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Maintaining robust size across environmental conditions is achieved through plastic growth dynamics in the central nervous system of *Drosophila melanogaster*

Ansa E. Cobham[1*], Brent Neumann[2], Christen K. Mirth[1*]

[1]School of Biological sciences, Monash University, Melbourne, Australia
[2]Neuroscience Program, Monash Biomedicine Discovery Institute and Department of Anatomy and Developmental Biology, Monash University, Melbourne, Australia

***Correspondence**: ansacobham@yahoo.com, christen.mirth@monash.edu

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Running Title: Different growth dynamics regulate robustness in brain size

Abstract

Organ growth is tightly regulated across environmental conditions to generate appropriate final size. While the size of some organs is free to vary, others need to maintain constant size to function properly. This poses a unique problem: how is robust final size achieved when environmental conditions alter the rates and the duration of growth? Brain growth is known to be "spared" from the effects of the environment from humans to fruit flies. Here, we explore how this robustness in brain size is achieved by examining differences in growth dynamics between the larval body, the brain, and the mushroom bodies – a brain compartment – in *Drosophila melanogaster* across thermal and nutritional conditions. We identify key differences in patterns of growth between the whole brain and mushroom bodies, as well as differences in the signalling pathways implicated, that are likely to underlie robustness of final organ shape. Further, we show that these differences produce distinct brain shapes across environments.

Introduction

How are the shapes and sizes of growing organs regulated throughout development to generate fully-functional multicellular organisms with highly specialized parts? This seems particularly difficult to understand given that body parts initiate growth at different times, and further grow at different rates and with differing dynamics (Huxley, 1932; Andersen et al., 2013; Eder et al., 2017). In addition, some organs show exquisite sensitivity to environmental conditions, known as plasticity, changing their shape and size with changes in nutrition, temperature, and other conditions (Bateson, 2017). In contrast, other organs maintain relatively constant final sizes across conditions (Nijhout, 2002; Bateson, 2017). Properties that allow growth to resist perturbations in environmental conditions contribute to robustness in development (Nijhout,

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2002; Bateson, 2017; Mirth & Shingleton, 2019). As a result of differing degrees of plasticity or robustness across organs, organisms that develop in different environments, such as rich versus poor nutritional environments, will differ in their body size and shape (Mirth & Shingleton, A. W., 2012). Understanding the properties of organ growth that allow them to be either plastic or robust to environmental conditions is key to uncovering how correct, functional body form is achieved.

Extensive studies in insects have described how the patterns of growth across organs generate variation in size and shape of the adult body (Mirth & Shingleton, A. W., 2012; Andersen et al., 2013; Nijhout et al., 2014; Mirth & Shingleton, 2019). Organs differ in their growth dynamics because they grow at different rates, grow with different dynamics, or grow for differing amounts of time. This can occur either at the level of an individual organ or through coordinating growth processes among organs relative to the growing body (Huxley, 1932; Shingleton & Frankino, 2018). Environmental conditions can alter each of these growth properties. For instance, in lepidopterans like the tobacco horn worm *Manduca sexta* and the buckeye butterfly *Junonia coenia,* and in fruit flies like *Drosophila melanogaster,* poor nutrition results in reduced growth rates in the developing wing and also extends the larval development period (Miner et al., 2000; Shingleton et al., 2008; Shingleton et al., 2009; Nijhout & Grunert, 2010). Poorly nourished larvae give rise to adults with small wings because wing growth is sufficiently suppressed that the extended growth period cannot compensate to restore wing size to fully-fed levels (Shingleton et al., 2009). In *D.* melanogaster, rearing larvae at lower temperatures also reduces wing growth rates, albeit to a lesser degree than poor nutrition, and extends developmental time. However, in this case even though growth rates are slower, the extended developmental period results in adults with larger wings (McDonald et al., 2018). These findings illustrate that if we want to understand how organs achieve either plasticity or robustness in their final size, we first need to understand how these properties of organ growth change across environmental conditions.

Across a wide variety of animals, including mammals and insects, the brain is generally less sensitive to changes in environmental conditions than other organs of the body (Cusick & Georgieff, 2016). This is commonly referred to as brain sparing. In humans, reduced nutrient

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availability or reduced oxygen supply during late fetal life reduces fetal growth (Cohen et al., 2015). However, oxygen and nutrient supplies to the brain are maintained at the expense of the rest of the body. As such, at birth newborns have reduced weight and body sizes and disproportionately large heads (Cox & Marton, 2009; Cohen et al., 2015). This illustrates that the brain has built-in mechanisms to ensure its size is not compromised.

The larval central nervous system of the fruit fly *Drosophila melanogaster*, hereafter referred to as the brain, provides an excellent system for how organ growth can be robust to environmental perturbation. *Drosophila* larvae reared under poor nutrient conditions have proportionally larger brains than those reared under nutrient rich conditions (Cheng et al., 2011). In *Drosophila*, the brain differentiates in the embryo, a stage that is protected from nutrient restriction as the embryos not feed. However, reactivation of quiescent neuroblast and glial stem cells occurs early in the first larval stage of development and requires cell autonomous nutrient signals (Yuan et al., 2020). After reactivation, most brain growth occurs in the three larval stages, and nutrition plays an important role. Poor nutrition, especially in the later stages of larval development, produces in small sized adults (Mirth & Shingleton, 2012), but brain growth is spared against poor nutrition via the action of a tyrosine kinase-like insulin receptor called Alk and its ligand Jelly Belly (Jeb). Jeb is secreted by glial cells under poor nutrient conditions (Cheng et al., 2011). Once bound to its ligand, Alk activates downstream effectors of the insulin signalling pathway and also regulates downstream targets of the TOR kinase bypassing amino-acid sensing in the absence of nutrient cues. By maintaining the insulin and TOR pathways active, Jeb ensures that the size of the brain is maintained even when larvae are starved, and that the composition of cells in the brain is maintained for proper functioning (Cheng et al., 2011; Lanet & Maurange, 2014).

While these findings highlight a genetic mechanism through which brain sparing occurs, they do not explain how brain growth adjusts with extended larval growth periods caused by poor nutrition. If Alk caused growth rates to remain as high in starved larvae as it does in fed, the extension in developmental time caused by starvation would cause brains to overgrow. Because this does not happen, it suggests that the brains of starved larvae adjust their growth dynamics to avoid overshooting their size with longer growth periods.

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This could happen in several ways. Firstly, when larvae were starved, their brains could maintain constant growth rates but then stop growing once they reach a target size (Figure 1A). This could be modelled using non-linear models that approach an asymptote. Alternatively, brains from starved larvae could delay the time at which they initiate growth, but then maintain constant rates once growth is initiated (Figure 1A). A change in the lag term of a lagged-exponential model between starved and fed larval brains would indicate that this was the method through which brains adjust with environmental conditions. Finally, Jeb and Alk might not act to ensure insulin and TOR signalling are maintained at constant levels. Instead this pathway might tune growth rates, and the timing at which growth is initiated, to adjust for the extended growth period (Figure 1A). This would result in changes in both the lag and the growth rate terms from a lagged-exponential model. These differences in growth dynamics are important, as each implies a different mechanism for adjusting brain size with environmental conditions.

Like all brains, the *Drosophila* larval brain is composed of distinct functional compartments, each containing specific populations of neurons (Truman & Bate, 1988; Prokop & Technau, 1991). The growth properties of neurons are known to differ across compartments (Hartenstein, V. et al., 2008). The neuroblasts undergo three waves of proliferation at distinct developmental stages - embryonic, larval, and pupal stages - to generate all the neurons in the larval and adult brain (Hartenstein, Volker et al., 1985). Neuroblast progenitors produced during the embryonic wave go into quiescence (Hartenstein, Volker et al., 2008), and are reactivated during larval development to form the neural cells that will contribute to the adult brain. The timing of reactivation of neuroblasts varies between different populations of cells in the brain, determined by both the activity of proneural genes (Hartenstein, V. & Wodarz, 2013) and by environmental conditions (Chell & Brand, 2010; Speder & Brand, 2018)

Supporting this idea, different neuronal subclasses change their rates of cell division in response to nutrition and other environmental conditions like temperature, light, and population densities during larval stages of growth, although the overall brain size is still maintained (Prokop & Technau, 1994; Heisenberg et al., 1995; Lin et al., 2013; Wang et al., 2018). While other neuronal populations enter quiescence in the early larval stages, the mushroom body neurons –

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paired neuronal structures important for olfactory processing and learning – continue to divide from the first instar (L1) stage onwards, differentiating two distinct lineages across larval development: the γ and α'/β' lineages (Kunz et al., 2012). A third lineage, the α/β lineage, is established during pupal development (Kunz et al., 2012). When faced with extremely poor nutritional conditions that reduce larval growth, the mushroom body neurons maintain division of the same neuronal lineage – the γ lineage – over longer developmental periods (Lin et al., 2013; Rossi et al., 2017). In contrast, the optic lobe neurons, which receive sensory input from the visual system, are activated late in larval development and highly sensitive to changes in the nutritional environment, and the size of the neuron pool involved in initial proliferation depends on nutrient availability (Lanet & Maurange, 2014). This suggests that specific brain regions differ in how they protect the brain from environmental perturbations.

These findings allow us to further propose a model of how the mushroom body compartments of the brain might remain constant in size in the face of changing environmental conditions. Firstly, because neuroblasts such as those that give rise to the mushroom body neurons begin proliferating much earlier than the majority of the brain neuroblasts, and proliferate throughout the larval instars, we might expect the size of these structures to increase constantly, or linearly, throughout larval development (Figure 1B). Most of the remaining neuroblasts of the brain initiate proliferation late in the second instar. Thus, we would expect a period of little or no discernible growth across the whole brain in the first two instars, punctuated by a rapid onset of growth in later development that would be best characterized by exponential growth with a time lag to its onset (Figure 1B). This would mean that growth dynamics in the mushroom body could differ significantly from that of the whole brain. Differences in their dynamics could indicate that growth is mediated by differing mechanisms between compartments, potentially dictating their response to environmental cues. As an alternative hypothesis, the dynamics of mushroom body growth could also be exponential but could have a much shallower slope.

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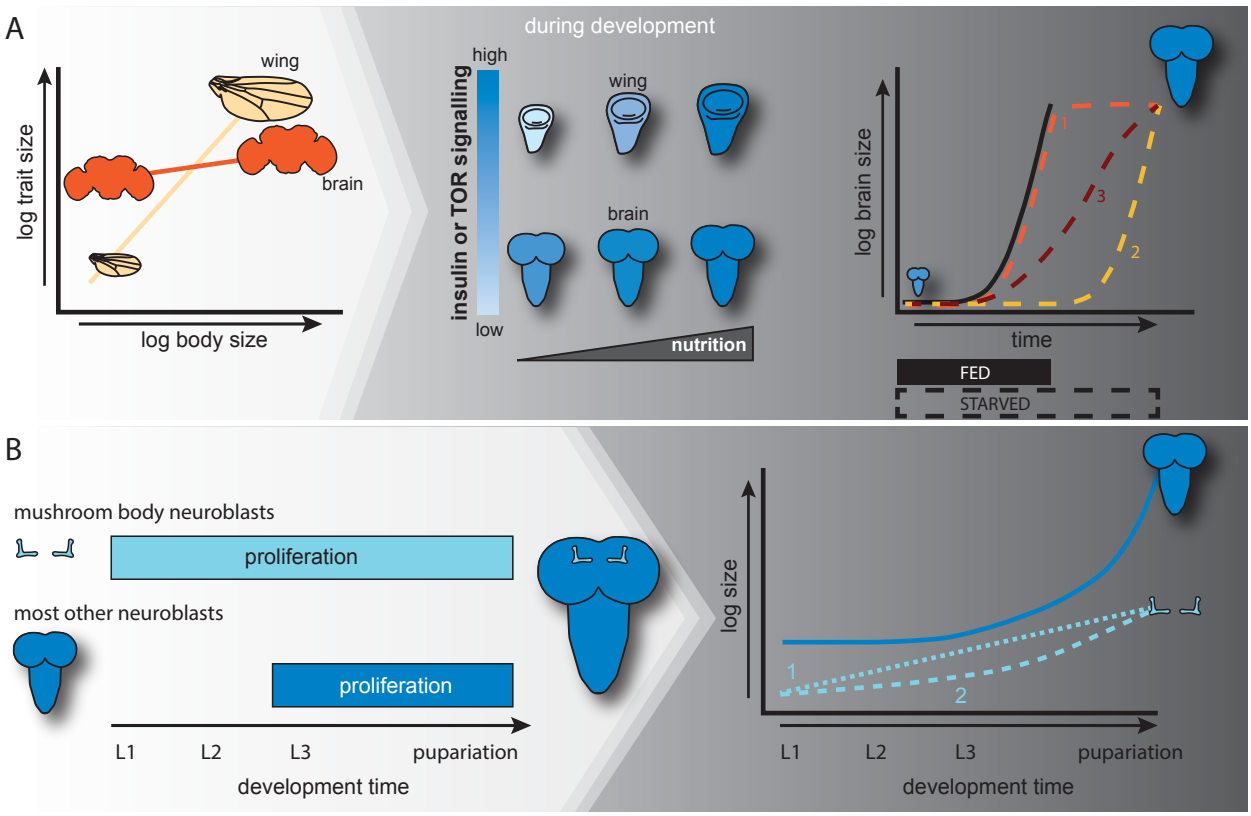

Figure 1: **How do the growth dynamics of the whole brain and the mushroom bodies vary.** (A) Hypothesis 1: Mushroom bodies proliferate throughout larval development, while most of the other neuroblasts in the brain remain quiescent and reinitiate proliferation in the late second instar (L2). These differences in proliferation could results in differences in the dynamics of mushroom body growth when compare to the whole brain. While we expect that the whole brain would show a lag period where it does not growth, followed by a period of exponential growth, the mushroom body might show constant (linear) increases in size across the larval stages of development (dashed line 1). Alternatively, the mushroom body might show similar growth dynamics, with shallower increase in growth rate in later development (dashed line 2). Differences in growth dynamics between the mushroom bodies and the whole brain would suggest that they are regulated in distinct manners under changing environmental conditions. (B) In comparison to other organs like the wing, adult brain size changes little with changes in body size. The reason that this is thought to occur is that insulin and target of rapamycin (TOR) signalling is kept high in the brain even under poor nutrient conditions via the action of Jeb/Alk. High levels of insulin or TOR signalling would suggest that brains would maintain constant growth rates even across environmental conditions – like starvation – that induce prolonged larval development. To maintain constant size, this would mean that the brain would either need to grow at constant rates until it reached its target size and then stop (orange dashed line 1), or else delay the onset of growth until later (yellow dashed line 2). Alternatively, Jeb/Alk could tune insulin or TOR signalling levels such that the rate of growth was reduced to compensate for the extended development time (red dashed line 3).

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This work aims to determine how brains regulate their growth to ensure robust size across different environmental conditions. To address this, we first compared the growth patterns of whole brains and mushroom bodies, relative to the larval body, under standard rearing conditions. We then used altered nutritional and thermal conditions to explore how the dynamics of brain growth respond to environmental change. Finally, we manipulated the activity of three signalling pathways known to regulate organ growth rates and the length of the larval growth period. These studies reveal differences in the way the mushroom body compartment regulates its growth when compared to the whole brain, and highlights how growth dynamics are tuned by nutrition and temperature within the mushroom body and across the whole brain. With these studies, we deepen our understanding of how different brain regions maintain robustness across environmental conditions.

RESULTS

Comparing the growth dynamics of the larval body, whole brain, and mushroom bodies across larval development

Given that the mushroom body neuroblasts show different patterns of growth to the majority of other neuroblasts in the brain, our first goal was to devise methods to compare mushroom body growth to whole brain and larval body growth across all three larval instars. To ensure that we compared the growth of the same structures across developmental time, we required a marker that would be expressed throughout all three instars. Using the expression data available from the Janelia FlyLight project (http://flweb.janelia.org/cgi-bin/flew.cgi), we selected lines with GAL4 expression in the mushroom bodies. We then crossed these GAL4 lines to UAS GFP lines, and selected animals immediately after hatch to the first instar (L1) larvae, or after the moult to the second instar (L2) and third instar (L3) larvae. We found that the GMR38E10 GAL4 line drove GFP expression in the vertical and medial lobes of the mushroom body neurons from hatch through to pupariation (Figure 2). In the late L3 stage, GFP expression was not apparent in the mushroom body calyx (Figure 2), which is the dendritic projections of Kenyon cell bodies (Supplementary Figure 1). Thus, to be able to compare measurements across all stages of development we excluded the calyx and peduncles from our analyses and measured only the ventral and medial lobes for mushroom body volume (Supplementary Figure 1).

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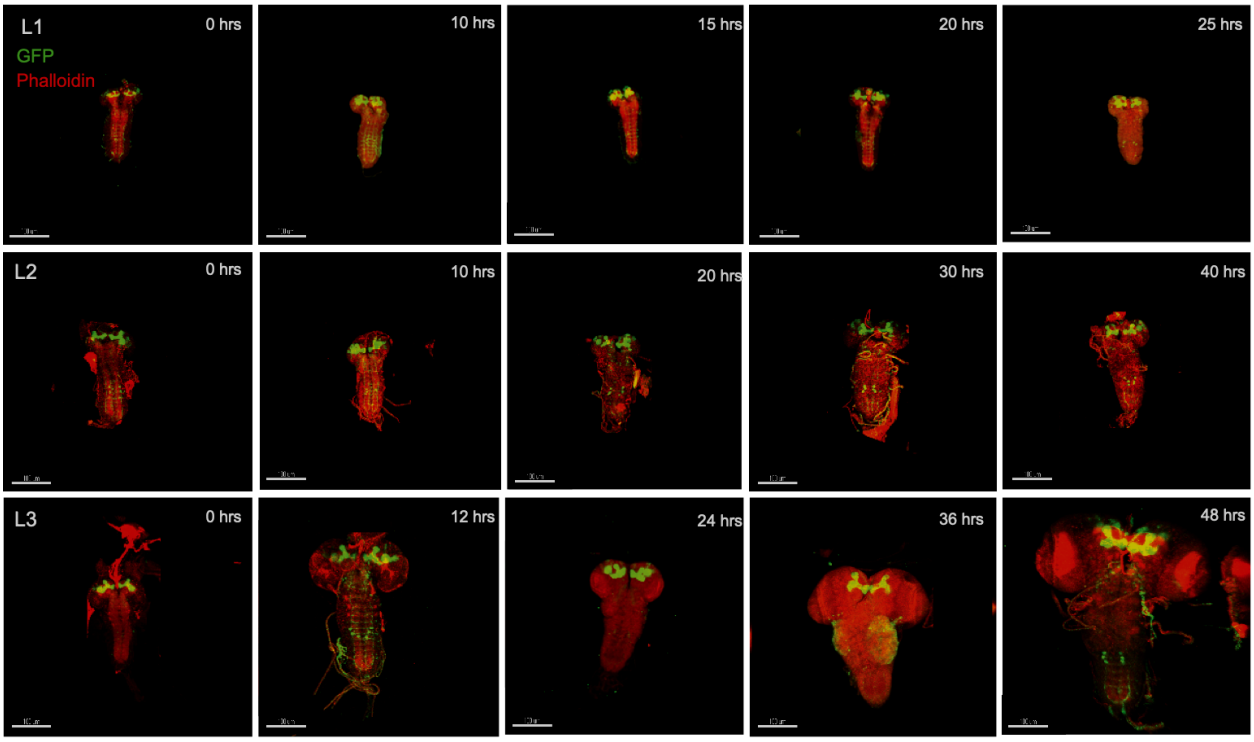

Figure 2: Changes in brain growth across larval stage of development. Larval brains expressing GFP in the neurons (green) of the mushroom body co-stained with phalloidin (red) across five developmental time points in the three larval stages. First instar (L1) A-E (0 hrs is relative to hatching), the second instar (L2) F-J (0 hours relative to the moult to L2) and the third instar (L3) K-P (0 hours relative to the moult to L3). At L3, the last two time points correspond to wandering and white prepupal stages. (Scale bar: 100µm)

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We next sought to compare the dynamics of larval, whole brain, and mushroom body growth. For each sample collected, we imaged the larvae before dissecting out their brains. By quantifying larval length and width, we could approximate larval volume using the formula length x width^2. Counter-staining the brains with phalloidin, which marks the actin cytoskeleton of all cells, allowed us to derive whole brain volume and mushroom body volume in the same sample.

Because growth is typically exponential, the standard method of looking at growth dynamics is to log-transform the data. In the simplest of cases, this linearizes the data allowing us to use linear regression analyses to explore growth dynamics. Non-linear growth dynamics in log-transformed data indicate that growth rates change with time.

Larval growth increased steadily throughout the first, second, and third instar stages (Figure 3A-C, Table 1). Linear models explain 68%, 55%, and 78% of the variation in larval volume over time for L1, L2, and L3 respectively (Table 1, adjusted R^2 values). Similarly, the mushroom body displayed steady linear growth throughout all three instars (Figure 3G-I, Table 1), with linear models explaining 43%, 55%, and 77% of the variance in mushroom body volume over time for the L1, L2, and L3 respectively (Table 1, adjusted R^2 values). In contrast, for whole brain volume we observed a slight, but significant, decrease in whole brain volume with time in the L1 (Figure 3D, Table 1). In this case, the linear model explained only 4% of the variance in whole brain volume in the L1 (Table 1, adjusted R^2 values). There was no significant change in brain volume with time across the L2 stage (Figure 3E, Table 1). In the L3, whole brain volume shows a non-linear relationship with time, curving upwards. This suggests that whole brain growth speeds up as the third instar progresses (Figure 3F). Curiously, at 0 hours after the moult to both L2 and L3, brain volume appears to increase despite no evidence of positive growth during the L2 instar. We cannot tell whether this is a random sampling effect or if this results from a burst of growth during the moult cycle itself, which we could not accurately sample.

Our results thus far suggest that whole brain growth is regulated differently to that of the larval body and mushroom bodies. To formally test this, we fit our growth data with both linear models and a range of non-linear models commonly used to describe growth dynamics, including second order polynomial, exponential, lagged exponential, and power models (Karkach, 2006). Each of these models infers something different about growth. The second order polynomial

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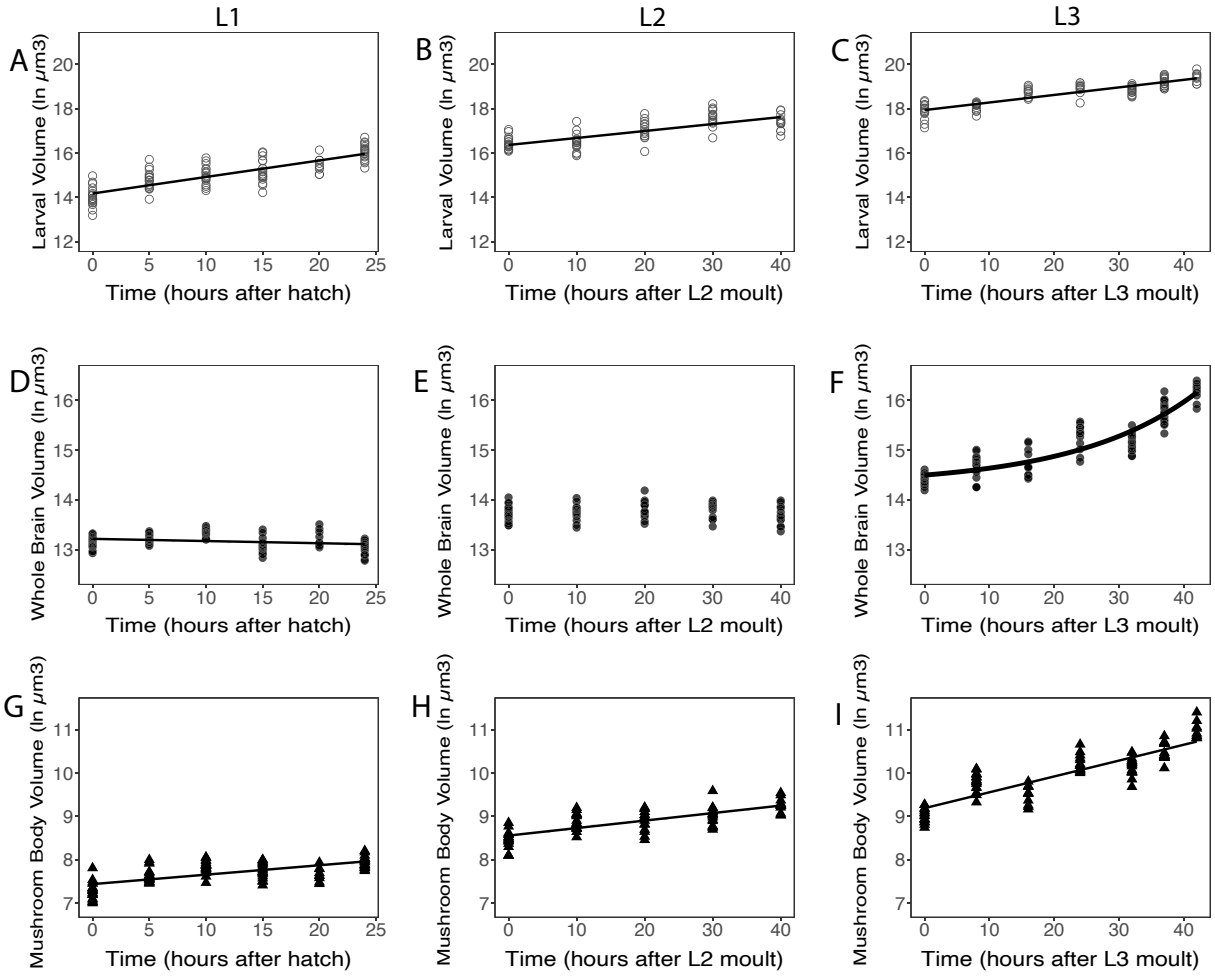

Figure 3: Growth patterns of larval body, brain and mushroom body. The volume of the larval body (A-C), whole brain (D-F), and mushroom body (G-I) at L1 stage (A, D, G), L2 stage (B, E, H) and at L3 stage (C, F, I) measured from 0 hrs after hatching/ larval moult to the end of the larval instar. At L3, the last two timepoints correspond to wandering and white prepupae larval stages. Each point shows individuals measured.

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Tables

Table 1: Linear regression models of larval volume, brain and mushroom body volume across the first, second and third instar stages of development.

Trait	Stage	F stat	p value	R^2 Adj
Larval Volume	L1	224.44	< 2.2e-16	0.6762
	L2	99.333	1.28E-15	0.5514
	L3	374.65	< 2.2e-16	0.7806
Brain Volume	L1	6.1501	0.01472	0.04592
	L2	0.0045	0.9468	-0.0126
	L3	372.81	< 2.2e-16	0.7798
MB Volume	L1	83.835	4.45E-15	0.4364
	L2	99.097	1.35E-15	0.5508
	L3	354.69	< 2.2e-16	0.7711

R^2 Adj: Adjusted R^2. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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model assumes that there are both linear and quadratic components to growth over time; exponential models describe growth that speeds up exponentially over time; lagged exponential models are similar to exponential models, but infer the presence of a slow period of growth followed by a switch to exponential growth; and the power model implies that growth increases according to a power function. We assessed which model best fit our growth data for each trait using two different model selection methods: Akaike's Information Criteria (AIC) and Bayesian Information Criteria (BIC), both of which estimate the quality of each model relative to the others, penalizing models with a higher number of parameters to avoid overfitting the data. The model with the lowest AIC and BIC values provides the best fit for the data. Where these values were close between models, we selected the simplest model (i.e. the model with the fewest parameters). We restricted these comparisons to L3 growth, since the whole brains did not show significant positive growth in the L1 and L2 stages.

For growth in the larval body and mushroom body, we found that linear models provided the best fit to our data (Table 2). This means that the growth rates in the larval body and mushroom body do not change over time in the third instar. Whole brain growth, on the other hand, was best fit with a lagged exponential model. This indicates that in the early stages of the third instar the whole brain grew slowly. After this lag phase, the rate of whole brain growth increased exponentially. Taken together, these data suggest that while the larval body and mushroom body growth rates do not change with time over the third instar, the whole brain undergoes a period of little growth, followed by a second phase of rapidly increased growth in the L3.

Developmental time and growth dynamics are modulated by changes in nutrition and temperature

We next sought to determine how brain size remains robust when developmental time was extended as a result of altered environmental conditions. To do so, we reared larvae on one of three food dilutions, 10%, 25%, or 100%, and at one of two temperatures, 25ºC or 29ºC. We compared growth rates in the L3 across these six environmental conditions. Changing the diet and/or rearing temperature altered the time it took for animals to initiate metamorphosis at pupariation (white pre-pupae). Compared to animals grown under standard conditions (25ºC and

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253 Table 2: Akaike's Information Criterion (AIC) and Bayesian Information Criteria (BIC) for
254 modelling larval volume, brain and mushroom body volume in the third instar (L3) stage of
255 development. Values for best fit are in blue.

Trait	Fit	AIC	BIC
Body	Volume.lmL3	20.35881	28.34913
	Volume.lmL3poly	19.63951	30.29327
	Volume.expL3	30.84764	
	Volume.explagL3_100		38.83796
	Volume.powerL3	118.6141	126.6044
Brain	Brain.lmL3	36.96086	44.95118
	Brain.lmL3poly	18.70613	29.35988
	Brain.expL3	19.51189	27.5022
	Brain.explagL3_100	12.13046	22.78422
	Brain.powerL3	156.5104	164.5007
Mushroom Body	MB.lmL3	40.93267	48.92299
	MB.lmL3poly	42.85683	53.51059
	MB.expL3	42.70775	50.69807
	MB.explagL3_100	42.80673	53.46049

256 lm: linear model, poly: polynomial, exp: exponential model, explag: lagged exponential model.

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100% food), animals reared on food with only 10% of the normal caloric content took the longest to pupariate (90 and 80 hours after the moult at 25ºC and 29ºC respectively, compared to 42 hours at 25ºC on 100% food). At 25ºC, pupariation was delayed to 50 hours after the moult when larvae were reared on 25% food. Development time was similar between the 25% and 100% food conditions at 29ºC (42 hours from moult to white pre-pupae).

Given these differences in development time across nutritional and thermal conditions, we next defined how this changed growth dynamics of the mushroom body, whole brain, and larval body. For each condition, we sampled 5-7 time points across the L3 stage, with the last 2 time points corresponding to the wandering and white prepupal stages, respectively. Diluting the food reduced growth rates of the larval body at both temperatures (Figure 4A, B, Table 3). Overall, the larval body grew more slowly when larvae were reared at 29ºC compared to 25ºC (Figure 4A, B, Table 3). Larvae grew slowest on 10% food at 29ºC and fastest on 100% food at 25ºC (Figure 4A, B, Table 3), resulting in a significant interaction between time, food, and temperature. These data provide a convenient proof-of-principle that we can alter growth dynamics by manipulating food and temperature.

Changing developmental time allowed us to directly test our different models. We predicted that brain structures would remain robust to changes in developmental time in one of three ways (Figure 1A). Our first model predicted that when developmental time was extended, brain structures would maintain their growth rates, grow to their final size, and then stop growing and remain the same size until pupariation. This would be modelled best using an asymptotic regression, but could also be approximated by a negative quadratic term from a second order polynomial regression – indicating growth is flattening out. In our second model, we predicted that brain structures would remain robust against changes in developmental time by altering the time at which growth initiated, but maintaining constant growth rates. This hypothesis would be best supported by a change in the lag constant of a lagged exponential regression. Our final hypothesis proposed that brain structures would carefully tune both their rates of growth and the time they initiated exponential growth, supported by a change in both the scaling and lag constants of a lagged exponential regression or by a change in slope in a linear regression in the case of the mushroom bodies.

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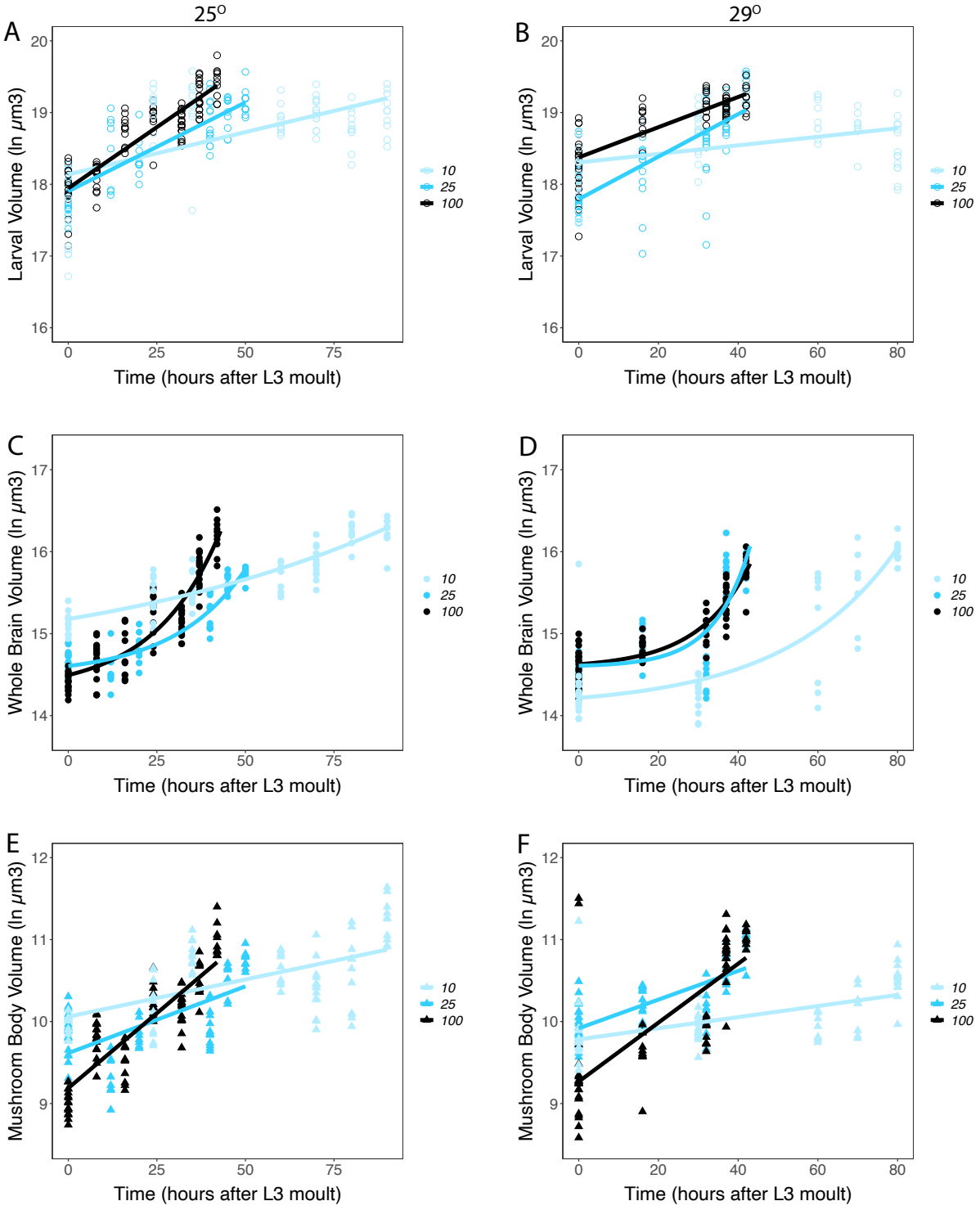

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Figure 4: Growth rates of the larval body (A, B), whole brain (C, D), and mushroom bodies (E, F) over time from the moult prior to third instar through pupariation under different dietary and thermal conditions. Panels (A), (C) and (E) show three dietary conditions (10%, 25%, and 100% food) at 25°C. Panels (B), (D) and (F) show the three dietary conditions (10%, 25%, and 100% food) at 29°C.

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Table 3: Growth rates of the larval body, brain and mushroom bodies depend on nutritional and thermal conditions. Larval body and mushroom body volumes were fit with linear models (lm). Brain volumes were fit with second order polynomial models with Time as (Time, 2, raw = TRUE).

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
Time	58.358	1	402.9612	<2.2e-16 ***
Food	19.295	2	66.6153	< 2.2e-16 ***
Temp	0.051	1	0.3500	0.554400
Time x Food	16.805	2	58.0189	< 2.2e-16 ***
Time x Temp	1.358	1	9.3796	0.002328 **
Food x Temp	0.429	2	1.4828	0.228125
Time x Food x Temp	1.755	2	6.0605	0.002532 **
Brain Volume				
(Time, 2, raw = TRUE)	89.118	2	601.0508	< 2.2e-16 ***
Food	2.620	2	17.6721	4.178e-08 ***
Temp	5.342	1	72.0532	3.331e-16 ***
(Time, 2, raw = TRUE) x Food	13.364	4	45.0669	< 2.2e-16 ***
(Time, 2, raw = TRUE) x Temp	6.363	2	42.9132	< 2.2e-16 ***
Food x Temp	14.659	2	98.8697	< 2.2e-16 ***
(Time, 2, raw = TRUE) x Food x Temp	2.207	4	7.4441	8.318e-06***
Mushroom Body Volume				
Time	51.040	1	371.8073	< 2.2e-16 ***
Food	2.806	2	10.2220	4.576e-05 ***
Temp	0.012	1	0.0880	0.7669
Time x Food	25.315	2	92.2044	< 2.2e-16 ***
Time x Temp	0.081	1	0.5931	0.4416
Food x Temp	6.580 2	2	23.9649	1.318e-10 ***
Time x Food x Temp	0.104 2	2	0.3788	0.6849

D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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In the mushroom body, we found that diluting the food reduced growth rates (Figure 4E, F, Table 3), but that rearing temperature did not affect the rate of growth in this structure. This resulted in a significant decrease in growth rates for larvae grown on 10% food when compared to 25% food, as well as reduced growth rates on 25% food when compared to 100% food at both temperatures. Under all conditions, the mushroom bodies maintained linear growth trajectories. This best supports our model that at least the mushroom body compartment of the brain achieves robustness of size by carefully tuning its growth rates to adjust for changes in developmental time.

Because the whole brain showed non-linear growth patterns, we initially modelled whole brain growth using second order polynomials (Figure 4C, D, Table 2). Similar to the larval body and mushroom bodies, diluting the food reduced the growth rates of the whole brain with the slowest growth on 10% food for both temperatures. Rearing temperature also reduced growth rates in the whole brain (Figure 4C, D, Table 3). In addition, the way that food affects growth rates depends on the rearing temperature. For larvae reared at 25ºC, growth rates differed depending on whether they were given 25% or 100% food. At 29ºC, there was no difference in growth rate between the 25% and 100% food. Thus, the whole brain shows complex responses to the combined effects of temperature and diet.

These models allowed us to further distinguish between our hypotheses. If whole brains grew to a target size and then stopped, we would expect the quadratic terms from our polynomial regressions to be negative as growth flattens out. In all cases where the quadratic term was significant in our models, we found that the value was positive (Table 4). This suggests that our first model – that brains should grow to a target size then stop – is not supported by our data.

We can distinguish between our second and third models using the lagged exponential growth models using the formula $\ln(whole\ brain) = a + e^{(\frac{Time-b}{c})}$, where a is the intercept, b is the lag constant, and c is the scaling constant. If brains remain robust to changes in developmental time by altering the time at which they turn on growth (hypothesis 2), we would expect the lag constant (b) to change, but not the scaling constant (c). Hypothesis 3 would be supported if both the lag constant (b) and scaling constant (c) changed with altered developmental time. We fit our whole brain growth data with lagged exponential curves and

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323 Table 4: Model to test hypothesis 1 that brains maintain growth rate to target size when
324 developmental time is extended

Brain growth rate	df	R^2 value	T value	P value
Food = "25", Temp = "25"				
Model 1	65	0.8754	-1.325	0.19
Model 2	65	0.8754	6.288	3.07e-08 ***
Food = "10" & Temp ="25"				
Model 1	74	0.7718	2.425	0.0178 *
Model 2	74	0.7718	1.858	0.0671 .
Food == "25" & Temp == "29"				
Model 1	67	0.5775	-1.312	0.194096
Model 2	67	0.5775	3.700	0.000437 ***
Food == "10" & Temp == "29")				
Model 1	57	0.7683	-1.939	0.0575 .
Model 2	57	0.7683	5.447	1.13e-06 ***

325 D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001. To support this
326 hypothesis, model 1 should fit the brain/mushroom body data better than model 2 in poorer food conditions
327

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328 explored whether the lag and scaling constants differed across our six environmental conditions
329 (Table 5). We then conducted pairwise comparisons between whole brain growth curves either
330 at the same temperature but across different diets, or on the same diet but across the two
331 temperatures. We asked whether fitting specific lag and scaling constants for the curves for each
332 condition improved the fit to the data. For the comparisons between the 10% food and either
333 the 25% or the 100% food, the lag constants were too dissimilar to find a common coefficient,
334 resulting in a failure to resolve a null model. While this suggests that the lag constants differ in
335 these comparisons, we cannot formally test for this. However, we find definitive evidence that
336 both the lag constants (1 instance) and the scaling constants (5 instances) differ significantly
337 between conditions for whole brain growth (Table 5). Taken together, our data best supports a
338 model where both the timing at which exponential growth begins and the growth rate are
339 carefully tuned to adjust for differences in developmental time.

340 **Changing environmental conditions affects size traits in the prepupae**

341 We have shown that the growth dynamics of the larval body, mushroom body, and whole
342 brain are all sensitive to environmental perturbation, but that they respond in different ways to
343 changes in diet and temperature. We next extended these findings by examining the effects of
344 changed environmental conditions on their final size at pupariation.

345 Pupal body volume decreased as the food was diluted and also decreased at the higher
346 temperature (Figure 5A, Table 6). This is what we would have expected given previously
347 published data on the effects of diet and temperature on pupal body size (Loeb & Northrop,
348 1917; Davidowitz et al., 2003; Couret et al., 2014). At pupariation, we did not observe a significant
349 effect of diet on its own for whole brain volume (Figure 5B, Table 6). However, whole brains were
350 smaller at 29ºC than at 25ºC, and there was a significant temperature by diet interaction (Figure
351 5B, Table 6). This is due to the fact that at 25ºC larval diet had no effect on brain volume while at
352 29ºC, brain volume decreased with diet concentration. Mushroom body volumes at pupariation
353 varied with diet and temperature, with increasing food concentrations and increasing
354 temperatures negatively impacting mushroom body volume (Figure 5C, Table 6). The significant
355 interaction between diet and temperature results from the fact that while food concentration

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Table 5: Model to test that brains remain robust to changes in developmental time by changing the time at which they turn on growth (hypothesis 2) or by changing both growth rates and the time at which they turned on growth changed (hypothesis 3).

Comparison		**Any constant differs**	**Lag or Scaling Constant Differ**	**Lag Constant Differs**	**Scaling Constant differs**	**Intercept Differs**
Temperature (ºC)	Food (%)	F value (all constants the same)	(a varies)	(a and c varies)	(a and b varies)	(b & c varies)
25	25 & 100	43.315***	48.136***	4.6947	0.0854	-
25	10 & 25	does not resolve	does not resolve	does not resolve	2.772***	3.4892***
25	10 & 100	does not resolve	does not resolve	does not resolve	3.673***	2.1648*
29	25 & 100	0.8435	-	-	-	-
29	10 & 25	33.158***	16.125***	does not resolve	8.7448**	4.9836
29	10 & 100	45.503***	14.132***	does not resolve	3.0147	2.8911
25 & 29	10	64.844***	29.453***	0.1876	5.2161*	-
25 & 29	25	10.522***	13.738***	1.2351	7.3676**	-
25 & 29	100	15.285***	15.828***	6.8819***	1.4653	-
25 & 29	10	64.844***	29.453***	0.1876	5.2161*	-
25 & 29	25	10.522***	13.738***	1.2351	7.3676**	-
25 & 29	100	15.285***	15.828***	6.8819***	1.4653	-
25 & 29	10	64.844***	29.453***	0.1876	5.2161*	-

D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1. To support hypothesis 2, the lag constant (b) should change, but not the scaling constant (c) and hypothesis 3, if both (b) and (c) changes with altered developmental time. We applied Holm's adjustment to the p-values to account for multiple tests.

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correlates negatively with mushroom body volume at 25ºC, it correlates positively with mushroom body volume at 29ºC.

These differences in the way the whole brain and mushroom body volumes respond to diet and temperature has interesting implications for brain shape. While mushroom body volumes are remarkably robust in size on 25% and 100% foods, on 10% food they are larger for their brain size at 25ºC and smaller for their whole brain size at 29ºC (Figure 5D, Table 6). This highlights that brain shape changes across environmental conditions, as compartments of the brain differ in how they grow in response to these conditions. This leads us to ask how these differences in response across brain compartments are achieved.

The role of nutrition-sensitive signalling pathways in regulating whole brain and mushroom body growth

Developmental timing is ultimately regulated by ecdysone (Yamanaka et al., 2013), and the timing of ecdysone production is modified by the activity of nutrition-sensitive pathways including insulin and TOR signalling (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Koyama et al., 2014). Ecdysone, insulin, and TOR pathways are also known to regulate the growth of the larval body and of the brain (Ikeya et al., 2002; Chell & Brand, 2010; Sousa-Nunes et al., 2011; Lanet & Maurange, 2014; Rossi et al., 2017; Syed et al., 2017). We sought to understand which of these pathways changes growth dynamics in the structures of the brain, thus potentially uncovering the signalling mechanisms through which the whole brain and mushroom bodies adjust their growth with dietary and thermal conditions.

<u>Ecdysone affects developmental timing but not growth rates of the brain structures</u>

We first explored whether ecdysone signalling changed growth dynamics in the larval body, whole brain, and mushroom body. To understand the role of ecdysone in regulating growth acros nutritional conditions, we supplemented either 100% or 10% food with 50 ng/mg of 20-Hydroxyecdysone (20E). As a control, we added the same volume of the vehicle (ethanol). Animals were staged at 0 h L3 and transferred from the standard fly food (100% food) to the treatment diets. L3 staged animals were left in each diet until they reached the white prepupal stage. As in previous experiments, we analysed five time points from the L3 moult through to

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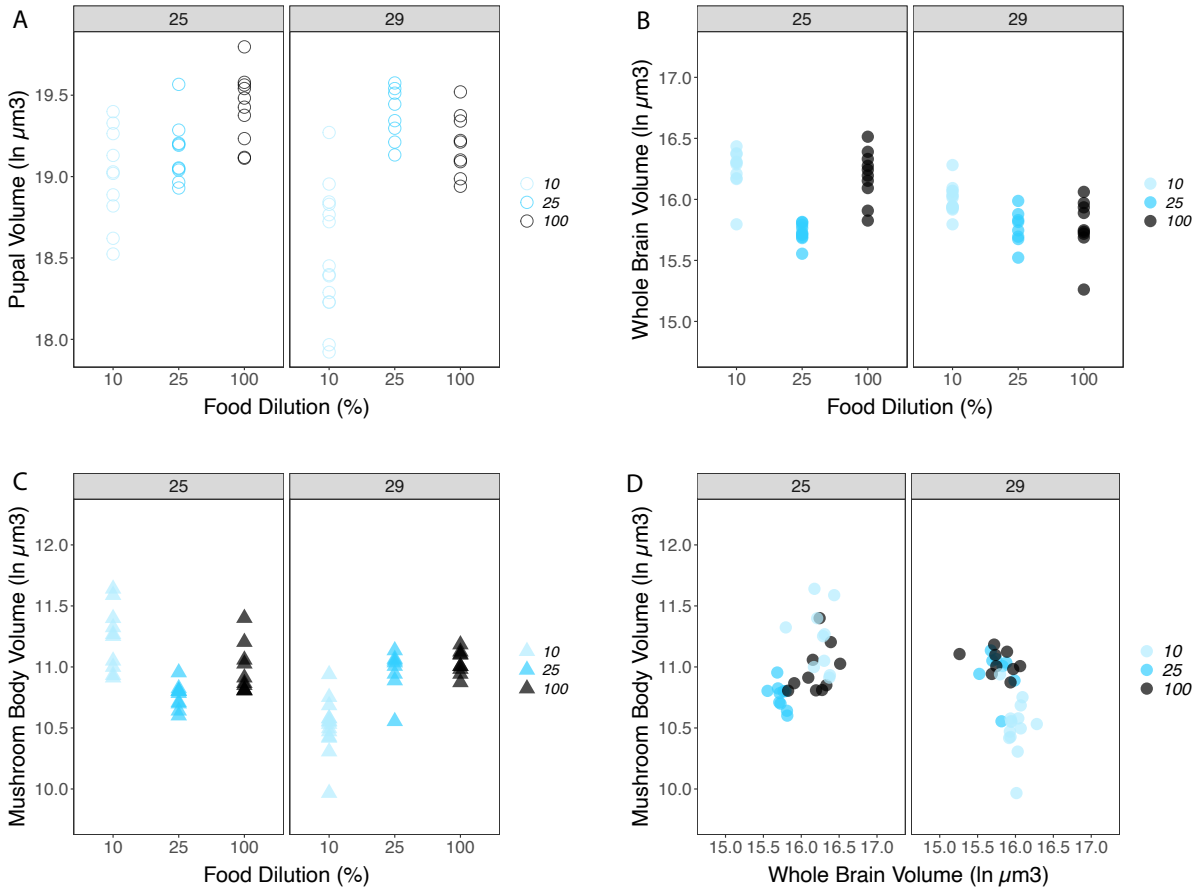

Figure 5: The prepupal volume of (A), whole brain volume (B) and the mushroom body volume (C) across nutritional (10%, 25% and 100%) and thermal conditions (25°C and 29°C). The relationship between whole brain and mushroom body volume is shown in (D).

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Table 6: The final relationship between whole brain and body size depends only on temperature whereas the mushroom body/body size relationship depends on both diet and temperature, with a significant two – way interaction.

	Sum Sq	Df	F value	P value
Whole Brain Volume				
Prepupal Volume	0.04509	1	0.8123	0.371511
Food	0.00164	1	0.0295	0.864238
Temp	0.43744	1	7.8803	0.006978 **
Prepupal Volume x Food	0.10216	1	1.8404	0.180657
Prepupal Volume x Temp	0.02148	1	0.3870	0.536537
Food x Temp	0.03836	1	0.6911	0.409514
Prepupal Volume x Food x Temp	0.04492	1	0.8092	0.372417
Mushroom Body Volume				
Prepupal Volume	0.4282	1	7.1508	0.009941 **
Food	0.1020	1	1.7032	0.197510
Temp	0.3106	1	5.1860	0.026831 *
Prepupal Volume x Food	0.0162	1	0.2713	0.604640
Prepupal Volume x Temp	0.1316	1	2.1969	0.144211
Food x Temp	0.2992	1	4.9964	0.029637 *
Prepupal Volume x Food x Temp	0.0614	1	1.0247	0.316011
Mushroom Body Volume by Whole Brain Volume				
Brain	0.17491	1	3.6288	0.0622218.
Food	0.03948	1	0.8191	0.3695460
Temp	0.60644	1	12.5816	0.0008246 ***
Brain x Food	0.04823	1	1.0006	0.3217034
Brain x Temp	0.95303	1	19.7724	4.495e-05 **
Food x Temp	0.62825	1	13.0341	0.0006789 ***
Brain x Food x Temp	0.11305	1	2.3453	0.1316064

D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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pupariation. We did not have sufficient data to fit lagged exponential models for the brain volume growth curves. In addition, we found that for these experiments linear models fit the data better than second order polynomial models, and we therefore opted for the simpler models (Supplementary table 1).

Supplementing 20E in the diet resulted in significant reductions in the time to pupariation when compared to animals fed with the vehicle control. Animals placed on control 100% food reached pupariation at 52 h after L3 moult while those on 100% food supplemented with 20E reached pupation at 40 h after L3 moult. This 12-hour increase in the control treatment appearred to be due to ethanol addition. As expected, in the 10% diet condition, the addition of 20E significantly reduced the time to pupariation. Animals that were reared on the 10% food supplemented with 20E reached pupariation at 60 h after L3 moult, but pupariated at 80 h when reared on the 10% food with ethanol alone. (Figure 6).

As in our previous experiment, the larval body, the whole brain, and the mushroom bodies grew at slower rates on the 10% food supplemented with ethanol. Adding 20E to the food significantly reduced larval volume on both 10% and 100% diets (Figure 6A, Table 7), corroborating previous findings that ecdysone suppresses the growth of larval tissues (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). In contrast, 20E had no significant effects on the growth rates of either the whole brain or the mushroom body volumes on either diet (Figure 6B, C, Table 7).

Diet however, did have a significant effect on whole brain volume at pupariation (Figure 6B, Table 7). Further, because it reduced total development time adding 20E to the diets reduced pupal volume, and the whole brain and mushroom body volumes in the white prepupae (Supplementary Figure 3, Table S3). Taken together, while 20E can control the size of brain structures by regulating total developmental time, ecdysone signalling is unlikely to control changes in growth rates in response to diet and temperature in the brain.

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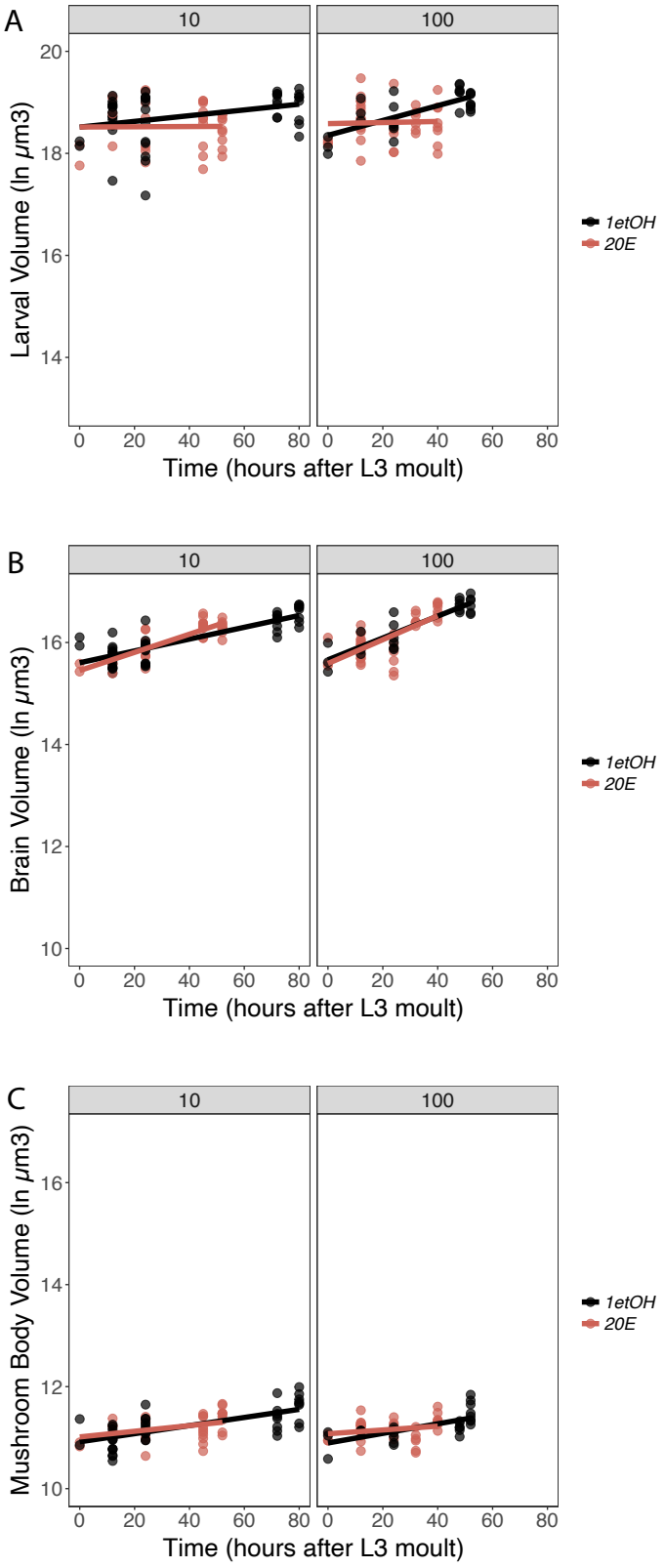

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Figure 6: Growth rate of larva, brain and mushroom body following ecdysone administration. Ecdysone affects the growth rate larval growth (A) but not that of the whole brain and mushroom bodies (B and C) under two diet conditions (10% and 100%).

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428 Table 7: The effect of ecdysone on whole brain and body size depends on diet whereas the
429 relationship between mushroom body and body size depends only on temperature.

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
Time	2.0610	1	12.3093	0.000614 ***
Treatment	0.8889	1	5.3087	0.0227 *
Food	0.4510	1	2.6934	0.103
Time x Treatment	0.7230	1	4.3182	0.0396 *
Time x Food	0.4386	1	2.6196	0.108
Treatment x Food	0.0016	1	0.0098	0.921
Time x Treatment x Food	0.1617	1	0.9660	0.327
Whole Brain Volume				
Time	14.6501	1	264.32	< 2.2e-16 ***
Treatment	0.0218	1	0.394	0.53146
Food	3.9987	1	72.15	3.358e-14 ***
Time x Treatment	0.2150	1	3.879	0.05095 .
Time x Food	0.9310	1	16.79	7.168e-05 ***
Treatment x Food	0.0312	1	0.5621	0.45471
Time x Treatment x Food	0.0708	1	1.2776	0.26036
Mushroom Body Volume				
Time	3.4587	1	66.3139	2.359e-13 ***
Treatment	0.0093	1	0.1782	0.6736
Food	0.0120	1	0.2297	0.6325
Time x Treatment	0.1357	1	2.6021	0.1091
Time x Food	0.0015	1	0.0282	0.8669
Treatment x Food	0.0012	1	0.0236	0.8782
Time x Treatment x Food	0.0262	1	0.5080	0.4772

430 D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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Insulin affects the growth rates of the larval body and mushroom body, while TOR signalling regulates the growth rates of the whole brain

We next tested whether inhibiting either the insulin or TOR signalling pathways could change whole brain and mushroom body growth rates. We first performed dose response curves for both the inhibitors of insulin (wortmannin) and the TOR (rapamycin) to determine the minimum dose that would affect body size and developmental timing (Supplementary Figure 4). From these results, we selected doses of 100µM of wortmannin/ml of food and 30µM of rapamycin/ml of food, which were dissolved in ethanol and supplemented into the standard 100% food. We did not have sufficient data to accurately fit lagged exponential models for the brain volume growth curves. Thus, for each measure of size, we tested whether linear or second order polynomials best fit the data, and selected the simpler model in cases where the fit was similar.

Adding Wortmannin to the food extended larval development time from 52 (control) to 72 hours. Wortmannin decreased the growth rate of the larval body, as indicated by a significant treatment by time interaction (Figure 7A, Table 8), resulting in decreased prepupal volumes (Supplementary Figure 5, Supplementary Table 3). When we observed the whole brain, the prepupal volume was also significantly decreased in animals treated with wortmannin (Supplementary Figure 5, Supplementary Table 3). However, we could not detect significant decreases in growth rate of the whole brains from wortmannin-treated larvae (Figure 7B, Table 8). In contrast, the growth rate of the mushroom bodies was significantly affected the by the addition of wortmannin, resulting in smaller prepupal mushroom bodies than controls. Indeed, the mushroom body volumes appeared to shrink after the larvae were transferred to wortmannin-supplemented food. This suggests that the growth of the larval body and mushroom bodies is more sensitive to insulin signalling than that of the whole brain.

Rapamycin treatment did not affect larval body growth rates (Figure 8A, Table 9). Because the addition of rapamycin to the food extended larval development time to 86 hours, these larvae pupariated with larger larval body volumes (Supplementary Figure 6, Supplementary Table 4). Similarly, mushroom body growth rates were not significantly affected by rapamycin treatment, although the treated larvae had lower mean mushroom body volumes across larval stages (Figure 8C, Table 9), the mushroom body volumes in the prepupal stage was not affected with rapamycin

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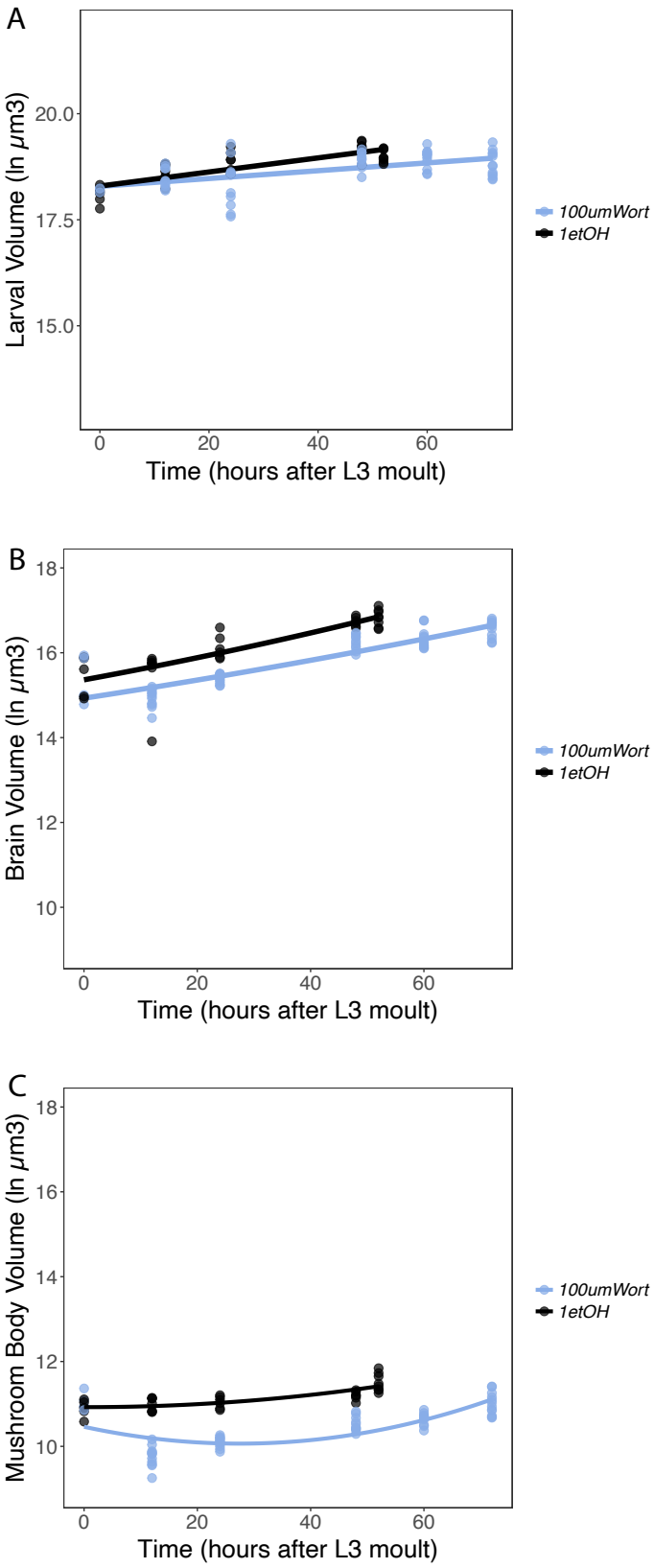

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Figure 7: Differences in growth rate following supplementation of 100μM/ml of Wortmannin in food. Growth rates of (A) larval body (P<0.1), and (C) mushroom body (p<0.05) is affected by Wortmannin treatment. but not that of the whole brain (B)

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462 Table 8: Wortmannin affects the rate of growth of the whole brain and mushroom body. Brain
463 and larval volumes were fit with second order polynomial models.

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
(Time, 2, raw = TRUE)	7.0727	2	40.5955	3.848e-13 ***
Treatment	0.7562	1	8.6806	0.004138 **
(Time, 2, raw = TRUE) x Treatment	0.4567	2	2.6216	0.078493 .
Whole Brain Volume				
(Time, 2, raw = TRUE)	31.764	1	275.3451	< 2.2e-16 ***
Treatment	7.102	1	61.5592	9.551e-12 ***
(Time, 2, raw = TRUE) x Treatment	0.223	1	1.9358	0.1676
Mushroom Body Volume				
(Time, 2, raw = TRUE)	8.7666	2	49.398	5.197e-15 ***
Treatment	14.6143	1	164.696	< 2.2e-16 ***
(Time, 2, raw = TRUE) x Treatment	0.7276	2	4.100	0.01992 *

464 D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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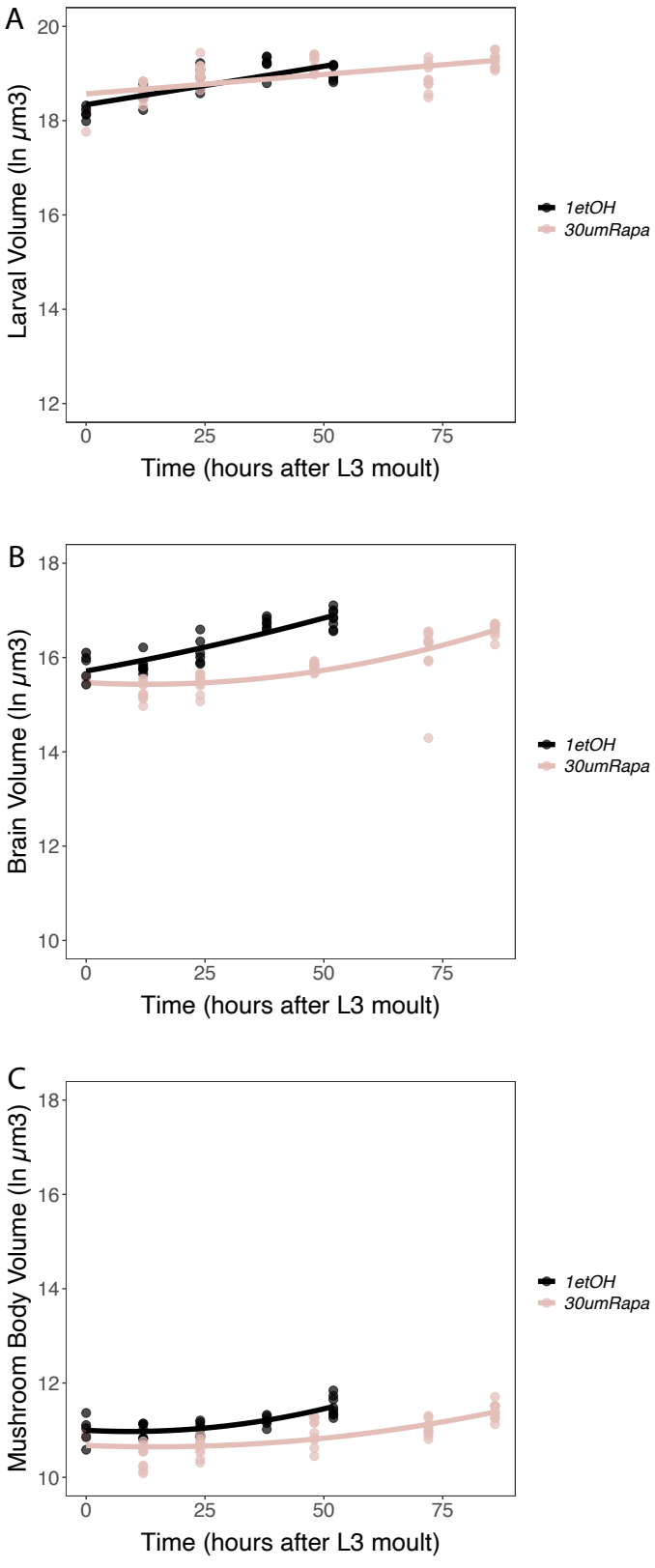

Figure 8: Growth rates following administration of 30μM/ml of Rapamycin in food. Treatment (does not affect the growth rates of (A) larval volume and mushroom body volume (C) but significantly alters (B) the volume of the whole brain ($p<0.001$).

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467 Table 9: Effects of rapamycin on the rate of growth of the whole brain and mushroom body.

468 Brain and mushroom body volumes were fit with second order polynomial models.

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
(Time, 2, raw = TRUE)	1.888	1	0.5985	0.4413
Treatment	3.895	1	1.2348	0.2697
(Time, 2, raw = TRUE) x Treatment	1.939	1	0.6147	0.4352
Whole Brain Volume				
(Time, 2, raw = TRUE)	14.7557	2	78.6562	< 2.2e-16 ***
Treatment	9.7800	1	104.2657	3.275e-16 ***
(Time, 2, raw = TRUE) x Treatment	1.6593	2	8.8448	0.0003355 ***
Mushroom Body Volume				
(Time, 2, raw = TRUE)	6.0522	2	46.9374	2.905e-14 ***
Treatment	3.9633	1	61.4741	1.547e-11 ***
(Time, 2, raw = TRUE) x Treatment	0.2965	2	2.2998	0.1068

469 D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.' p<0.1

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treatment (Supplementary Figure 6, Supplementary Table 4). Again, this is in contrast to what we expect as increasing developmental time and keeping the growth rate constant should result in an increase in the final size, as organs continue to grow at the same rate for longer durations than the control group. For the whole brain, rapamycin significantly reduced growth rates when compared to controls (Figure 8B, Table 9) resulting in smaller prepupal brain volumes (Supplementary Figure 6, Supplementary Table 4). These results indicate that TOR signalling plays a more important role in regulating whole brain growth rates than in regulating the growth rates of the larval body and mushroom body.

Discussion

Individual organs vary in their response to the environmental conditions that affect adult body size (Shingleton et al., 2009). Organs like the brain and genital discs are known to be nutritionally insensitive when compared to organs like the wing (Chell & Brand, 2010; Shingleton, 2010; Cheng et al., 2011; Tang et al., 2011; Shingleton & Frankino, 2018). While we have some understanding of the genetic mechanisms underpinning robustness in size in these organs, our understanding of how these mechanisms affect the dynamics of growth was poorly understood. Further, the brain is a complex organ whose compartments do not all behave in the same manner. Functional compartments like the *Drosophila* mushroom body differ in their growth patterns as well as their nutritional plasticity from the rest of the brain. In this study, we aimed to test our predictions that the differences in proliferation between neurons of the whole brain and mushroom bodies would confer distinct growth dynamics, which could impart differences in their sensitivity to environmental conditions.

Previous studies had suggested that brain sparing occurs under stressful conditions because Jeb/Alk maintain high levels of insulin and TOR signalling in the neuroblasts (Cheng et al., 2011). These same conditions also affect development time of the larva (Beadle et al., 1938; Robertson & Reeve, 1952; Robertson, 1962, 1966; Partridge et al., 1994; Nunney & Cheung, 1997). If insulin and TOR levels are at the same level in the brains of starved and fed larvae, then the brain must have additional mechanisms to prevent overgrowth when development time is extended. In this study, we altered development time by changing both nutrition and temperature. We proposed

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three hypotheses that would allow brain size to remain robust against environmental conditions. These posited that in response to changes in total development time the brain would either 1) grow to a target size and stop growing for the remainder of the growth period, 2) delay the onset of its growth, but maintain constant growth rates even under stressful conditions, or 3) regulate both its growth rate and the time at which it switches growth on to adjust for changes in developmental time. Our data supports our third hypothesis, that robustness of brain size is possible because both the time at which exponential growth is initiated and the rates of growth of the brain have been altered.

Our results imply that Jeb/Alk signalling, which is responsible for brain sparing in *Drosophila*, plays a more nuanced role than previously described. Rather than simply maintaining high levels of insulin and/or TOR signalling, signalling from Alk could be acting to adjust growth rates of the brain to match changes in developmental time. Precisely how this occurs is unknown, however given that both insulin and ecdysone signalling are key regulators of the length of the growth period (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Shingleton et al., 2005; Koyama et al., 2014), these systemic cues could be regulating the concentration of Jeb secreted by the glial cells in accordance with the degree to which development is delayed. Other organs that show robustness in final size could be responding to environmental conditions in a similar fashion. For example, we would predict the genital discs maintain robust final size by tuning their growth rates to account for extended growth periods under poor nutrition or thermal stress.

While the size of the pupal brain is robust against environmental conditions, this does not mean that brain growth is insensitive to environmental stress. Nutritional signals are important for neuroblasts to exit quiescence and re-initiate proliferation in the larval stages (Britton & Edgar, 1998; Chell & Brand, 2010; Yuan et al., 2020). Cues from the fat body, the insect equivalent of the adipose tissue and liver, signal to glial cells in the brain, which in turn produce insulin-like peptides that induce the neuroblasts to recommence cell divisions (Britton & Edgar, 1998; Chell & Brand, 2010; Yuan et al., 2020). Starving larvae in early instars causes most neuroblasts and glia, with the exception of the mushroom body neuroblasts, to remain quiescent (Britton & Edgar, 1998; Chell & Brand, 2010; Yuan et al., 2020). This is owing to the cell-autonomous and non-autonomous growth coordination activity of PI3Kinase in the early larval stages of development

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(Yuan et al., 2020). After they exit quiescence, neuroblasts no longer depend on nutritional cues to maintain proliferation (Cheng et al., 2011; Lanet & Maurange, 2014). It is unclear how this change in the impact of nutrition relates to the growth dynamics measured throughout larval development in the current study, however it is likely to be responsible for reduced rates of exponential growth in the third instar on poor diets.

While the growth dynamics of the whole brain change to accommodate additional developmental time, our findings also demonstrate that not all compartments of the brain should be expected to respond in the same way. Our comparisons between whole brain and the mushroom bodies highlight how the growth dynamics of specific brain compartments can differ from the patterns observed across the brain as a whole. Some of these differences arise simply due to differences in the timing of neuroblast reactivation. While the neurons of the mushroom body continue to proliferate throughout larval development, most other neuroblasts reinitiate proliferation after the late second instar. This alone should be sufficient to generate differences in growth dynamics between the mushroom bodies and the rest of the brain.

However, differences in growth patterns are not unique to the mushroom body. Unlike most of the other regions of the brain, the optic lobe shows extensive plasticity in size with nutritional conditions (Lanet et al., 2013; Lanet & Maurange, 2014). This is presumably to compensate for changes in eye size across environmental conditions, and is facilitated due to their unique mode of development. Instead of arising from embryonic neuroblasts, the optic lobe is formed from neuroepithelium that continues to divide and expand until early in the third instar (Farkas & Huttner, 2008; Brand & Livesey, 2011). Proliferation of the optic lobe neuroepithelium remains sensitive to nutrition until the early third instar, where a small pulse of ecdysone induces the cells in this neuroepithelium to become neuroblasts (Lanet et al., 2013). After this transition, starvation no longer impacts cell divisions in this brain region, and each neuroblast proceeds to divide and generate the full complement of neuronal cells types necessary for the function of the optic lobe (Lanet et al., 2013). This ensures that while the total number of neurons in the optic lobe is plastic, the diversity of cell types is held constant (Lanet et al., 2013). Given its mode of development and persistent sensitivity to nutrition, we would expect that the optic lobes would also exhibit different growth dynamics from the whole brain.

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The growth of the whole brain and mushroom bodies also differed in their sensitivity to insulin and TOR. We observed that while mushroom body growth appeared to be more sensitive to insulin signalling, the growth of the whole brain was more sensitive to TOR signalling. Our results for whole brain growth are consistent with other studies in *Drosophila* (Sousa-Nunes et al., 2010; Yuan et al., 2020), but also in mammals (Cloetta et al., 2013), where TOR signalling controls cell cycle progression and neuronal exit from quiescence respectively, ultimately regulating final brain size.

Our results showing that the mushroom bodies were sensitive to reductions in insulin signalling is contrary to previous research (Sipe & Siegrist, 2017). Sipe and Siegrit (2017) found that the Pax-6 orthologue, Eyeless, is specifically expressed in the mushroom body. Eyeless expression allows mushroom body neurons to continue proliferating independent of PI3Kinase activity, a central regulator of insulin signalling, under conditions of poor nutrition (Sipe & Siegrist, 2017). This discrepancy in results is likely to be one of degree: while eyeless undoubted plays a role in maintaining proliferation, insulin signalling in the mushroom body neurons has its own independent effects on proliferation and in controlling the size of the arbour.

Finally, the majority of studies of brain growth have focused on nutritional stress. However, a number of other conditions are known to extend developmental time, including temperature, oxygen limitation, and larval density (Partridge et al., 1994; Peck & Maddrell, 2005; Mirth & Shingleton, A. W., 2012). The mechanisms regulating extended developmental time under these conditions are less well understood, but ultimately culminate in changing the rate of ecdysone production and secretion. While we found that ecdysone did not change growth rates in our experiments, it did change larval, whole brain, and mushroom body size by truncating the length of the larval period. Previous studies have documented that reducing or eliminating ecdysone or ecdysone signalling also reduces brain size (Lanet et al., 2013; Herboso et al., 2015). It is likely that the conditions under which we reared larvae were insufficient to reduce ecdysone concentrations enough to affect growth rates.

Conclusion

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In this research, we sought to understand how organs achieve robust final size by exploring the growth dynamics of the brain across nutritional and thermal conditions. We found that at least one compartment of the brain can differ in its growth patterns from the rest of the brain, and speculate that this might be true of other compartments. These distinct growth patterns allow specific brain regions to vary their response to changing environmental conditions via modulating the action of different molecular mechanism that regulate their growth. Taken together, our findings demonstrate that brain compartments achieve robustness in final size via different trajectories. Furthermore, by probing the growth dynamics of organs under environmental stress, we fill in important gaps in our knowledge of how these organs achieve robustness of final size.

Materials and Methods

Fly strains and husbandry

Drosophila stocks were reared at 25°C with 65% humidity, on a 12-hour light/dark cycle and maintained on sucrose-yeast (SY) diet (detailed below). To drive the expression of green fluorescent protein (GFP) in the mushroom body neurons, we used the R21B06-GAL4 line (BDSC 68318), known to be expressed in the mushroom bodies of larval and adult brains (http://flweb.janelia.org/cgi-bin/flew.cgi; (Pfeiffer et al., 2008; Jenett et al., 2012). This line was crossed with a membrane-tagged GFP reporter (w[*]; P[y[+t7.7] w[+mC]=10XUAS-IVS-myr::GFP]attP2). These stocks were obtained from the Bloomington Drosophila Stock Center, Indiana University, Bloomington.

Media

Sucrose/Yeast (SY) diet was prepared as reported by (Toivonen et al., 2007), with 100g autolyzed Brewer's Yeast, 50g sucrose, 10g agar, 1.5ml propionic acid, 15ml Nipagin M solution dissolved in one liter of distilled water. In addition to the standard diet (100% SY), we exposed larvae to additional experimental diets, which contained 10% and 25% of the caloric content of the standard SY diet. These diets were made by adding appropriate amounts of the original Brewer's yeast and sucrose to the same concentration of agar and water. 25% food contained

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25g autolyzed Brewer's Yeast and 12.5g sucrose, while 10% food contained 10g autolyzed Brewer's Yeast and 5g sucrose, dissolved in one litre of distilled water. All diets were allowed to cool to 60^{o} before the preservatives (propionic acid and Nipagin M) were added and the food dispensed.

Treatment with ecdysone and inhibitors

The concentrations of both inhibitors, Wortmannin and Rapamycin, to be used was derived following a dosage response curve on pupal volumes (Supplementary Figure 4). For Wortmannin, doses ranging from 1 – 80µM/ml of food produced no significant effect on pupal volumes, but 1000µM/ml of food significantly reduced pupal volumes, and at 120µM/ml of food, we observed a significant increase in larval lethality. Similarly, there was no significant effect of doses ranging from 1 – 25µM/ml of food, but 30µM/ml of Rapamycin in food significantly reduced pupal volumes, and doses higher than 40µM/ml of food, produced significant larval lethality. This way, we selected 30µM/ml of food of Rapamycin and 100µM /ml of food of Wortmannin for the experiment.

The standard medium was then supplemented with solutions containing one of the following substances dissolved in 100% ethanol:

- 30µM Rapamycin (Sigma-Aldrich, USA), TOR signalling inhibitor
- 100µM wortmannin (Sigma-Aldrich, USA), PI3Kinase inhibitor (Neri et al., 1999)
- 50ng/mg 20E (Sigma-Aldrich, USA) Ecdysone supplement.

Body size measurement, organ dissection, and immunocytochemistry

Animals picked at the relevant time points were first placed in cold PBS solution, to immobilize them, and then imaged using a Zeiss Stemi 508 dissecting microscope before dissection. These images were analyzed using the FIJI (ImageJ, Version 2.0.0-rc-69/1.52i, 2019) software. The length and width of the larva or pupa was measured using the straight-line tool, and volume was calculated using the formula lw^2 (length x width^2).

After measuring each larva/pupa, we dissected out their brain in cold 1x Phosphate Buffered Saline (1xPBS) under a Leica S9E dissecting microscope according to methods previously described (Hafer & Schedl, 2006; Daul et al., 2010). Isolated brains were fixed overnight in 4%

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643 paraformaldehyde at 4°C. After four washes in a solution of cold 0.3% Triton X-100 in PBS (PBT)
644 for 20 minutes each, samples were incubated in 2% normal donkey serum block solution prior to
645 immunostaining. The blocked tissue samples were then transferred to Acti-stain ™ 670 Phalloidin
646 (1:1200, Cytoskeleton Cat#: PHDN1) reagent diluted in PBT and normal donkey serum, and
647 incubated on a rocking platform shaker in the dark for 2-3days at 4°C. Prior to imaging, samples
648 were rinsed in cold PBS, and PBS was replaced with 25% KY jelly in water solution. Samples were
649 imaged using the Leica SP8 HyD microscope with 40x water immersion objective.

650 **Image processing and statistical analysis**

651 Images were analyzed using the Imaris© (Bitplane) software. Image normalization was
652 performed to ensure standardized measurements across images with different signal intensities,
653 and 3D analysis of the brain was done by software's in-built wizard. Images were rendered as
654 volumes using the surface analysis tool on Imaris.

655 Data analyses were carried out in R Studio (Version 1.2.5019© 2009-2019 RStudio, Inc.). We
656 fit our body and organ size data with both linear, using the *lm* function, and non-linear models,
657 using the *nls* package (Baty et al., 2015). We used AIC and BIC to assess model fit, selecting the
658 simplest models when these values were similar. Data visualization was conducted using the
659 ggplot package (Wickham, 2016) in R Studio (Version 1.2.5019© 2009-2019 RStudio, Inc.).

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666

667 **Author contributions**

668 AEC, BN and CKM conceived and designed the experiments. AEC performed the experiments.
669 AEC and CKM analysed the data. All authors contributed to interpretation of data and final
670 manuscript preparation.

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Declaration of interests

The authors declare no competing interests.

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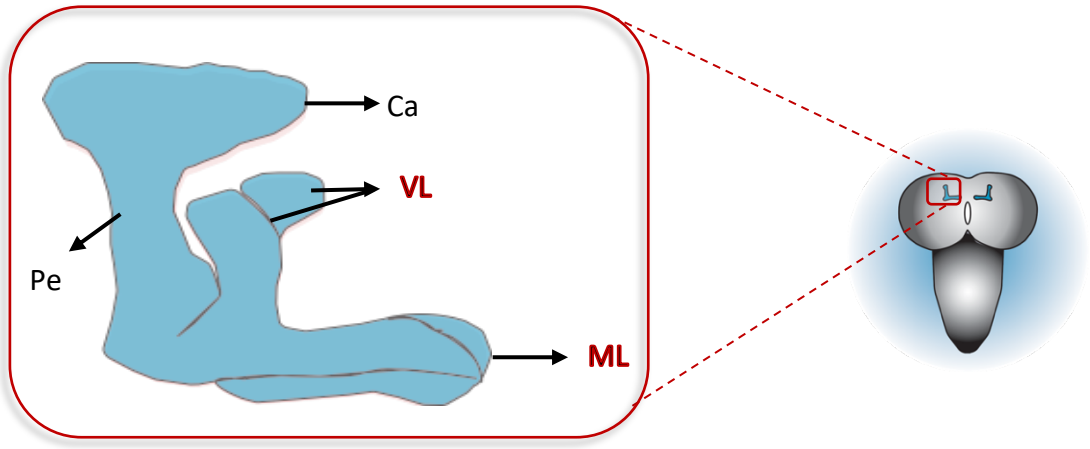
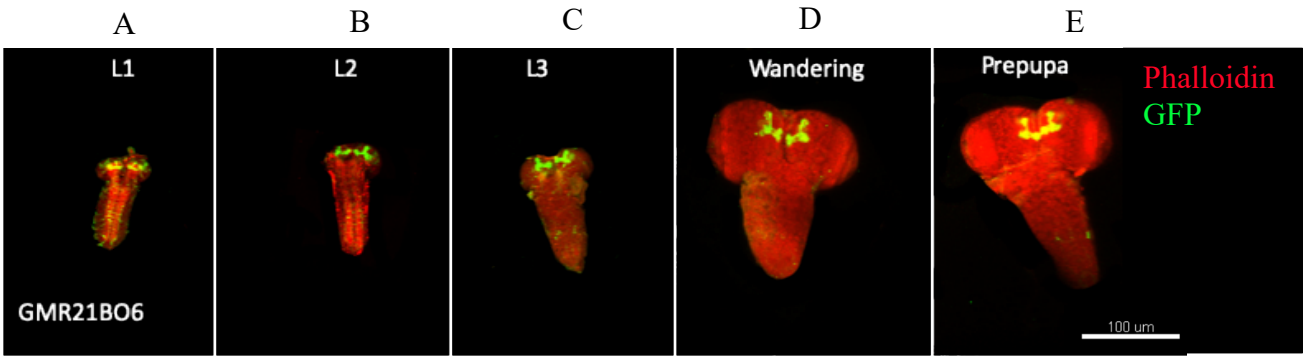
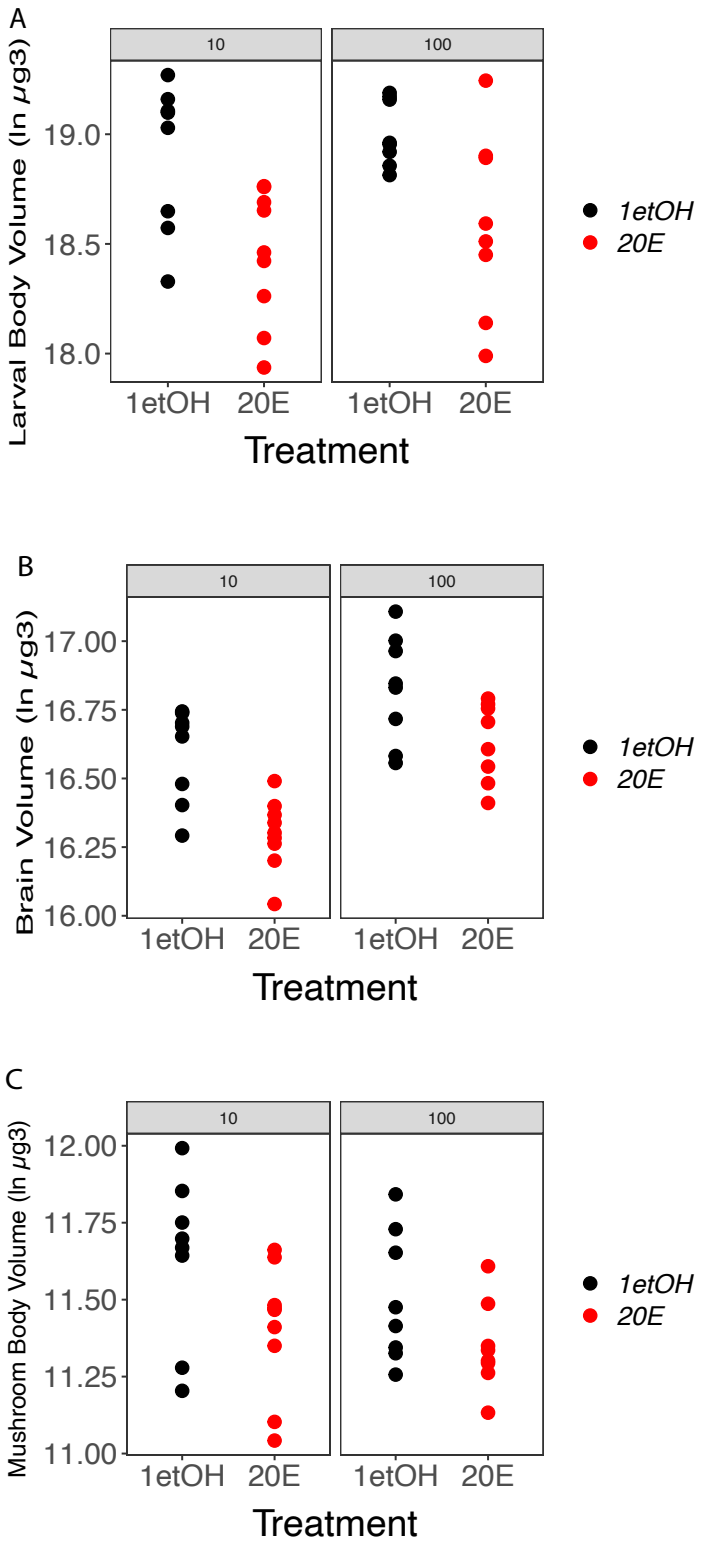
SUPPLEMENTARY MATERTIAL



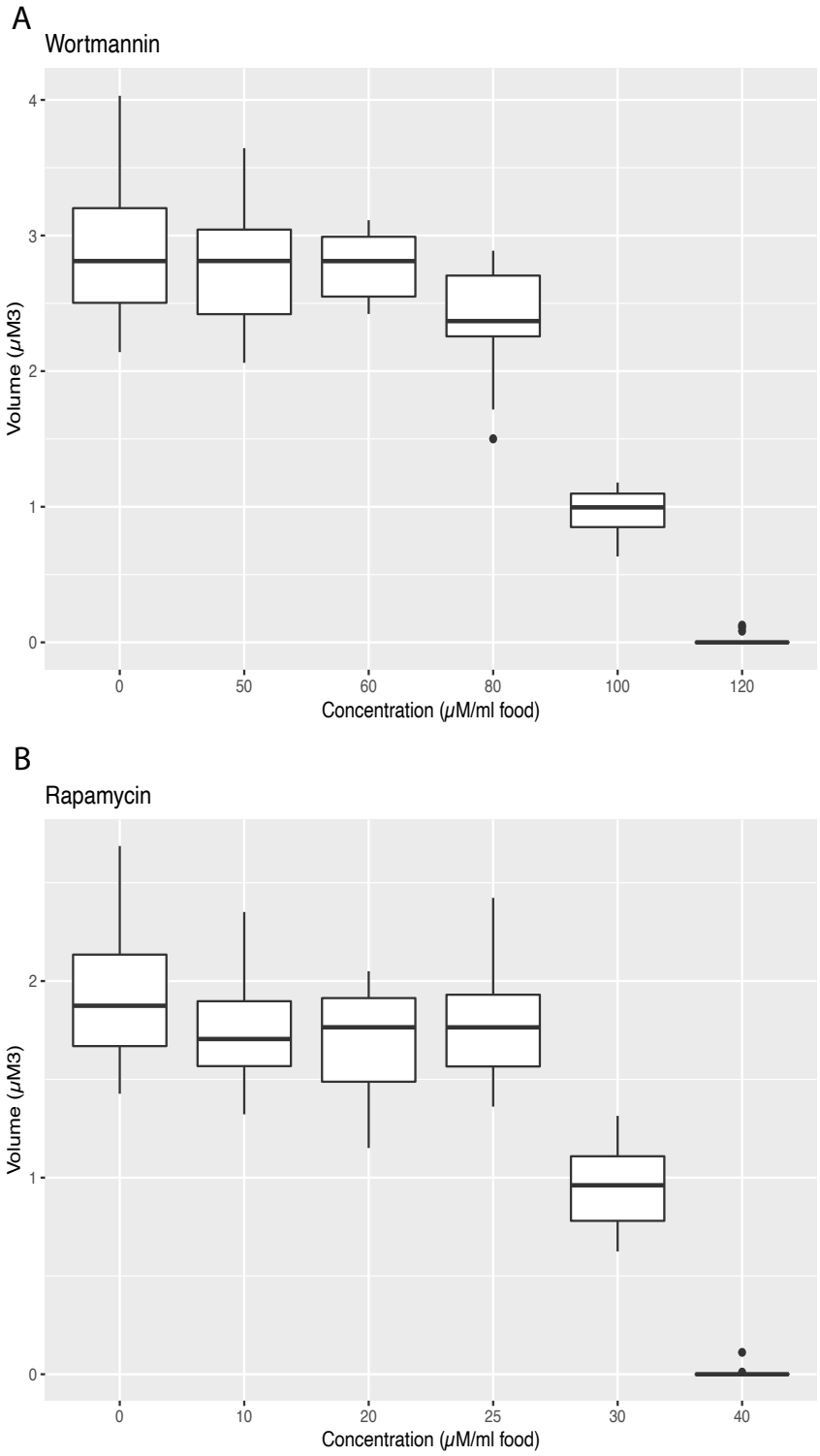
Supplementary Figure 1: Schematic of the dorsal view of mushroom body neuropil in the left hemisphere of brain of *Drosophila melanogaster* showing the Calyx (Ca), Peduncle (Pe), Vertical lobe (VL) and Medial lobe. Regions included in this study are labelled in red, the vertical and medial lobes only.



Supplementary Figure 2: Larval brains expressing GFP in the neurons (green) of the mushroom body co-stained with phalloidin (red) across five different stages. (a-e) shows brains at 0hr of L1, L2, L3, wandering and white prepupae larval stages respectively.



Supplementary Figure 3. Prepupal volume of (A) larval body, whole brain (B) and the mushroom body (C) following treatment of 50ng/mg Food of Ecdysone



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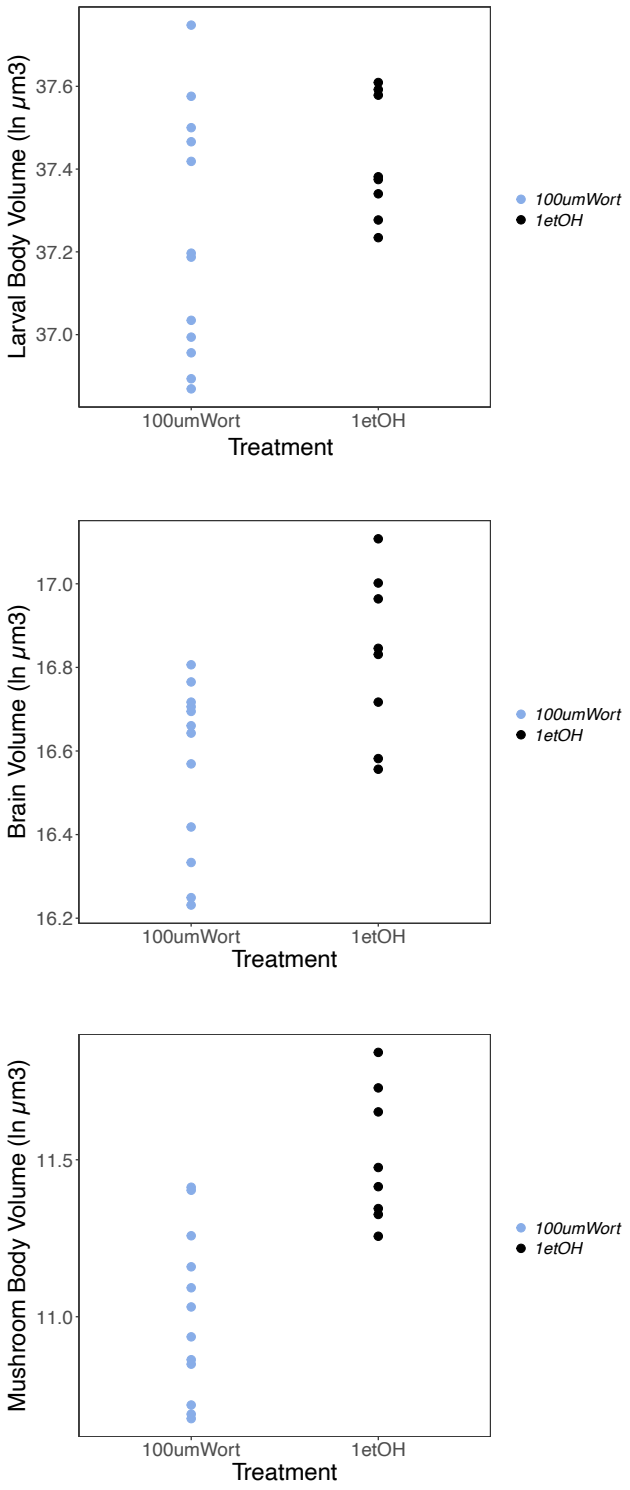
Supplementary Figure 4: Dosage response curve to determine the concentration of (A) Wortmannin and (B) Rapamycin used in the experiment.



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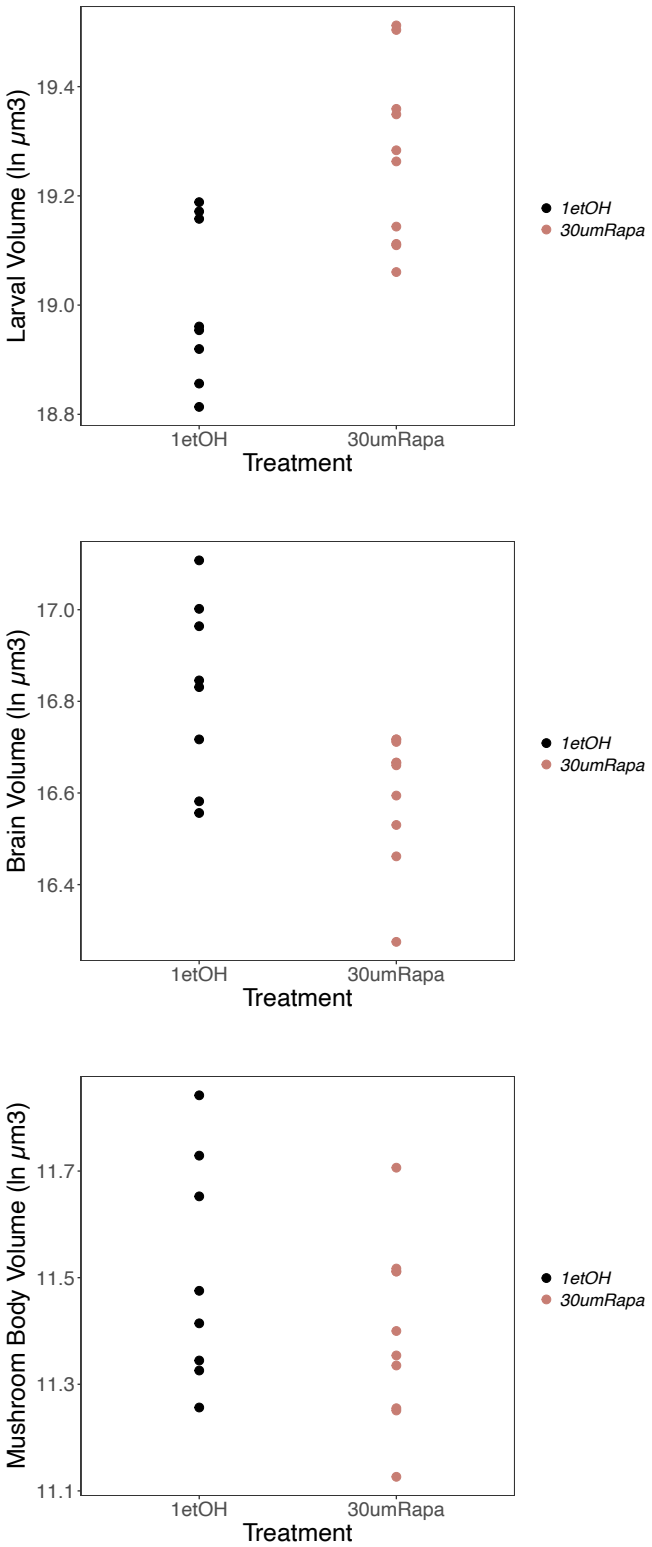
Supplementary Figure 5: Prepupal volume of (A) larval body, whole brain (B) and the mushroom body (C) following treatment of 100µM Wortmannin



924

Supplementary Figure 6: Prepupal volume of (A) larval body, whole brain (B) and the mushroom body (C) following treatment of 30µM Rapamycin

925 Supplementary Table 1: Akaike's Information Criterion (AIC) and Bayesian Information Criteria
926 (BIC) for modeling larval volume, brain and mushroom body volume following administration of
927 50ng/mg food of ecdysone. Values for best fit are in blue.

Trait	Fit	AIC	BIC
Body	Volume.lmL3	158.9679	185.5703
	Volume.lmL3poly	161.3162	199.742
Brain	Brain.lmL3	1.978233	28.58068
	Brain.lmL3poly	5.966518	32.45923
Mushroom Body	MB.lmL3	-6.652748	19.9497
	MB.lmL3poly	-0.4347325	37.99102

928 lm: linear model, poly: polynomial, exp: exponential model, explag: lagged exponential model.
929

Supplementary Table 2: Ecdysone has a significant effect on final volume of larval body, whole brain and mushroom body. This effect is not observed on the different diet groups (10% and 100%)

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
Treatment	1.55487	1	15.5662	0.0004639 ***
Food	0.12468	1	1.2482	0.2730607
Treatment x Food	0.00375	1	0.0375	0.8477163
Brain Volume				
Treatment	0.48304	1	18.517	0.0001747 ***
Food	0.68105	1	26.107	1.871e-05 ***
Treatment x Food	0.01925	1	0.738	0.3973628
Mushroom Body Volume				
Treatment	0.31938	1	6.9793	0.01315 *
Food	0.07097	1	1.5509	0.22297
Treatment x Food	0.01139	1	0.2489	0.62165

D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.'p<0.1

936 Supplementary Table 3: Wortmannin does not affect final larval volume but affects mushroom
937 body and whole brain volumes.
938

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
Treatment	0.16755	1	2.6868	0.1185
Brain Volume				
Treatment	0.32335	1	7.9357	0.01141 *
Mushroom Body Volume				
Treatment	1.1888	1	19.928	0.0002999 ***

939 D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.'p<0.1

Supplementary Table 4: Rapamycin affect the final volume of larval body and whole brain but does not affect the mushroom body.

Larval Volume	**Sum Sq**	**Df**	**F value**	**P value**
Treatment	0.31664	1	12.873	0.002461 **
Brain Volume				
Treatment	0.22643	1	7.9501	0.01233 *
Mushroom Body Volume				
Treatment	0.05202	1	1.4578	0.2448

D.f: degrees of freedom. Sum Sq: Sum of squares. Significance codes: * p<0.05, ** p<0.01, *** p<0.001, '.'p< 0.1