirth CK, Shingleton AW, Suzuki Y, Callier V. The
985 developmental control of size in insects. WIREs Developmental Biology 2014,
986 3:113-134.

987 30. Mirth CK, Shingleton AW. Integrating body and organ size in *Drosophila*: recent
988 advances and outstanding problems. Frontiers in Endocrinology 2012, 3:1-13.

989 31. Azevedo RB, French V, Partridge L. Temperature modulates epidermal cell size
990 in *Drosophila melanogaster*. Journal of Insect Physiology 2002, 48:231–237.

991 32. David JR, Moreteau B, Gauthier JP, Pétavy G, Stockel A, et al. Reaction norms
992 of size characters in relation to growth temperature in *Drosophila melanogaster*:
993 an isofemale lines analysis. . Genetics Selection Evolution 1994, 26:229-251.

994 33. French V, Feast M, Partridge L. Body size and cell size in *Drosophila*: the
995 developmental response to temperature. Journal of Insect Physiology 1998,
996 44:1081-1089.

997 34. James AC, Azevedo RB, Partridge L. Genetic and environmental responses to
998 temperature of *Drosophila melanogaster* from a latitudinal cline. Genetics 1997,
999 146:881-890.

1000 35. Partridge L, Barrie B, Fowler K, French V. Evolution and development of body
1001 size and cell size in *Drosophila melanogaster* in response to temperature.
1002 Evolution 1994, 48:1269-1276.

1003 36. Grunert LW, Clarke JW, Ahuja C, Eswaran H, Nijhout HF. A quantitative analysis
1004 of growth and size regulation in Manduca sexta: the physiological basis of
1005 variation in size and age at metamorphosis. PLoS ONE 2015, 10(5):e0127988-
1006 0127923.

1007 37. Reynolds S, Notthingham SF. Effects of temperature on growth and efficiency of
1008 food utilization in fifth-instar caterpillars of the tobacco hornworm, Manduca
1009 sexta. Journal of Insect Physiology 1985, 31:129-134.

1010 38. Thomas RH. Ecology of body size in *Drosophila buzzatii*: untangling the effects
1011 of temperature and nutrition. Ecological Entomology 1993, 18:84-90.

1012 39. Callier V, Nijhout HF. Control of body size by oxygen supply reveals size-
1013 dependent and size-independent mechanisms of molting and metamorphosis.
1014 Proceedings of the National Academy of Science USA 2011, 108:14664-14669.

1015 40. Callier V, Shingleton AW, Brent CS, Ghosh SM, Kim J, Harrison JF. The role of
1016 reduced oxygen in the developmental physiology of growth and metamorphosis
1017 initiation in *Drosophila melanogaster*. Journal of Experimental Biology 2013,
1018 216:4334-4340.

1019 41. Glendinning JI. How do herbivorous insects cope with noxious secondary plant
1020 compounds in their diet? Entomologia Experimentalis et Applicata 2002,
1021 104(1):15-25.

1022 42. Oliveira MM, Shingleton AW, Mirth CK. Coordination of wing and whole-body
1023 development at developmental milestones ensures robustness against

1024 environmental and physiological pertubations. PLoS Genetics 2014,
1025 10:e1004408.

1026 43. Wu Q, Brown MR. Signaling and function of insulin-like peptides in insects.
1027 Annual Review of Entomology 2005, 51(1):1-24.

1028 44. Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, et al. An evolutionarily
1029 conserved function of the *Drosophila* insulin receptor and insulin-like peptides in
1030 growth control. Current Biology 2001, 11:213-221.

1031 45. Ikeya T, Galic M, Belawat P, Nairz K, Hafen E. Nutrient-dependent expression of
1032 insulin-like peptides from neuroendocrine cells in the CNS contributes to growth
1033 regulation in *Drosophila*. Current Biology 2002, 12:1293-1300.

1034 46. Chen C, Jack J, Garofalo RS. The *Drosophila* insulin receptor is required for
1035 normal growth. Endocrinology 1996, 137:846-856.

1036 47. Yenush L, Fernandez R, Myers MGJ, Grammer TC, Sun XJ, et al. The
1037 *Drosophila* insulin receptor activates multiple signaling pathways but requires
1038 insulin receptor substrate proteins for DNA synthesis. Molecular and Cellular
1039 Biology 1996, 16:2509-2517.

1040 48. Géminard C, Rulifson EJ, Léopold P. Remote control of insulin secretion by fat
1041 cells in *Drosophila*. Cell Metabolism 2009, 10(3):199-207.

1042 49. Weinkove D, Leevers SJ. The genetic control of organ growth: insights from
1043 *Drosophila*. Current Opinion in Genetics & Development 2000, 10(1):75-80.

1044 50. Caldwell PE, Walkiewicz M, Stern M. Ras activity in the *Drosophila* prothoracic
1045 gland regulates body size and developmental rate via ecdysone release. Current
1046 Biology 2005, 15:1785-1795.

1047 51. Colombani J, Andersen DS, Leopold P. Secreted peptide Dilp8 coordinates
1048 *Drosophila* tissue growth with developmental timing. Science 2012, 336:582-585.

1049 52. Mirth CK, Truman JW, Riddiford LM. The role of the prothoracic gland in
1050 determining critical weight for metamorphosis in *Drosophila melanogaster*.
1051 Current Biology 2005, 15:1796-1807.

1052 53. Koyama T, Rodrigues MA, Athanasiadis A, Shingleton AW, Mirth CK. Nutritional
1053 control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis.
1054 eLife 2014, 3:e03091.

1055 54. Shingleton AW, Das J, Vinicius L, Stern DL. The temporal requirements for
1056 insulin signalling during development in *Drosophila*. PLoS Biology 2005, 3:e289.

1057 55. Stieper BC, Kupershtok M, Driscoll MV, Shingleton AW. Imaginal discs regulate
1058 developmental timing in *Drosophila melanogaster*. Developmental Biology 2008,
1059 321(1):18-26.

1060 56. Herboso L, Oliveira MM, Talamillo A, Pérez C, González M, Martín D, Sutherland
1061 JD, Shingleton AW, Mirth CK, Barrio R. Ecdysone promotes growth of imaginal
1062 discs through the regulation of Thor in *D. melanogaster*. Scientific Reports 2015,
1063 5:12383.

1064 57. Gokhale RH, Hayashi T, Mirque CD, Shingleton AW. Intra-organ growth
1065 coordination in *Drosophila* is mediated by systemic ecdysone signaling.
1066 Developmental Biology 2016, 418:135-145.

1067 58. Dye NA, Popović M, Spannl S, Etournay R, Kainmüller D, Ghosh S, Myers EW,
1068 Jülicher F, Eaton S. Cell dynamics underlying oriented growth of the *Drosophila*
1069 wing imaginal disc. Development 2017, 144:4406-4421.

1070 59. Mirth CK, Truman JW, Riddiford LM. The Ecdysone Receptor controls the post-
1071 critical weight switch to nutrition-independent differentiation in *Drosophila* wing
1072 imaginal discs. Development 2009, 136(14):2345-2353.

1073 60. Mendes CM, Mirth CK. Stage-specific plasticity in ovary size is regulated by
1074 insulin/insulin-like growth factor and ecdysone signalling in *Drosophila*. Genetics
1075 2016, 202:703-719.

1076 61. Gancz D, Lengil T, Gilboa L. Coordinated regulation of niche and stem cell
1077 precursors by hormonal signaling. PLoS Biology 2011, 9:e1001202.

1078 62. Shingleton AW, Mirth CK, Bates PW. Developmental model of static allometry in
1079 holometabolous insects. Proceedings of the Royal Society Series B 2008,
1080 275:1875-1885.

1081 63. McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. Spatiotemporal rescue
1082 of memory dysfunction in *Drosophila*. Science 2003, 302:1765-1768.

1083 64. McGuire SE, Mao Z, Davis RL. Spatiotemporal gene expression targeting with
1084 the TARGET and gene-switch systems in *Drosophila*. Science Signalling 2004,
1085 2004:16.

1086 65. Nijhout HF, Wheeler DE. Growth models of complex allometries in
1087 holometabolous insects. The American Naturalist 1996, 148:40-56.

1088 66. Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C,
1089 Antoniewski C, Carre C, Noselli S, Leopold P. Antagonistic actions of ecdysone
1090 and insulins determine final size in *Drosophila*. Science 2005, 310(5748):667-
1091 670.

1092 67. Mirth C, Truman JW, Riddiford LM. The role of the prothoracic gland in
1093 determining critical weight for metamorphosis in *Drosophila melanogaster*.
1094 Current biology : CB 2005, 15(20):1796-1807.

1095 68. Warren JT, Yerushalmi Y, Shimell MJ, O'Connor MB, Restifo LL, Gilbert LI.
1096 Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval
1097 development of *Drosophila melanogaster*: correlations with changes in gene
1098 activity. Developmental Dynamics 2006, 235:315-326.

1099 69. Riddiford LM. Hormones and *Drosophila* Development. In: *The Development of*
1100 *Drosophila melanogaster.* Edited by Bates M, Martinez-Arias A, vol. 2. Plainview:
1101 Cold Spring Harbor Laboratory Press; 1993: 899-939.

1102 70. Mirth CK, Tang HY, Makohon-Moore SC, Salhadar S, Gokhale RH, Warner RD,
1103 Koyama T, Riddiford LM, Shingleton AW. Juvenile hormone regulates body size

1104 and perturbs insulin signaling in *Drosophila*. Proceedings of the National
1105 Academy of Sciences USA 2014, 111:7018-7023.

1106 71. Parker NF, Shingleton AW. The coordination of growth among *Drosophila* organs
1107 in response to localized growth-perturbation. Developmental Biology 2011,
1108 357:318-325.

1109 72. Testa ND, Ghosh SM, Shingleton AW. Sex-specific weight loss mediates sexual
1110 size dimorphism in *Drosophila melanogaster*. PLoS One 2013, 8:e58936.

1111 73. Strassburger K, Lutz M, Müller S, Teleman AA. cdysone regulates *Drosophila*
1112 wing disc size via a TORC1 dependent mechanism. Nature Communications
1113 2021, 12:6884.

1114 74. Lee GJ, Han G, Yun HM, Lim JJ, Noh S, Lee J, Hyun S. Steroid signaling
1115 mediates nutritional regulation of juvenile body growth via IGF-binding protein in
1116 *Drosophila*. Proceedings of the National Academy of Sciences 2018,
1117 115(23):5992.

1118 75. Miner AL, Rosenberg AJ, Nijhout HF. Control of growth and differentiation of the
1119 wing imaginal disk of *Precis coenia* (Lepidoptera: Nymphalidae). Journal of
1120 Insect Physiology 2000, 46:251-258.

1121 76. Parker J, Struhl G. Scaling the *Drosophila* wing:TOR-dependent target gene
1122 access by the Hippo pathway transducer Yorkie. PLoS Biology 2015,
1123 13:e1002274.

1124 77. Ohhara Y, Kobayashi S, Yamanaka N. Nutrient-Dependent Endocycling in
1125 Steroidogenic Tissue Dictates Timing of Metamorphosis in Drosophila
1126 melanogaster. PLoS Genetics 2017, 13(1):e1006583.

1127 78. Mirth CK, Saunders TE, Amourda C. Growing up in a changing world:
1128 environmental regulation of development in insects. Annual Reviews of
1129 Entomology 2021, 66:81-99.

1130 79. Moreteau B, David JR. Phenotypic plasticity and reaction norms of abdominal
1131 bristle number in *Drosophila melanogaster*. Journal of Bioscience 2005, 30:689-
1132 697.

1133 80. Moreteau B, Gibert P, Delpuech J-M, Petavy G, David JR. Phenotypic plasticity
1134 of sternopleural bristle number in temperate and tropical populations of
1135 *Drosophila melanogaster*. Genetical Research 2003, 81:25-32.

1136 81. David JR. Le nombre d'ovarioles chez *Drosophila melanogaster*: relation avec la
1137 fécondité et valeur adaptive. Archives de Zoologie Expérimentale e Générale
1138 1970, 111:357-370.

1139 82. Delpuech JM, Moreteau B, Chiche J, Pla E, Vouidibio J, David JR. Phenotypic
1140 plasticity and reaction norms in temperate and tropical populations of *Drosophila*
1141 *melanogaster* - Ovarian size and developmental temperature. Evolution 1995,
1142 49(4):670-675.

1143 83. Green DA, Extavour CG. Insulin signalling underlies both plasticity and
1144 divergence of a reproductive trait in *Drosophila*. Proceedings of the Royal Society
1145 B: Biological Sciences 2014, 281:20132673–20132673.

1146 84. Hodin J, Riddiford LM. Different mechanisms underlie phenotypic plasticity and
1147 interspecific variation for a reproductive character in Drosophilids (Insecta:
1148 Diptera). Evolution 2000, 54(5):1638-1653.

1149 85. Debat V, Bégin M, Legout H, David JR. Allometric and nonallometric components
1150 of *Drosophila* wing shape respond differently to developmental temperature.
1151 Evolution 2003, 57:2773-2784.

1152 86. Debat V, Debelle A, Dworkin I. Plastitcity, canalization, and developmental
1153 stability of the *Drosophila* wing: joint effects of mutation and developmental
1154 temperature. Evolution 2009, 63(11):2864-2876.

1155 87. Outomuro D, Adams DC, Johansson F. Wing shape allometry and aerodynamics
1156 in calopterygid damselflies: a comparative approach. BMC Evolutionary Biology
1157 2013, 13(1):118.

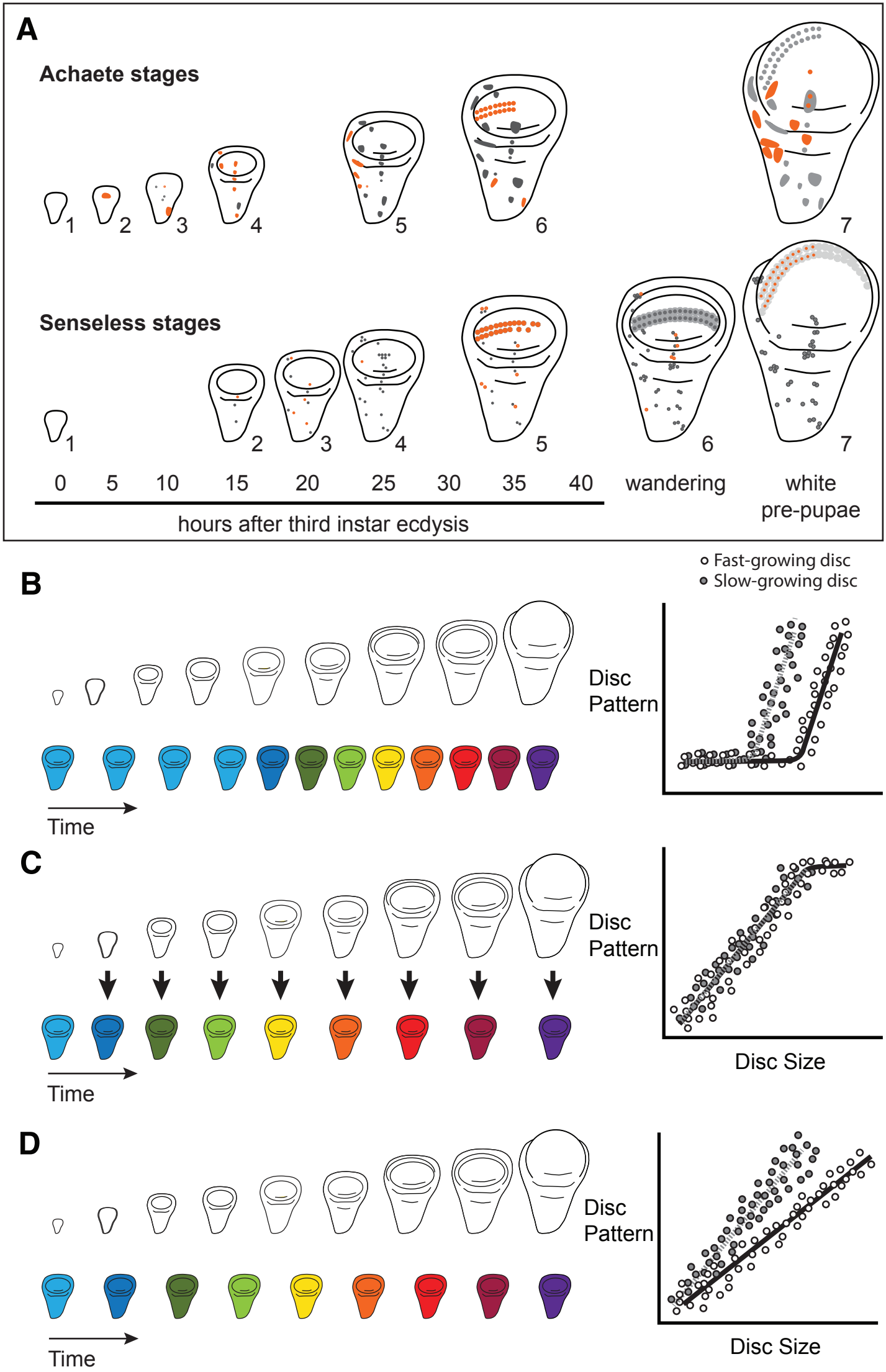
1158 88. Bitner-Mathé BC, Klaczko LB. Size and shape heritability in natural populations
1159 of *Drosophila mediopunctata*: temporal and microgeographical variation.
1160 Genetica 1999, 105:35-42.

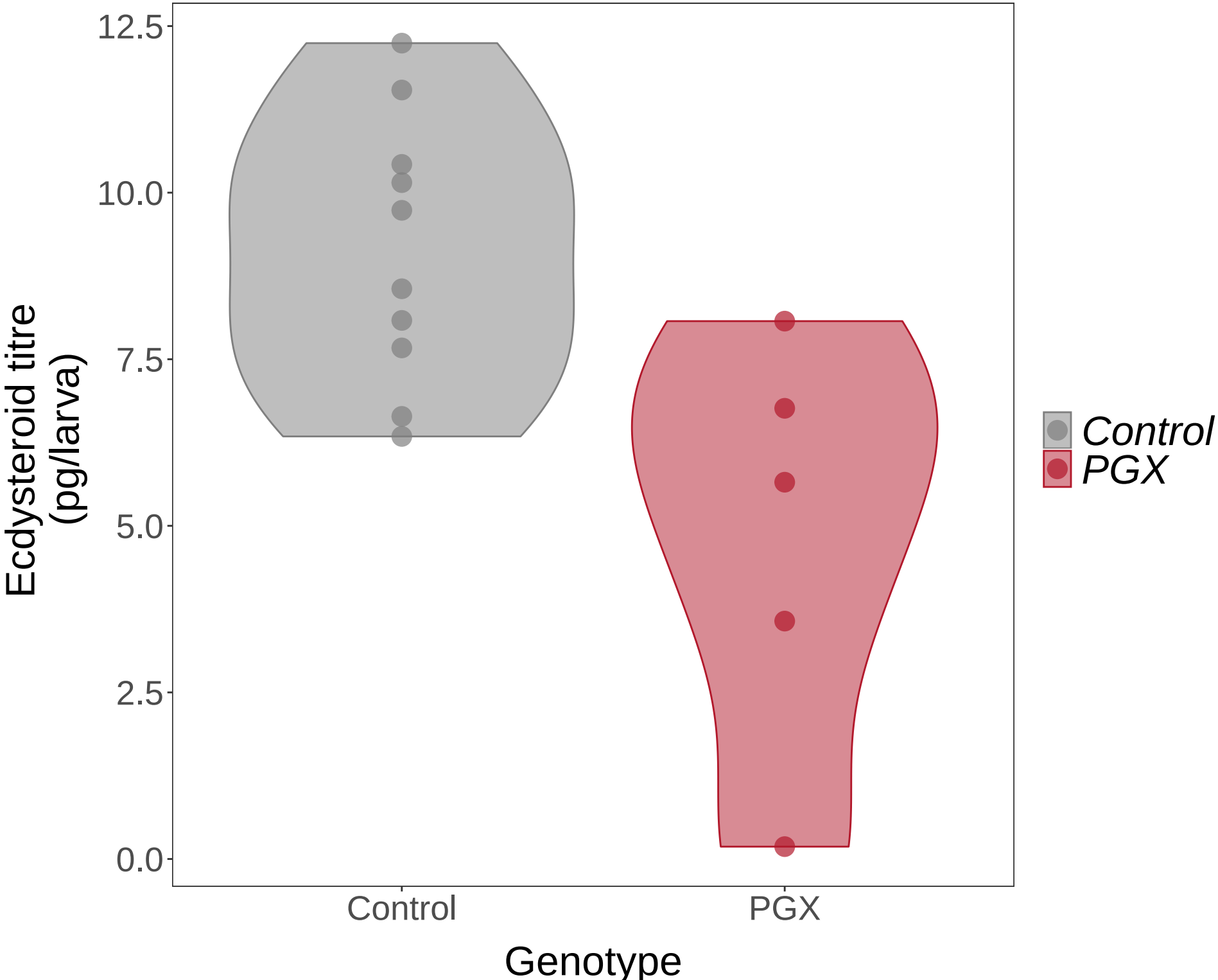
1161 89. Matamoro-Vidal A, Salazar-Ciudad I, Houle D. Making quantitative morphological
1162 variation from basic developmental processes: Where are we? The case of the
1163 Drosophila wing. Developmental Dynamics 2015, 244(9):1058-1073.

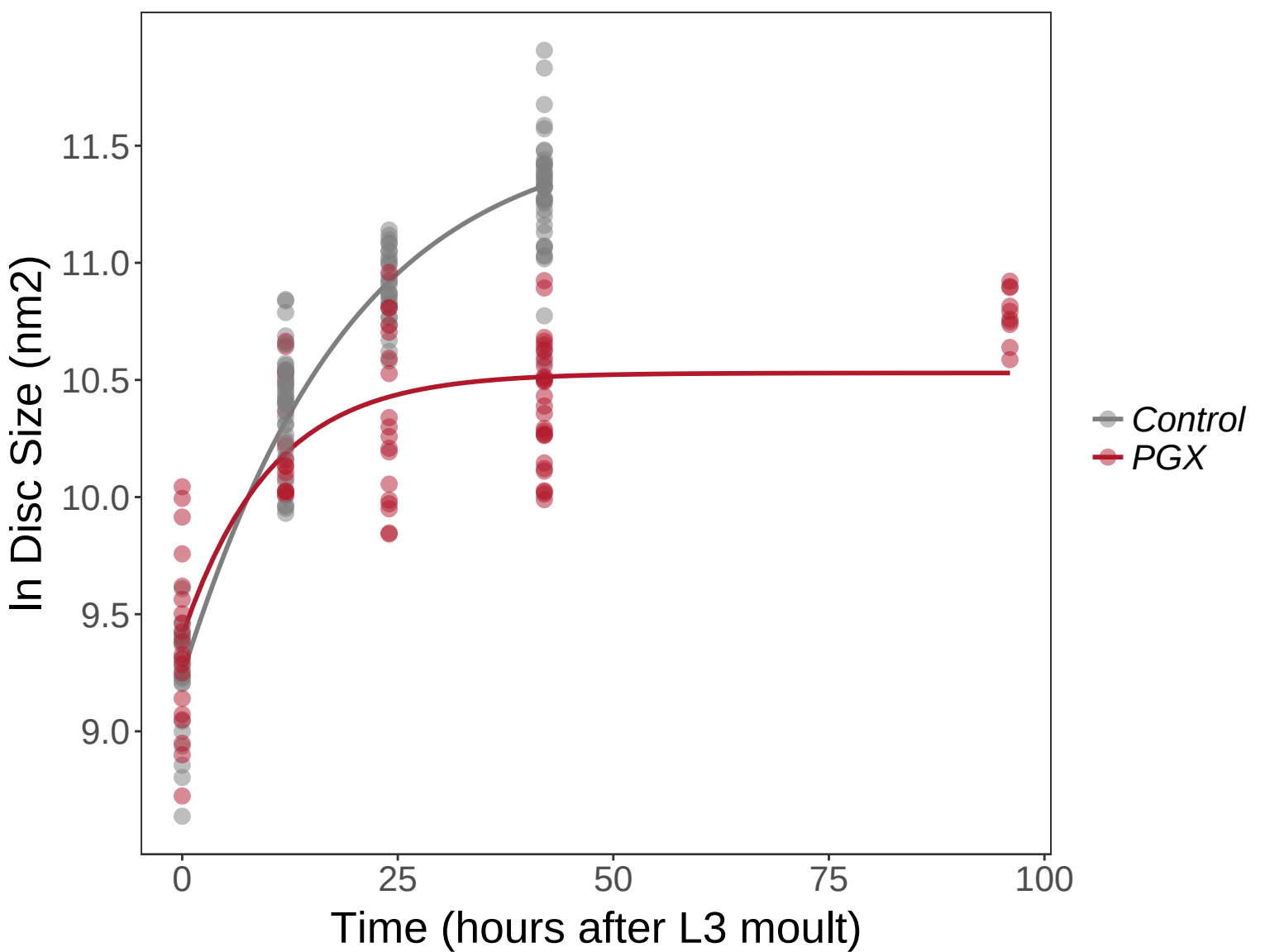
1164 90. Cobham AE, Mirth CK. The development of body and organ shape. BMC
1165 Zoology 2020, 5(1):14.

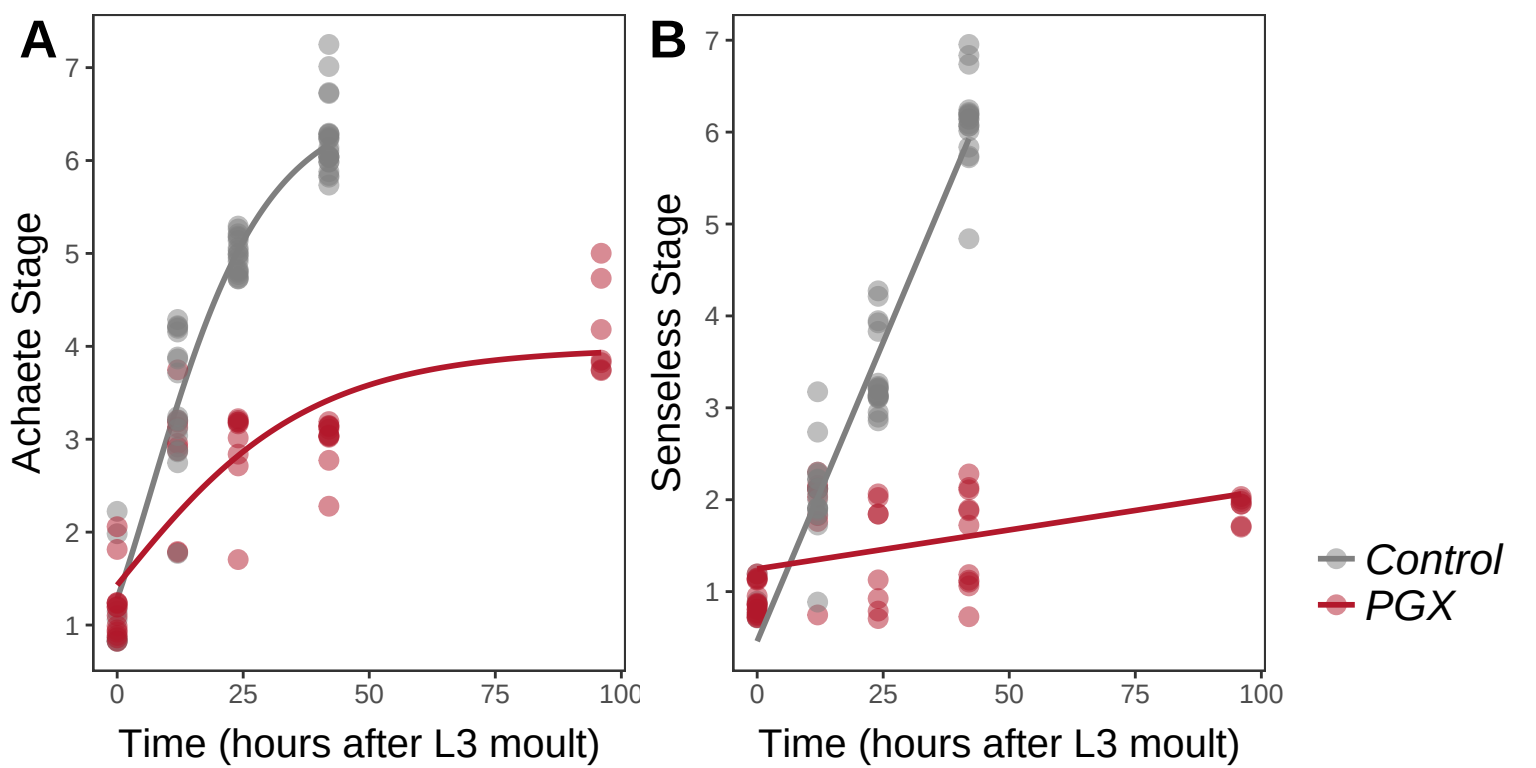
1166 91. Lenth R. emmeans: Estimated Marginal Means, aka Least-Squares Means. R
1167 package version 152-1 https://CRANR-projectorg/package=emmeans 2020.

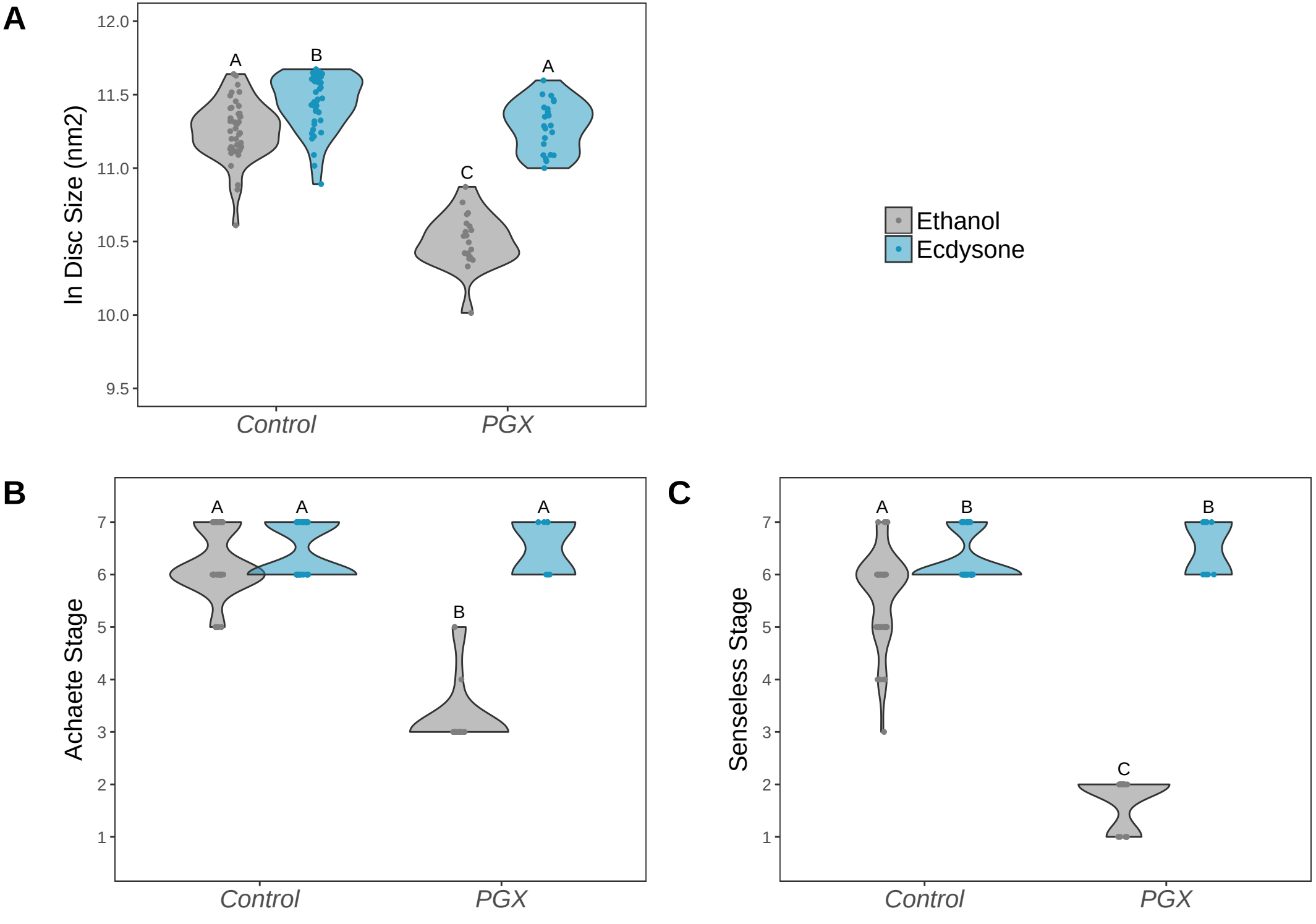
1168

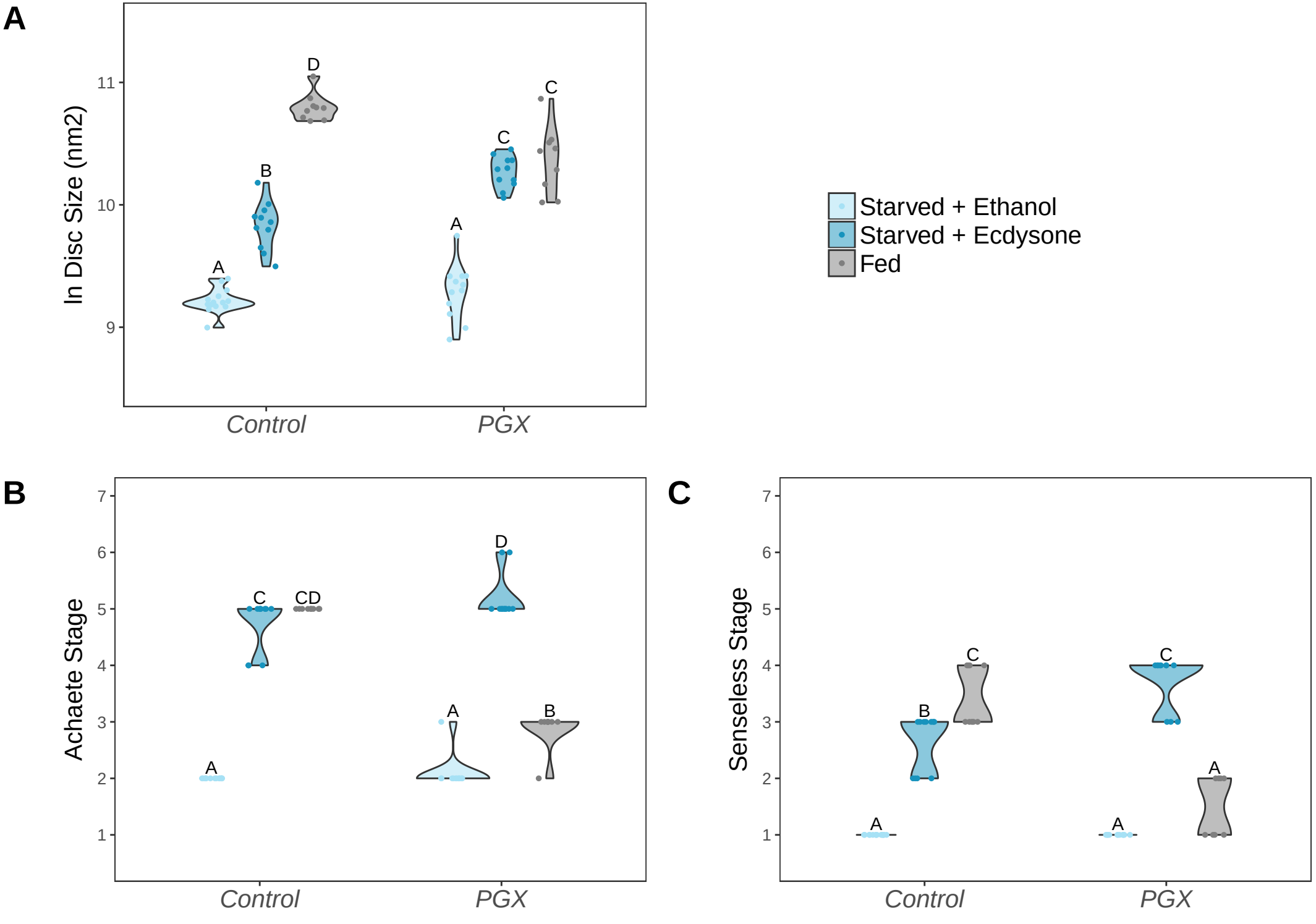

A
Achaete stages
1
2
3
4
5
6
7
Senseless stages
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
wandering
white pre-pupae
hours after third instar ecdysis
B
Fast-growing disc
Slow-growing disc
Disc Pattern
Time
C
Disc Pattern
Disc Size
Time
D
Disc Pattern
Disc Size
Time

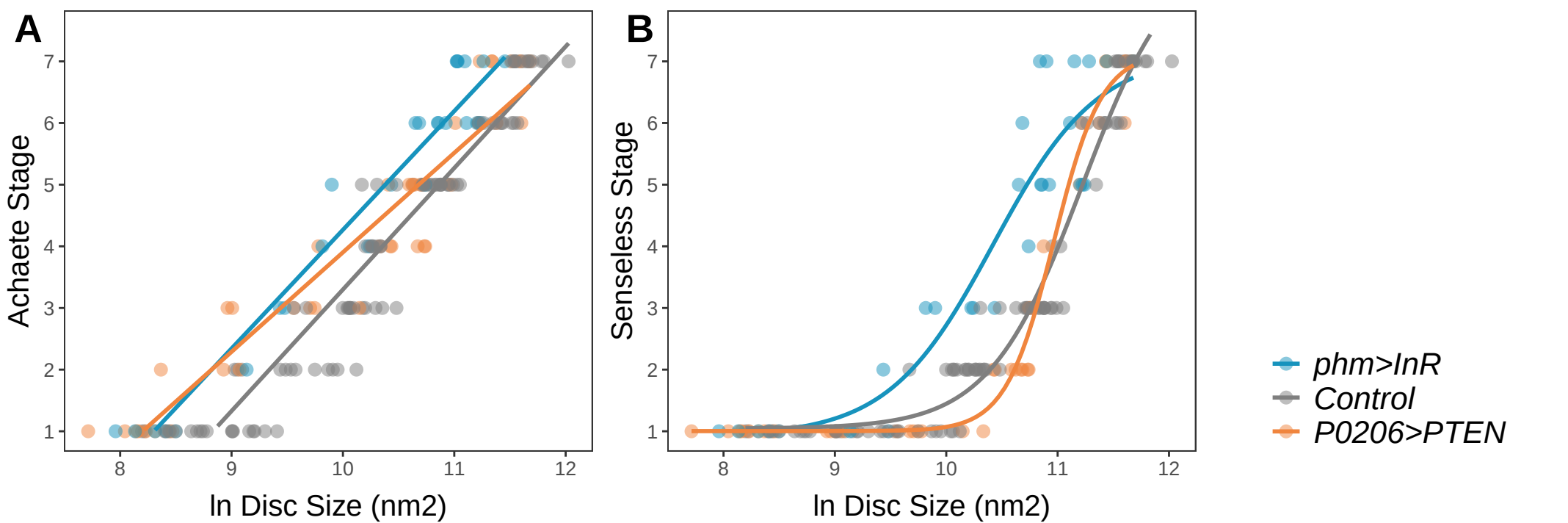

12.5
10.0
7.5
5.0
2.5
0.0
Ecdysteroid titre
(pg/larva)
Control
PGX
Genotype
Control
PGX

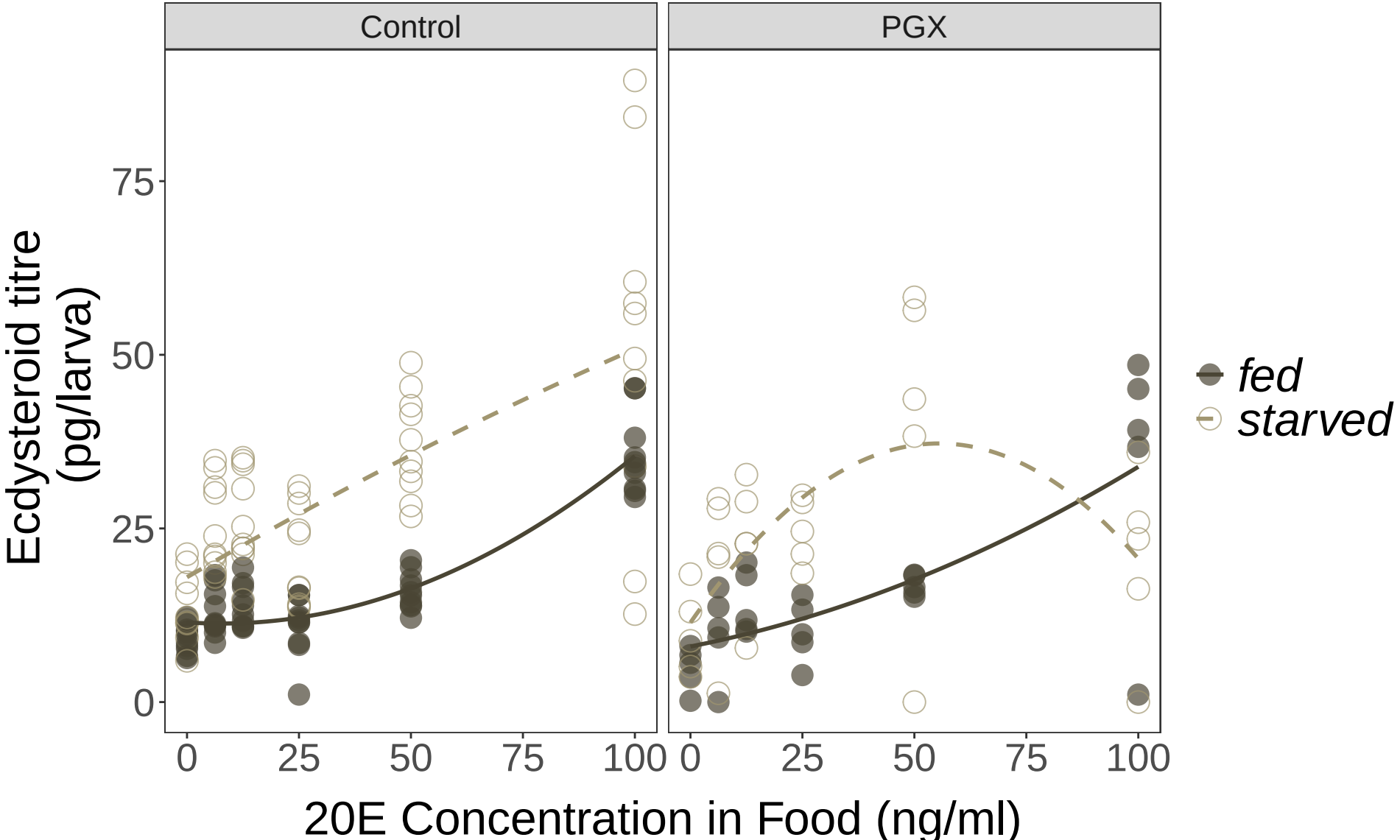

ln Disc Size (nm2)
11.5
11.0
10.5
10.0
9.5
9.0
0
25
50
75
100
Time (hours after L3 moult)
Control
PGX

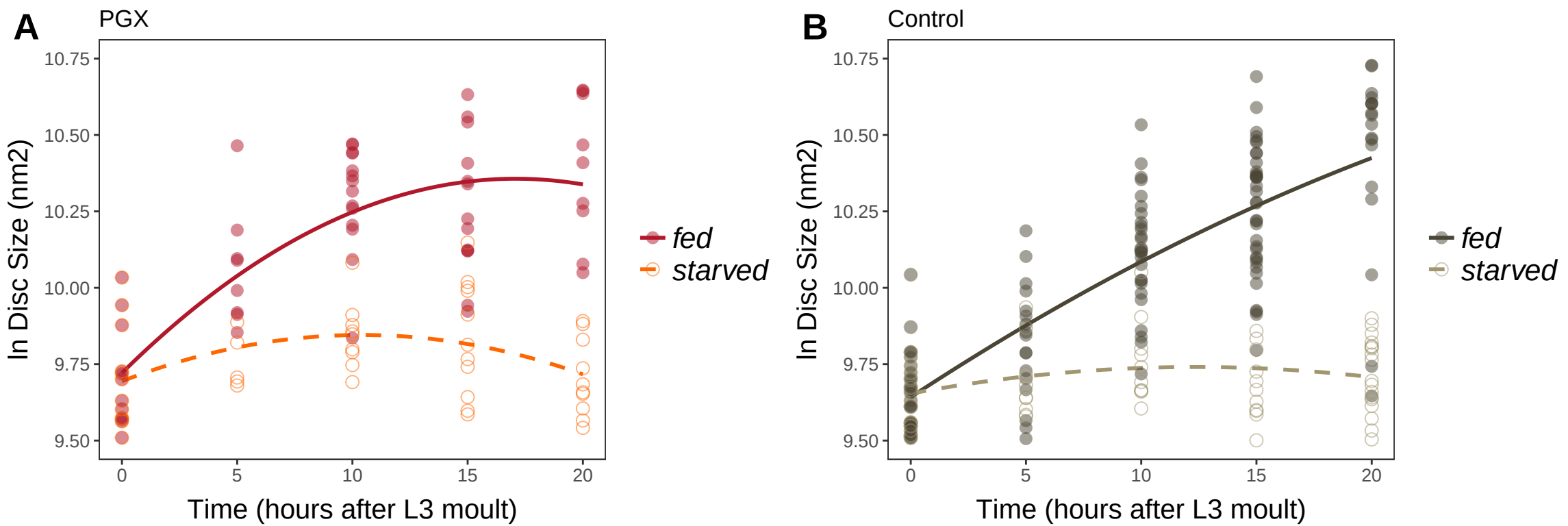

A
B
Achaete Stage
Senseless Stage
Time (hours after L3 moult)
Time (hours after L3 moult)
0
25
50
75
100
1
2
3
4
5
6
7
Control
PGX

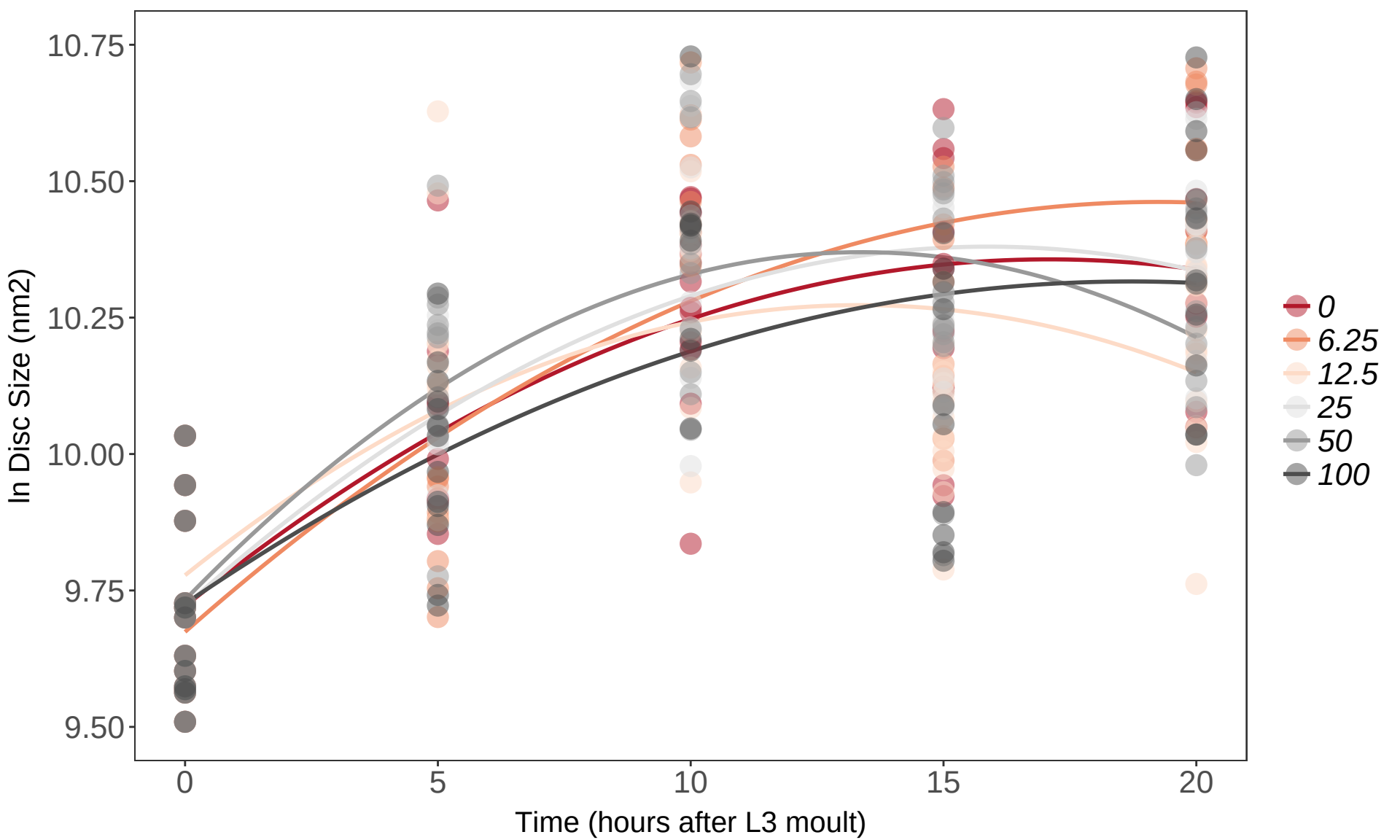

A
ln Disc Size (nm2)
12.0
11.5
11.0
10.5
10.0
9.5
A
B
C
A
Control
PGX
Ethanol
Ecdysone
B
Achaete Stage
7
6
5
4
3
2
1
A
A
B
A
Control
PGX
C
Senseless Stage
7
6
5
4
3
2
1
A
B
C
B
Control
PGX

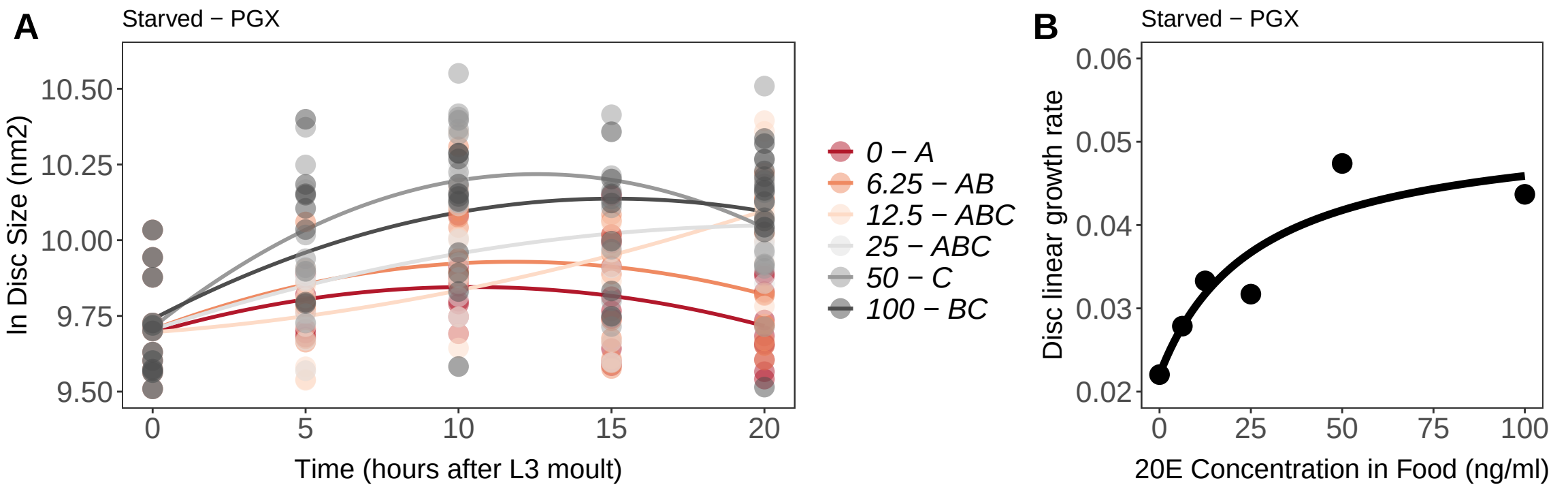

A
ln Disc Size (nm2)
11
10
9
D
B
A
C
C
A
Control
PGX
Starved + Ethanol
Starved + Ecdysone
Fed
B
Achaete Stage
7
6
5
4
3
2
1
C
CD
A
D
A
B
Control
PGX
C
Senseless Stage
7
6
5
4
3
2
1
C
B
A
C
A
A
Control
PGX

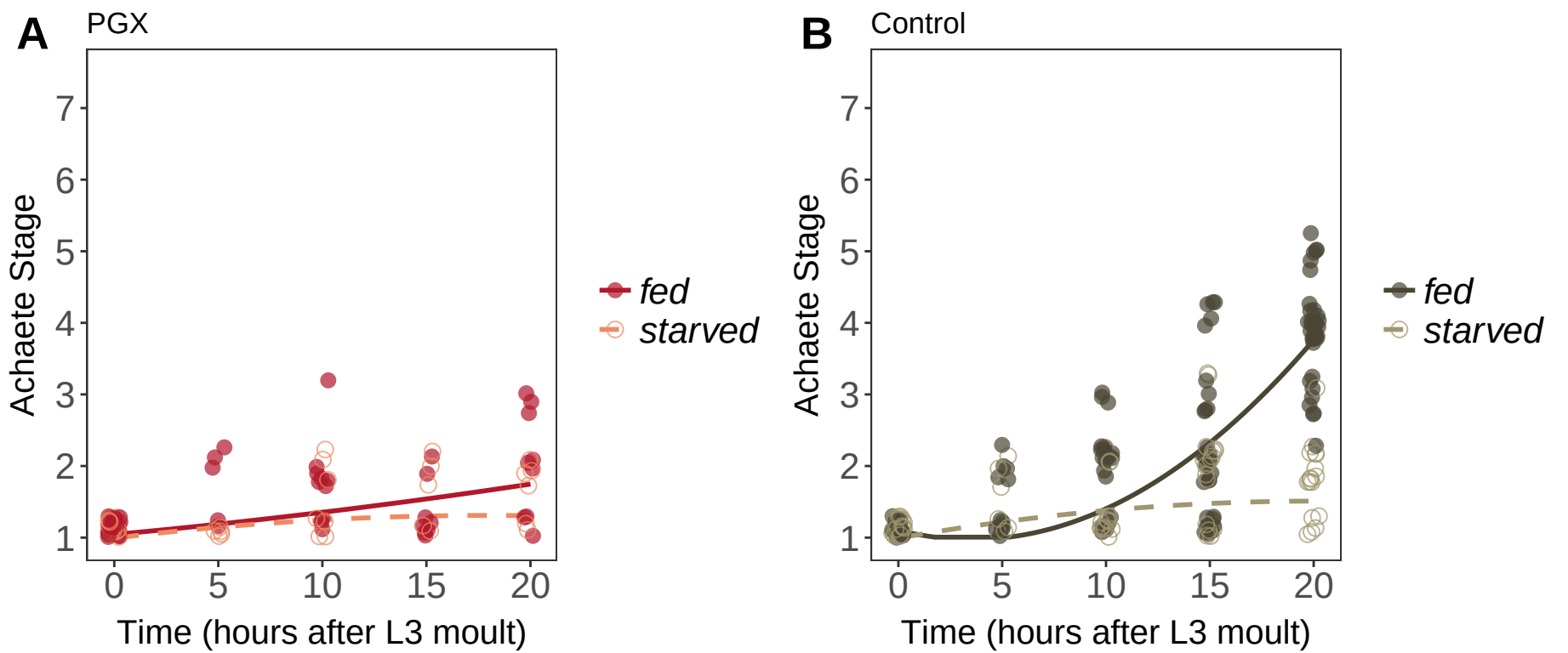

A
Achaete Stage
ln Disc Size (nm2)
B
Senseless Stage
ln Disc Size (nm2)
phm>InR
Control
P0206>PTEN

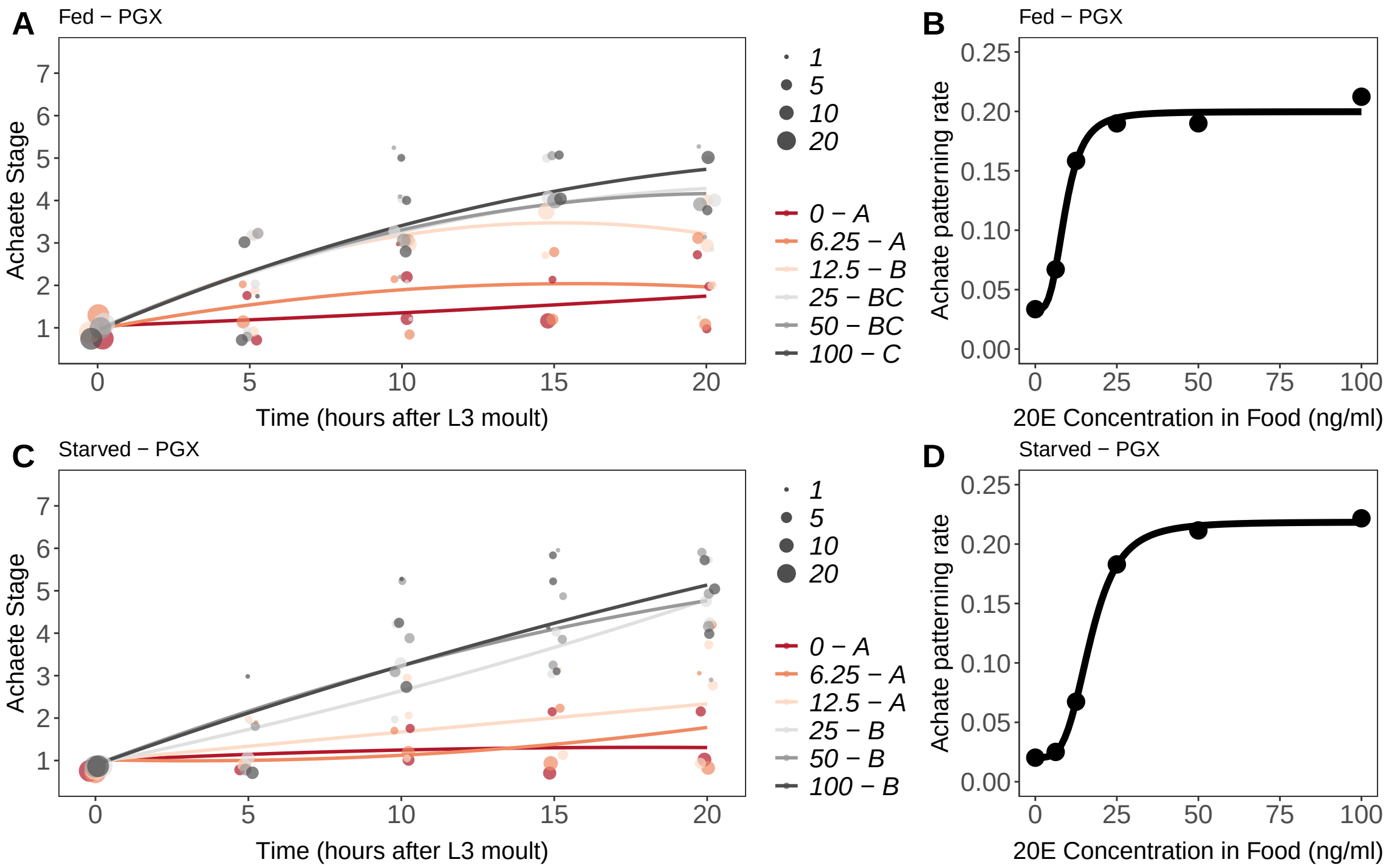

Control
PGX
Ecdysteroid titre
(pg/larva)
0
25
50
75
0
25
50
75
100
0
25
50
75
100
20E Concentration in Food (ng/ml)
fed
starved

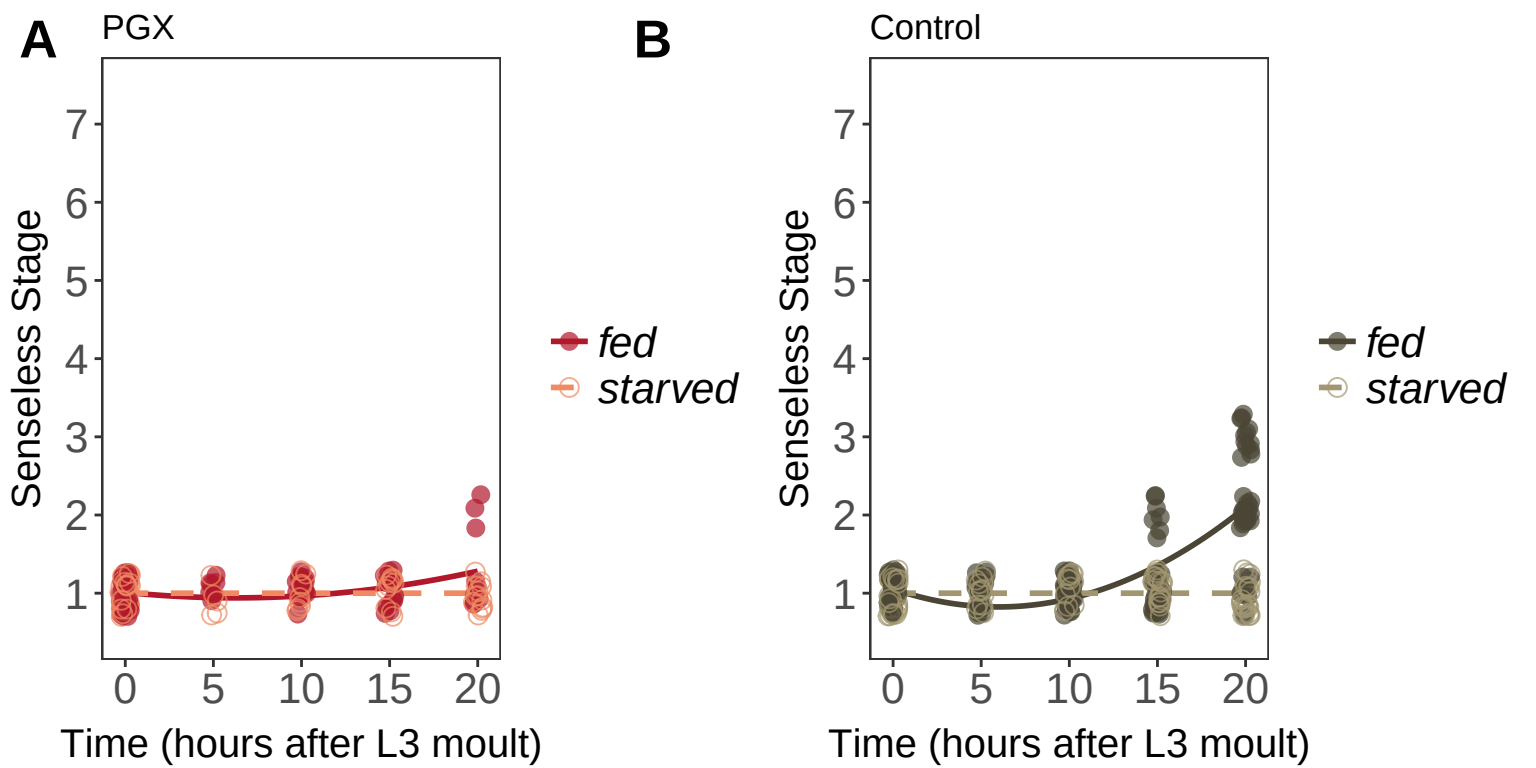

A
PGX
B
Control
ln Disc Size (nm2)
10.75
10.50
10.25
10.00
9.75
9.50
0
5
10
15
20
Time (hours after L3 moult)
fed
starved

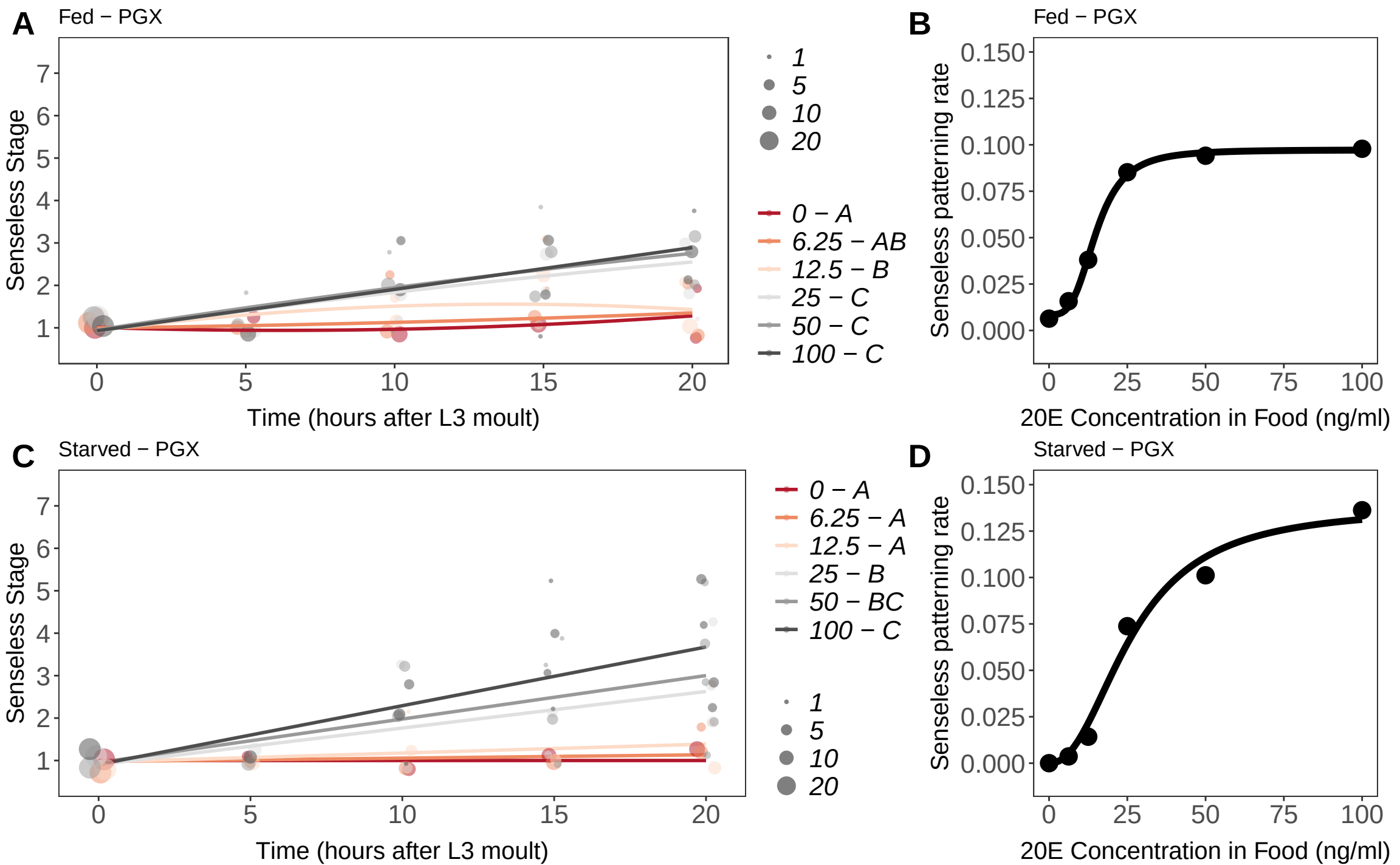

10.75
10.50
10.25
10.00
9.75
9.50
ln Disc Size (nm2)
0
5
10
15
20
Time (hours after L3 moult)
0
6.25
12.5
25
50
100


A
Starved − PGX
ln Disc Size (nm2)
10.50
10.25
10.00
9.75
9.50
0
5
10
15
20
Time (hours after L3 moult)
0 − A
6.25 − AB
12.5 − ABC
25 − ABC
50 − C
100 − BC
B
Starved − PGX
Disc linear growth rate
0.06
0.05
0.04
0.03
0.02
0
25
50
75
100
20E Concentration in Food (ng/ml)


A
PGX
Achaete Stage
7
6
5
4
3
2
1
0
5
10
15
20
Time (hours after L3 moult)
fed
starved
B
Control
Achaete Stage
7
6
5
4
3
2
1
0
5
10
15
20
Time (hours after L3 moult)
fed
starved


A
Fed − PGX
Achaete Stage
Time (hours after L3 moult)
1
5
10
20
0 − A
6.25 − A
12.5 − B
25 − BC
50 − BC
100 − C
B
Fed − PGX
Achate patterning rate
20E Concentration in Food (ng/ml)
C
Starved − PGX
Achaete Stage
Time (hours after L3 moult)
1
5
10
20
0 − A
6.25 − A
12.5 − A
25 − B
50 − B
100 − B
D
Starved − PGX
Achate patterning rate
20E Concentration in Food (ng/ml)


A
PGX
B
Control
Senseless Stage
7
6
5
4
3
2
1
0
5
10
15
20
Time (hours after L3 moult)
fed
starved


A
Fed − PGX
Senseless Stage
Time (hours after L3 moult)
1
5
10
20
0 − A
6.25 − AB
12.5 − B
25 − C
50 − C
100 − C
B
Fed − PGX
Senseless patterning rate
20E Concentration in Food (ng/ml)
C
Starved − PGX
Senseless Stage
Time (hours after L3 moult)
0 − A
6.25 − A
12.5 − A
25 − B
50 − BC
100 − C
1
5
10
20
D
Starved − PGX
Senseless patterning rate
20E Concentration in Food (ng/ml)