Stage-specific plasticity in ovary size is regulated by insulin/insulin-like growth factor and ecdysone signalling in *Drosophila*

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Abstract

Animals from flies to humans adjust their development in response to environmental conditions through a series of developmental checkpoints, which alter the sensitivity of organs to environmental perturbation. Despite their importance, we know little about the molecular mechanisms through which this change in sensitivity occurs. Here we identify two phases of sensitivity to larval nutrition that contribute to plasticity in ovariole number, an important determinant of fecundity, in *Drosophila melanogaster*. These two phases of sensitivity are separated by the developmental checkpoint called critical weight; poor nutrition has greater effects on ovariole number in larvae before critical weight than afterwards. We find that this switch in sensitivity results from distinct developmental processes. In pre-critical weight larvae, poor nutrition delays the onset of terminal filament cell differentiation, the starting point for ovariole development, and strongly suppresses the rate of terminal filament addition and the rate of increase in ovary volume. Conversely, in post-critical weight larvae, poor nutrition only affects the rate of increase in ovary volume. Our results further indicate that two hormonal pathways, the insulin/insulin-like growth factor and the ecdysone signalling pathways, modulate the timing and rates of all three developmental processes. The change in sensitivity in the ovary results from changes in the relative contribution of each pathway to the rates of TF addition and increase in ovary volume before and after critical weight. Our work deepens our understanding of how hormones act to modify the sensitivity of organs to environmental conditions, thereby affecting their plasticity.

Introduction

Developmental plasticity, the ability of an organism to adjust its developmental trajectory in response to environmental variation, is a widespread property of multicellular organisms. Trait plasticity depends not only on the trait itself and the environmental conditions considered (Mirth and Shingleton 2012), but also on windows of environmental sensitivity, known as critical periods, during which plastic responses are possible (Nijhout 2003; Koyama *et al.* 2013). In the most extreme cases, an environmental cue within a critical period triggers a developmental switch between alternative developmental trajectories, giving rise to distinct phenotypes, such as dramatic seasonal differences in the pigmentation of butterfly wing patterns and the

different body sizes and shapes seen in the castes of the honeybee (Brakefield *et al.* 1996; Smith *et al.* 2008). Although significant progress has been made in uncovering the molecular pathways underlying developmental plasticity in body and organ size (Gotoh *et al.* 2011, 2014; Beldade *et al.* 2011; Emlen *et al.* 2012; Koyama *et al.* 2013; Xu *et al.* 2015), there is still a fundamental gap in our understanding of the molecular pathways through which organs change in sensitivity to environmental conditions over developmental time.

Nutrition is an important determinant of body and organ size and its effects have been extensively studied in insects, particularly in the fruit fly *Drosophila melanogaster* (Nijhout 2003; Mirth and Shingleton 2012; Koyama *et al.* 2013). In *D. melanogaster*, and many other animals, nutrition modifies body and organ size through the action of the insulin/insulin-like growth factor signalling (IIS) pathway. In a well-nourished animal, neurosecretory cells in the brain synthesize and secrete insulin-like peptides (Rulifson *et al.* 2002; Ikeya *et al.* 2002). After being released into the insect bloodstream, these peptides act on target tissues by binding to the insulin receptor (InR) and activating the IIS pathway, thereby inducing tissue growth (Brogiolo *et al.* 2001; Britton *et al.* 2002). The amount of growth induced depends on tissue-specific sensitivity to insulin-like peptides and on the developmental stage of the larva (Shingleton *et al.* 2005; Tang *et al.* 2011). Most adult tissues develop as pouches of cells within the developing larva, called imaginal discs or tissues. The growth rate of wing imaginal discs, determined by changes in disc area, is more sensitive to nutrition and to changes in IIS activity early in the third larval instar than at later stages (Shingleton *et al.* 2008). This shift in sensitivity results from a developmental transition called critical weight (Mirth *et al.* 2005, 2009).

The developmental transition at critical weight regulates body and organ size by determining the length of the growth period (Beadle *et al.* 1938; Nijhout 1975, 2003). Starving larvae before reaching critical weight significantly delays the onset of metamorphosis (Beadle *et al.* 1938; Mirth *et al.* 2005; Stieper *et al.* 2008) and delays the patterning and growth of their wing imaginal discs (Shingleton *et al.* 2008; Mirth *et al.* 2009). Conversely, starvation after critical weight does not delay metamorphosis and allows continued patterning of the wing imaginal discs (Beadle *et al.* 1938; Mirth *et al.* 2005, 2009; Shingleton *et al.* 2008).

Critical weight is induced by a small nutrition-sensitive pulse of the steroid hormone ecdysone (Mirth *et al.* 2005; Warren *et al.* 2006; Koyama *et al.* 2014).

Activating or supressing IIS in the prothoracic glands, the glands that synthetize ecdysone, regulates the rate of ecdysone synthesis at critical weight (Caldwell *et al.* 2005; Mirth *et al.* 2005; Colombani *et al.* 2005; Layalle *et al.* 2008; Walkiewicz and Stern 2009), thereby affecting the progression of imaginal disc patterning and the timing of the onset of metamorphosis. Thus, the pulse of ecdysone at critical weight appears to reprogram the response of the imaginal discs to nutritional conditions.

Ecdysone exerts its effects by binding to the ecdysone receptor complex, a heterodimer between Ecdysone Receptor (EcR) and Ultraspiracle (Usp). In the absence of ecdysone, the EcR/Usp complex represses the transcription of a subset of ecdysone target genes (Schubiger and Truman 2000; Cherbas 2003; Schubiger *et al.* 2005; Brown *et al.* 2006). Once ecdysone binds to EcR/Usp, it induces target gene transcription either by relieving the repressive action of unliganded EcR/Usp, called derepression, or by inducing activation of gene transcription via EcR/Usp (Schubiger and Truman 2000; Cherbas 2003; Schubiger *et al.* 2005; Brown *et al.* 2006).

We can use the properties of the ecdysone receptor complex to understand how ecdysone regulates developmental processes. Overexpressing a dominant negative form of EcR that cannot bind to ecdysone induces constitutive EcR/Usp-mediated repression and also inhibits EcR/Usp activation function, thereby suppressing all ecdysone signalling (Cherbas 2003; Hu *et al.* 2003; Schubiger *et al.* 2005; Brown *et al.* 2006). Knocking down EcR induces derepression, mimicking part of the effects of ecdysone, but also inhibits EcR/Usp activation (Cherbas 2003; Hu *et al.* 2003; Schubiger *et al.* 2005; Brown *et al.* 2006). By comparing the phenotypes induced by dominant negative EcR and EcR knock down in tissues, we can infer the mechanism through which ecdysone regulates a given developmental process. In the ovaries and wing discs, overexpressing dominant negative EcR delays their patterning (Schubiger *et al.* 2005; Mirth *et al.* 2009; Gancz *et al.* 2011). In contrast, knocking down EcR in the ovaries and wing promotes precocious patterning (Schubiger *et al.* 2005; Mirth *et al.* 2009; Gancz *et al.* 2011). Because these manipulations result in opposing phenotypes, this suggests that ecdysone is likely to regulate the patterning of the ovaries and wing discs primarily through derepression.

Nutritional conditions during the larval stages also determine the size of the *Drosophila* ovary (Hodin and Riddiford 2000; Tu and Tatar 2003; Sarikaya *et al.* 2012; Green and Extavour 2014). Whether ovary development exhibits critical periods of nutritional sensitivity, and how this may influence its plastic response, is unclear. The

Drosophila ovary is composed of functional units called ovarioles, which are egg-producing structures in the insect ovary that directly affect female reproductive capacity (Boulétreau-Merle *et al.* 1982; R' kha *et al.* 1997; Klepsatel *et al.* 2013a; b). Ovariole development occurs during the third instar larval and early pupal stages (Kerkis 1931; King *et al.* 1968; King 1970) through the intercalation of terminal filament cells (TFCs) into stacks of seven to ten flattened cells, called terminal filaments (TFs) (Godt and Laski 1995; Sahut-Barnola *et al.* 1995, 1996). Each TF defines the position of one ovariole and thus, the number of TFs at pupariation is equivalent to adult ovariole number (Hodin and Riddiford 1998; Sarikaya *et al.* 2012; Sarikaya and Extavour 2015).

Both IIS and ecdysone signalling pathways regulate ovariole number (Hodin and Riddiford 1998; Gancz *et al.* 2011; Green and Extavour 2012, 2014; Gancz and Gilboa 2013), and IIS, in particular, underlies the plastic response of ovariole number to larval nutrition (Green and Extavour 2014). Based on previous studies, IIS and ecdysone signalling pathways are thought to regulate different developmental processes during ovariole development, with ecdysone primarily controlling the timing of TFC differentiation and IIS controlling ovary size (Gancz *et al.* 2011; Gancz and Gilboa 2013). This work provides an excellent springboard for detailed, quantitative explorations of ovary development over developmental time that specifically address how nutrition alters the rates of developmental processes, and how sensitivity to nutrition changes with developmental stage.

We first determined if ovariole number shows critical periods of sensitivity to nutrition in the third instar larval stage. Our results highlight a switch in nutritional sensitivity at critical weight. Next, we explored how the developmental processes that determine ovariole number are regulated by nutrition. We identified three developmental processes that are differentially affected by pre- and post-critical weight nutrition: the onset of TFC differentiation, the rate of TF addition, and the rate of increase in ovary volume. Finally, we altered either IIS or ecdysone signalling, and examined the effects on all three developmental processes. We demonstrate that complex, stage-specific interactions between ecdysone and IIS regulate the switch in sensitivity to nutrition in the developing ovary.

Material and Methods

Fly stocks

To assess the effects of larval nutrition on ovariole number, we used an outbred, wild caught population of *Drosophila melanogaster* founded and maintained as described in (Martins *et al.* 2013) and provided by Dr. Élio Sucena (Instituto Gulbenkian de Ciência). To genetically manipulate IIS and ecdysone signalling, we used *traffic jam*-GAL4 to drive expression in the somatic cells of the larval ovary. This driver line is a NP insertion line (P{GawB}NP1624) provided by Dr Lilach Gilboa (Weizmann Institute of Science, Rehovot, Israel). *Traffic jam*-GAL4 was crossed to w^{1118}, obtained from Dr. Lynn Riddiford (Janelia Research Campus – HHMI), and the F1 progeny is shown throughout the main text, figures, and tables as the genetic control (*tj-GAL4*). *Elav*-GAL4, *elav*-GAL80, UAS-*EcR.W650A TP3* (*UAS-EcR-DN*), UAS-*EcR RNAi CA104* (*UAS-EcR-IR*), UAS-*PTEN* and UAS-*InR29.4* (UAS-*InR*) were obtained from Dr. Lynn Riddiford (Janelia Research Campus – HHMI). *Hedgehog*-GAL4 and *patched*-GAL4 were provided by Dr. Florence Janody (Instituto Gulbenkian de Ciência, Oeiras, Portugal). *Bric-à-brác*-GAL4 was obtained from Dr. Cassandra Extavour (Harvard University, USA). *Nanos*-GAL4 was acquired from Dr. Rui Martinho (University of Algarve, Portugal). Fly stocks were maintained at 22ºC in bottles on standard fly food (4.5% molasses, 7.2% sugar, 7% cornmeal, 2% yeast extract, 1% agar and 2.5% Nipagin solution). All fly strains are available upon request.

Larval staging and dietary manipulations

Adults were allowed to lay eggs for two to six hours on fresh food plates (60 × 15 mm Petri dish filled with standard fly food - 45 g of molasses, 75 g of sucrose, 70 g of cornmeal, 20 g of yeast extract, 10 g of agar, 1100 ml of water, and 25 ml of a 10% Nipagin solution per liter of fly food). Egg density was controlled to prevent overcrowding (approximately 200 eggs per plate). Larvae were selected 0-2 hours after ecdysis to third instar (L3) and transferred onto new food plates (40-60 larvae per plate) to feed until they reached the appropriate age. For diet manipulations, 20-30 larvae of the appropriate age were transferred to vials containing either 20% sucrose and 0.5% agar medium (20% sucrose food), 1% sucrose and 0.5% agar medium (1% sucrose

food), or standard fly food (standard food) until the end of the feeding period. We chose 20% sucrose to 1) compare to previous studies on the effects of nutrition on the patterning of the wing discs and nervous system (Mirth *et al.* 2009; Lanet *et al.* 2013) and because it is close to the carbohydrate content of our standard fly medium (approximately 17% carbohydrates). The 1% sucrose medium was used to compare to previous studies by (Géminard *et al.* 2009). On 20% and 1% sucrose media, most larvae survive until pupariation and adult eclosion. To obtain L3 ovaries, larvae of the appropriate age were dissected and processed for immunocytochemistry. For 20E feeding experiments, 10-20 pre-critical weight larvae (5 h AL3E) were transferred to small vials containing either 20% sucrose food or standard food supplemented with 4.92 µl 20E (Sigma)/g of food (stock solution: 0.15 mg/ml 20E in ethanol). As a control, 10-20 pre-critical weight larvae (5 h AL3E) were transferred to small vials containing either 20% sucrose food or standard food supplemented with 4.92 µl ethanol/g of food. All experiments were performed at 25ºC.

Adult ovariole number and female weight

To count adult ovariole number, newly eclosed flies were maintained in vials on standard food until the time of dissection (4-6 days after eclosion). Ovaries were dissected in cold phosphate buffered saline containing 1% Triton X-100 (PBT) and ovarioles were teased apart and counted under a dissecting microscope. We used pharate weight as a proxy of adult body size (Mirth *et al.* 2005). Pharate adults were collected from food vials, sexed and individually weighed on a Sartorius SE2 ultramicrobalance (Sartorius, Goettingen, Germany).

Immunocytochemistry

Larvae were dissected in cold phosphate buffered saline (PBS) and fixed in 4% formaldehyde in PBS for 30 minutes at room temperature. Larvae were then washed three times for 20 minutes with PBT and blocked in 2% normal donkey serum in PBT for 30 minutes. We incubated the tissue overnight at 4°C in a primary antibody solution containing mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) diluted into 2% normal donkey serum in PBT. After washing three times for 20 minutes

in PBT, larvae were incubated in the dark with goat anti-mouse Alexa 568 (Invitrogen, 1:200) and TRICT-Phalloidin (Sigma, 1:200) diluted into 2% normal donkey serum in PBT overnight at 4°C. Larvae were rinsed with PBT and ovaries were mounted on a poly-L-lysine-coated coverslip using Fluoromount-G (SouthernBiotech).

Image Acquisition and Analysis

Samples were imaged using a Zeiss LSM 510 Meta confocal microscope using a 40x 1.3NA oil objective lens. During confocal image acquisition, the detection parameters were adjusted to avoid under- or overexposed pixels, and images were acquired through the full thickness of the ovary at 1µm. Images were processed and analysed using ImageJ (NIH) and Adobe Photoshop (Adobe Systems) softwares. For each time point/genotype/food treatment, forming terminal filaments (TFs) were identified by cell morphology and Engrailed expression and total number of forming TFs were counted. For ovary volume, the ImageJ Volumest plugin was used (Merzin 2008).

Statistical Analysis

All experiments were replicated at least twice. The distribution of residuals was tested for normality using Q-Q plots and the appropriate statistical test was applied. ANOVAs were performed followed by Tukey's multiple comparison test to evaluate all pairwise differences in means unless otherwise noted. Differences in the timing of the onset of TFC differentiation were tested with a Chi-squared test. To determine differences in the mean number of TFs and ovary volume, as well as the rates of TF addition and of increase in ovary volume between different genotypes/ food treatments, TF number and ovary volume were $\log_{10}$-transformed and analysed using linear models and ANCOVAs. When exploring the relative importance of larval age, ecdysone signalling, and IIS in determining TF number and ovary volume, we used linear models and the boot.relimp function, with lgm metrics, of the relaimpo package in R to calculate the relative contribution, and 95% confidence intervals, of each to the total R^2. All data analyses and statistics were conducted using R v3.1.2 (R Development Core

Team 2014). Plots were made using GraphPad Prism v6 (GraphPad Software). All data is available on Dryad (reference number to be provided).

Results

Effects of larval nutrition on ovariole number

To determine critical periods of nutritional sensitivity in ovariole number, we fed third instar (L3) larvae either on standard food or on 20% sucrose food at timed intervals starting between 0 h to 30 h after third instar ecdysis (AL3E) until the end of the larval development. We chose to feed larvae on 20% sucrose food because in this food they are starved of the protein, lipids, and other micronutrients present in yeast, and thus grow very slowly, yet show higher rates of survival than when starved completely. Larvae transferred to 20% sucrose food between 0 and 25 h AL3E showed a significant reduction in ovariole number when compared to the controls transferred to standard food (Figure 1A). In contrast, transferring larvae to 20% sucrose food at 30 h AL3E did not cause a significant reduction in ovariole number (Figure 1A). As expected, a reduction in ovariole number was correlated with a reduction in early fecundity, as determined by the number of eggs laid over the first three days after adult eclosion (Figure S1).

Interestingly, the effect of the 20% sucrose food on ovariole number depended on the timing at which larvae were transferred and/or the length of exposure to the 20% sucrose food (Figure 1A). To test for a significant change in the response to 20% sucrose food over time, we applied a bi-segmental linear regression model to the data and tested for a significant change in slope. The relationship between ovariole number and the age at transfer to 20% sucrose food (in h AL3E) has a significant change in slope around a single breakpoint (Davies' test for a change in the slope, $p<0.0001$) at 11.5 h AL3E (95% CI: 9.37–13.64 h AL3E) (Davies 1987; Muggeo 2003, 2007). This estimated breakpoint correlates with critical weight, suggesting that pre-critical weight ovaries are more sensitive to changes in larval nutrition than post-critical weight ovaries, similar to growth in the wing discs (Shingleton *et al.* 2008).

The effects of the 20% sucrose food on ovariole number could also be a direct consequence of different lengths of exposure to the 20% sucrose food. To test this hypothesis, we performed an experiment where L3 larvae were fed on 20% sucrose food

for 20 h starting either at 0 h AL3E or at 20 h AL3E and then returned them to standard food until the end of the feeding period. In pre-critical weight larvae fed first on 20% sucrose food between 0-20 h AL3E then transferred back to standard food, mean ovariole number was indistinguishable from that of larvae fed continuously on standard food (Figure 1B). In contrast, when post-critical weight larvae were fed on 20% sucrose food from 20 h to 40 h AL3E then transferred to standard food, ovariole number was significantly reduced (Figure 1B). This reduction in ovariole number was similar when compared to larvae transferred to 20% sucrose food at 20 h AL3E until the end of development (Figure 1A, B). These observations corroborate a previous study where re-feeding pre-critical weight larvae after a period of starvation delays pupariation, but does not affect final body size, measured as dry adult weight (Beadle *et al.* 1938). After critical weight, intervals of starvation do not affect the timing of pupariation and thus, larvae pupariate at smaller sizes (Beadle *et al.* 1938).

Developmental processes responding to nutrition during ovariole development

To determine how nutrition affects ovariole number, we examined the developmental processes that give rise to ovarioles at carefully timed intervals over the third instar. This approach allows us to precisely define the timing of developmental events, and also to determine the rate of developmental events in larvae reared on standard versus sucrose food. We first analysed the dynamics of TF addition and of ovary volume in L3 larvae from the outbred line raised on standard food. When TFCs differentiate from the surrounding ovarian somatic cells, they upregulate expression of the transcription factor Engrailed (En) (Forbes *et al.* 1996). Thus, we used En as a marker for TFC differentiation and TF addition. Consistent with previous studies, we did not observe TFCs in pre-critical weight ovaries (from 0-10 h AL3E) (Figure S2A) (Godt and Laski 1995). At 15h AL3E, TFCs appeared in the medial side of the ovary and a few forming TFs were visible (Figure S2A, B). New TFCs continued to emerge from the surrounding ovarian somatic cells and gradually intercalated into forming TFs. The addition of new TFCs occurs in a lateral direction (Figure S2A) (Godt and Laski 1995; Sahut-Barnola *et al.* 1995, 1996), and the rate of TF addition increased exponentially with time (Figure S2B). At the end of the L3, all of the approximately 18-

22 TFs have formed (Figure S2A, B) (Godt and Laski 1995; Hodin and Riddiford 1998; Sarikaya *et al.* 2012). Ovary volume also increased exponentially throughout the L3 (Figure S2C), confirming results previously found in (Kerkis 1931).

From our description of ovariole development, we hypothesized that larval nutrition regulates one or all of the three developmental processes in the developing ovary: 1) the onset of the differentiation of the first TFCs, representing the first step in ovariole development, 2) the rate at which new TFs emerge through intercalation of TFCs (referred to as the rate of TF addition), and 3) the rate of increase in ovary volume.

To test which of these processes respond to changes in nutrition, we fed outbred larvae on 20% sucrose food for 24 h, starting at 5 h intervals between 0 h to 25 h AL3E, and determined whether TFCs had begun differentiation, quantified the number of TFs, and measured ovary volume for each condition at the end of this one-day starvation period. When larvae were fed on 20% sucrose food before reaching critical weight (before 10 h AL3E), we failed to observe any En-positive cells in the ovaries indicating that the onset of TFC differentiation was delayed (Figure 2A-D, M). The wing discs and central nervous system of larvae staged before 10 h AL3E did show En expression, indicating that this antigen was detectable in other tissues (data not shown). In addition, the ovary volume was severely reduced relative to standard food controls in larvae fed on 20% sucrose food before 10 h AL3E (Figure 2N). Because high sucrose diets have been shown to rapidly induce insulin resistance in larvae (Musselman *et al.* 2011; Pasco and Léopold 2012), we repeated these experiments using 1% sucrose food. When pre-critical weight larvae (5-29 h AL3E) were fed on 1% sucrose food, the ovaries similarly did not show any TFCs (Figure S3A, B, E) and ovary volumes were even smaller than those from larvae fed on 20% sucrose between 5 h and 29 h AL3E (Figure S3F).

In contrast, when larvae were transferred to 20% sucrose food around the time of the critical weight transition (at 10 h AL3E), most ovaries had a few TFCs (Figure 2E-F), and in some ovaries TFCs were organized into forming TFs (Figure 2M). Ovary volume was still greatly reduced in these larvae (Figure 2N). Finally, ovaries from larvae transferred to 20% sucrose food after reaching critical weight (after 15 h AL3E), all had forming TFs (Figure 2G-L). Nevertheless, both TF number and ovary volume were moderately reduced when compared with larvae fed on standard food (Figure 2M, N). A stronger phenotype was obtained when larvae were transferred to 1% sucrose food after reaching critical weight (15-39 h AL3E) (Figure S3C, D-F); both TF number

and ovary volume were reduced when compared to post-critical weight larvae fed on 20% sucrose food during the same period of time (Figure S3E, F). These data suggest that all three developmental processes are affected by nutrition, but it does not resolve how the dynamics of these processes change over developmental time.

Although ovaries from pre-critical weight larvae fed on 20% sucrose food for 24 h did not contain any TFCs (Figure 2A-D, M), these larvae did eventually give rise to adults with functional ovaries (Figure 1A). Thus, in pre-critical weight larvae fed on 20% sucrose food TFC differentiation must eventually occur. We therefore postulated that the ovaries from pre-critical weight larvae might be more sensitive to nutrition because nutrition affects the timing of the onset TFC differentiation, as well as the rate of TF addition and/or the rate of increase in ovary volume. In contrast, feeding post-critical weight larvae on 20% sucrose does not delay the onset of TFC differentiation. We hypothesized that reduced ovariole number in these larvae arose from either a reduction in the rate of TF addition or a reduction in the rate of increase in ovary volume.

Both TF number and ovary volume increase exponentially with larval age (Figure S2B, C). Therefore, to explore how the dynamics of each of these processes change over developmental time, we $\log_{10}$-transformed the data to linearize the relationship with larval age (Shingleton *et al.* 2007; Tang *et al.* 2011), and performed an ANCOVA on the $\log_{10}$-transformed data. This allows us to characterize two features of each developmental process: 1) mean TF number and ovary volume, and 2) the rates of increase for each. Means for each developmental process can be estimated using their least squared means. We estimated the rates of increase using the slope of the relationship. If, for example, the least squared means for ovary volume differed between treatments, but their slopes were the same, this would mean that ovary volume differed between treatments at the first time point sampled, but that treatments increased in volume at the same rate within the sampling period. Additionally, this would mean that differences in ovary volume arose from differences in the rate of increase before the sampling period began. If the slopes differed between treatments, this means that the rate of increase differed between treatments for the time interval sampled. By analysing the data in this manner, we can precisely identify how nutrition affects each developmental process, and how this changes with developmental stage.

Indeed, in ovaries from pre-critical weight larvae fed on 20% sucrose food (starting at 5 h AL3E), TFCs and a few forming TFs were observed at 49 h AL3E

(Figure 3B, B′, D) and new TFs were still forming at 69 h AL3E (Figure 3D). Ovaries from pre-critical weight larvae fed on 20% sucrose showed significant reductions in TF number and TF addition rate when compared to ovaries from fed larvae (Table S1). For post-critical weight larvae fed on 20% sucrose food, ovaries showed significant differences in TF number, but showed a similar rate of TF addition (Figure 3A, A', C, C', D and Table S1) when compared to well-fed larvae. Both TF number and the rate of TF addition were higher in ovaries from post-critical weight larvae than in pre-critical weight larvae fed on 20% sucrose (Table S1).

Similar to what we found for TF number, in pre-critical weight larvae fed on 20% sucrose both ovary volume and the rate of increase in ovary volume was dramatically reduced, with no detectable increase in ovary volume over the time period sampled, when compared to ovaries from well-fed larvae or post-critical weight larvae fed on 20% sucrose food (Figure 3E and Table S1). Ovary volume was both smaller and showed a reduced rate of increase in post-critical weight larvae fed on 20% sucrose when compared to the ovaries of well-fed larvae (Figure 3E and Table S1).

We further hypothesized that the number of TFCs in a TF might contribute to changes in the rate of TF addition, thus final ovariole number. This would be especially relevant if more TFCs contributed to each TF in 20% sucrose fed larvae, thereby limiting the rate of TF addition. TFC number per TF in ovaries from pre-critical weight larvae fed on 20% sucrose food was significantly reduced at 69 h AL3E when compared to standard food controls (Table S2). However, we were unable to distinguish whether this reduction was due to an effect of nutrition on the mechanism regulating the sorting of TFCs, resulting in short and mature TFs, or merely to the delay in the developmental progression. In post-critical weight larvae fed on 20% sucrose, the number of TFCs per TF at 49 h AL3E was indistinguishable from that of larvae fed on standard food (Table S2). Because the number of TFCs per TF was either reduced, presumably due to developmental delays, or showed no difference between well-fed larvae and those fed on 20% sucrose, we excluded this parameter from further analyses.

Taken together, we can distinguish between the effects of nutrition on each developmental stage. In larvae fed on 20% sucrose before reaching critical weight, ovaries showed delayed onset of TFC differentiation, and reduced means and rates of TF addition and ovary volume. When larvae were fed on 20% sucrose after critical weight, TF number was reduced, but TF addition proceeded at normal rate. Because TF number is reduced at 29 h AL3E in post-critical weight larvae, this suggests that the rate

of TF addition was transiently reduced between 15 and 29 h AL3E, but returned to the same rates as fed larvae after 29 h AL3E. Both ovary volume and the rate of increase in ovary volume was significantly reduced in post-critical weight larvae fed on 20% sucrose food, albeit to a lesser degree than in pre-critical weight larvae.

Ovariole number is regulated by IIS and ecdysone signalling pathways

Given the differences in sensitivity to nutrition between pre- and post-critical weight larval ovaries, and in the developmental processes affected in each stage, we next hypothesized that these differences might arise if distinct signalling pathways regulated each process. Previous studies had shown that supressing IIS or ecdysone signalling in the whole organism or specifically in the somatic cells of the larval ovary reduces ovariole number (Hodin and Riddiford 1998; Green and Extavour 2012, 2014; Gancz and Gilboa 2013). To confirm these results, we manipulated the IIS and/or ecdysone signalling pathways in the somatic cells of the larval ovary using the *traffic jam*-GAL4 driver. At 0 h AL3E, *traffic jam*-GAL4 is expressed in all somatic cells of the larval ovary, but not in germ cells (Figure S4A-A"). By 40 h AL3E, its expression becomes restricted to the posterior part of the ovary (Figure S4C-C"). *Traffic jam*-GAL4 is also expressed in the larval brain (Figure S5A-B"). Co-expressing *elav*-GAL80 with *traffic jam*-GAL4 (*elav-GAL80, tj > GFP*) inhibits most of the expression in the larval brain, but not in the larval ovary (Figure S5C-D").

To determine whether suppressing IIS in somatic cells of the larval ovary reduces ovariole number, we used the *traffic jam*-GAL4 driver line (*tj-GAL4*) to overexpress Phosphatase and tensin homolog (PTEN) under the control of UAS (*tj > PTEN*). Adult ovariole number in *tj > PTEN* adult flies was significantly reduced (Figure S6A). Also, *tj > PTEN* larvae developed faster and gave rise to pupae with smaller pharate weights when compared to controls (Figure S6A, C).

To rule out the contributions of other cell types in regulating ovariole number, we overexpressed *PTEN* using different GAL4 driver lines that are expressed: 1) in TFCs (*hedgehog*-GAL4; *hh*-GAL4) (Gancz *et al.* 2011); 2) in anterior ovarian somatic cells (*patched*-GAL4; *ptc*-GAL4) (Gancz *et al.* 2011); 3) in ovarian somatic cells at early stages and later on in TFCs (*bric-à-brác*-GAL4*; bab*-GAL4) (Gancz *et al.* 2011; Sarikaya *et al.* 2012), or 4) in germ cells (*nanos*-GAL4*; nos*-GAL4). Adult ovariole

number was significantly reduced in *bab > PTEN* females when compared with control females (both *bab*-GAL4 and UAS-*PTEN* backgrounds) (Table S3). On the other hand, overexpressing *PTEN* under the control of the other GAL4 driver lines had no effect on ovariole number when compared to control females (both GAL4 driver lines and UAS-*PTEN* backgrounds) (Table S3). This suggests that IIS in the ovarian somatic cells at early stages of larval development is primarily responsible for determining ovariole number.

As *traffic jam*-GAL4 is expressed in the larval brain, we next determined whether the effects in ovariole number in *tj > PTEN* adult females were due to a reduction in IIS activity in the larval brain. To test this prediction, we used *elav-GAL80* to suppress GAL4 expression in the nervous system while simultaneously overexpressing *PTEN* specifically in ovarian somatic cells under the control of *traffic jam*-GAL4 (*elav-GAL80, tj > PTEN*). We also overexpressed *PTEN* in neuroblasts and neurons of the larval brain using the *elav*-GAL4 driver (*elav > PTEN*). As expected, ovariole number was significantly reduced in *elav-GAL80, tj > PTEN* females (Figure S6D). On the other hand, *elav > PTEN* females had a similar number of ovarioles as control females (both *elav*-GAL4 and UAS-*PTEN* backgrounds) (Figure S6D). These results indicate that suppressing IIS in the neuroblasts and neurons of the larval brain has no effect on ovariole number. Nonetheless, the reduction in ovariole number was stronger in *tj > PTEN* females than in *elav-GAL80, tj > PTEN* females ($p<0.001$; ANOVA). These differences are likely caused by differences in the genetic backgrounds. Interestingly, pharate weight was reduced in both *tj > PTEN* and *elav-GAL80, tj > PTEN* females (Figure S6C, E), but such reduction in pharate weight was not observed in *elav > PTEN* females (Figure S6C, E). Overall, these results indicate that the reduction in ovariole number in *tj > PTEN* females is due to the suppression of IIS in the ovarian somatic cells.

Ecdysone binds to EcR/Usp to induce two types of functions (Cherbas *et al.* 2003). First, for genes that are repressed by unliganded EcR/Usp, ecdysone relieves this repression (i.e. derepression) and allows gene transcription (Schubiger and Truman 2000; Schubiger *et al.* 2005; Brown *et al.* 2006). Secondly, by binding to EcR/Usp, ecdysone activates the transcription of target genes (Cherbas 2003; Hu *et al.* 2003). To determine the effects of supressing ecdysone signalling on ovariole number, we used *traffic jam*-GAL4 to overexpress a dominant negative EcR transgene, UAS-*EcRA.W650A* (*tj > EcR-DN*). Because *EcRA.W650A* bears a mutation in the ligand-

binding domain, it cannot bind to ecdysone. Thus, even in the presence of ecdysone, *EcRA.W650A* continues to repress its target genes and does not induce activation (Cherbas *et al.* 2003; Hu *et al.* 2003; Brown *et al.* 2006). Most *tj* > *EcR-DN* animals died in pupal stages. The few *tj* > *EcR-DN* females that eclosed had ovaries in which most ovarioles were fused and malformed, suggesting an incomplete separation of individual ovarioles. Ovariole number was severely reduced in *tj* > *EcR-DN* adult females (Figure S6A). Additionally, *tj* > *EcR-DN* larvae showed a slight but significant acceleration in their onset of metamorphosis, and gave rise to pupae with smaller pharate weights when compared to controls (Figure S6B, C).

Role of IIS pathway during ovary development

We next explored how IIS affects each of the nutrition-sensitive processes that contribute to variation in ovariole number: the onset of TFC differentiation, the rate of TF addition, and the rate of increase in ovary volume. First, we analysed the effects of manipulating IIS in the developing ovary in larvae reared on standard food. To decrease IIS in the ovarian somatic cells, we used the *tj*-GAL4 driver to overexpress a negative regulator of IIS, UAS-*PTEN* (*tj* > *PTEN*). We increased IIS in these cells using *traffic jam*-GAL4 to drive the expression of UAS-*InR* (*tj* > *InR*).

Reducing IIS in the somatic cells of the ovaries resulted in a moderate delay in the onset of TFC differentiation when compared to controls at 15 h AL3E (Figure 4A, B). In contrast, activating IIS in the ovarian somatic cells did not affect the timing of TFC differentiation in fed larvae (Figure 4A-C). However, activating IIS in the ovaries in larvae fed on 20% sucrose from 5 h AL3E onwards was sufficient to induce premature onset of TFC differentiation with respect to controls (Figure 4F, G, H). Overall, these results suggest that IIS plays a role in regulating the timing of TFC differentiation.

When we analysed the effects of IIS on TF number, we found that reducing IIS in the ovaries caused a significant decrease in both TF number and the rate of TF addition (Figure 4D and Table S4) with respect to control ovaries (*tj*-GAL4). Conversely, increasing IIS in the ovary increased TF number, but did not affect the rate of TF addition with respect to controls (Figure 4D and Table S4). In control larvae fed on 20% sucrose before reaching critical weight, we failed to detect any TFCs even at 39

h AL3E in the majority of the ovaries analysed (Figure 4F- F''). In larvae with increased IIS in the ovarian somatic cells, we detected significant differences in TF number and the rate of TF addition even when fed on 20% sucrose before reaching critical weight (Figure 4H and Table S4). These data indicate that IIS regulates both TF number and the rate of TF addition.

In terms of the effects of IIS on ovary volume, either decreasing or increasing IIS in the ovarian somatic cells altered ovary volume in fed larvae, but had no effect on the rate of increase in ovary volume when compared to ovaries from control larvae (Figure 4E and Table S4). Further, at the time of transfer to 20% sucrose (5 h AL3E), increasing IIS in the somatic cells of the ovary resulted in larger ovary volumes than that of ovaries from control larvae (Figure S7A, B, E). Despite their initial difference in size, ovaries from *tj > InR* larvae fed on 20% sucrose food did not change in volume and their rates of increase were not significantly different from similarly-treated controls (Figure 4I and Table S4). Taken together, this suggests that IIS regulates ovary volume, but not the rate of increase in ovary volume between 15 and 39 h AL3E. However, because ovaries from *tj > InR* larvae are larger in size at 5 and 15 h AL3E, IIS is likely to control the rate of increase in ovary volume before larvae reach critical weight.

Role of ecdysone signalling during ovary development

Critical weight itself is regulated by a small nutrition-sensitive ecdysone peak that occurs at around 8-10 h AL3E (Mirth *et al.* 2005; Warren *et al.* 2006; Koyama *et al.* 2014), around the same time that TFC differentiation begins. Moreover, both EcR and USP proteins are present in ovarian somatic cells during L3 larval stages (Hodin and Riddiford 1998) and ecdysone signalling has been previously shown to affect the timing of TFC differentiation and final ovariole number (Hodin and Riddiford 1998; Gancz *et al.* 2011). Thus, we reasoned that the peak of ecdysone at critical weight was likely to induce TFC differentiation, as well as potentially affect either TF number or ovary volume.

To test this hypothesis, we altered ecdysone signalling in the ovary, using the *traffic jam*-GAL4 line, in one of two ways: 1) we repressed ecdysone signalling using UAS-*EcRA.W650A* (*tj > EcR-DN*), or 2) we used an RNAi construct against *EcR*, UAS-

EcR-IR CA104 (*tj* > *EcR-IR*) to reduce both the repressive function of unliganded EcR/Usp and the activation function of this complex. The latter manipulation induces derepression while repressing ecdysone-mediated activation.

At 15 h AL3E, control ovaries (*tj*-GAL4) from larvae reared on standard food had TFCs and a few forming TFs (Figure 5A-A'', D). However, we only detected TFCs at 39 h AL3E when we suppressed ecdysone signalling in the ovaries of well-fed larvae (Figure 5B-B'', D). In well-fed conditions, knocking down EcR in the ovaries did not affect the timing of the onset of TFC differentiation (Figure 5A, C). But, when we knocked down EcR in the ovaries and fed these larvae on 20% sucrose food starting at 5 h AL3E, most ovaries already had differentiating TFCs at 15 h AL3E (Figure 5G, H). In control larvae fed on 20% sucrose, TFCs were not detected even at 39 h AL3E (Figure 5F-F'', H). This suggests that, like IIS, ecdysone signalling is important for regulating the timing of the onset of TFC differentiation.

When we examined the effects of ecdysone signalling on TF number, we found that suppressing ecdysone signalling in the ovaries of well-fed larvae severely reduced TF number and rate of TF addition (Figure 5D and Table S5). In contrast, in well-fed conditions, knocking down EcR in the ovaries did not result in significant changes in TF number or rate of TF addition when compared to controls (Figure 5D and Table S5). When fed on 20% sucrose, knocking down EcR in the ovaries resulted in increased TF number and rate of TF addition (Figure 5H and Table S5). Taken together, these data show that ecdysone signalling plays a role in determining both TF number and the rate of TF addition.

We also found that both ovary volume and the rate of increase in ovary volume was significantly reduced in ovaries in which ecdysone signalling was suppressed using a dominant negative EcR (Figure 5E and Table S5), suggesting that ecdysone is likely to be required to promote ovary growth. Although ovary volume was significantly reduced, the rate of increase in ovary volume was indistinguishable between *tj* > *EcR-IR* ovaries and controls from larvae fed either on standard food (Figure 5E and Table S5) or 20% sucrose food (Figure 5I and Table S5).

Although knocking down *EcR* in the larval ovaries induces the derepression, thereby inducing part of ecdysone function, it also suppresses the activation function of ecdysone (Schubiger and Truman 2000; Cherbas 2003; Schubiger *et al.* 2005; Brown *et al.* 2006). To investigate the full role of ecdysone signalling in regulating ovariole number plasticity, we fed wild-type, outbred larvae from 5 h to 29 h AL3E on either

standard food or 20% sucrose food supplemented with 0.15 mg/mL of the active ecdysone metabolite 20-hydroxyecdysone (20E). Control food was either standard food or 20% sucrose food supplemented with the same volume of ethanol. Adding 20E to the standard food had no effect on TF number (Figure 6A, C, E). However, larvae fed on 20E-supplemented 20% sucrose food initiated TFC differentiation earlier and had significantly more TFs at 29 h AL3E than larvae fed on 20% sucrose food plus solvent (ethanol) (Figure 6B, D, E). In addition, ovary volume significantly increased in larvae fed on both standard and 20% sucrose foods containing 20E relative to ethanol controls (Figure 6F). This experiment confirms that ecdysone is sufficient to induce TFC differentiation when pre-critical weight larvae are fed on 20% sucrose food. Because TFC differentiaton is precociously induced in sucrose-fed larvae both when knocking down EcR in the ovary and when feeding 20E, ecdysone likely regulates the onset of TFC differentiation via derepression. Finally, these data also demonstrate that ecdysone regulates the rate of increase in ovary volume, presumably through its activation function, even in starved conditions.

The interplay between IIS and ecdysone signalling pathways

Our results show that IIS and ecdysone overlap in regulating some, but not all, of the developmental processes that regulate final ovariole number. Whereas both IIS and ecdysone signalling are important for regulating the onset of TFC differentiation, TF number and rate of TF addition, and ovary volume, IIS appears only to regulate the rate of increase in ovary volume before critical weight while ecdysone signalling regulates its rate of increase throughout development. We next sought to understand how the interaction between these two signalling pathways might result in differences in the ovary's sensitivity to nutrition between pre- and post-critical weight larvae.

To understand how these two pathways interact to regulate each developmental process, we manipulated both pathways in combination in the developing ovary using *traffic jam*-GAL4. We downregulated IIS using UAS-*PTEN* and upregulated IIS using UAS-*InR*. For ecdysone signalling, we supressed ecdysone signalling using UAS-*EcR-DN* and induced the derepression function of ecdysone signalling using UAS-*EcR-IR*. We did all pairwise combinations of manipulations, and assessed the effects on the

timing of the onset of TFC differentiation, on TF number and rate of addition, and on ovary volume and rate of increase.

Suppressing ecdysone signalling in ovaries of well-fed larvae always resulted in delays in the onset of TFC differentiation, regardless of whether IIS was downregulated or upregulated (Figure 7A-C, A'-C'). In contrast, knocking down EcR while upregulating IIS resulted in precocious TFC differentiation, with TFCs appearing as early as 5 h AL3E (Figure S8A). This onset of TFC differentiation was not only earlier than that of control larvae, it was also significantly earlier than the onset of TFC differentiation in *tj > InR* and *tj > EcR-IR* ovaries (Figure S8B, C; $p<0.0001$, $\chi^2 = 45$, df = 3, Chi-Square Test). In contrast, we did not notice any effects of knocking down EcR while downregulating IIS in the ovary on the timing of the onset of TFC differentiation (Figure 7A, E, A', E'). These data suggest that ecdysone signalling acts primarily downstream of IIS in regulating the onset of TFC differentiation.

Because we upregulated and downregulated the activity of both pathways in the ovarian somatic tissue, and quantified the effects of this manipulation over time, we can explore the relative contribution of each in determining TF number. Variation in larval age, ecdysone signalling, IIS, and the interaction between ecdysone signalling and IIS explain 78% of the total observed variation in TF number. We next calculated the relative contribution of each parameter to the total R^2, bootstrapping their 95% confidence intervals, to estimate the relative importance of each on TF number. The 95% confidence interval for larval age, ecdysone signalling, IIS, and the interaction between both pathways showed that they contributed to 33-54%, 30-49%, 8-21%, and 1-6% of the total R^2 respectively. Thus, ecdysone signalling appears to contribute more to variation in TF number than IIS.

The analysis above provides an indication of how much each variable contributes to total TF number. We next assessed whether this explained variation was due to TF number or rates of addition. Simultaneously repressing both ecdysone signalling and IIS resulted in the lowest TF numbers and lowest rates of addition, with very few TFs forming between 15 and 39 h AL3E (Figure 7F and Table S6). Repressing ecdysone signalling while upregulating IIS increased both TF number and addition rate in comparison to ovaries in which both pathways were repressed (Figure 7F and Table S6). The reverse manipulation, knocking down EcR while downregulating IIS in the ovary increased TF number and addition rate relative to the previous two treatments, although these values were still lower than control. The highest rates of TF addition

were found in control ovaries and in ovaries where both pathways were upregulated, although these were not significantly distinguishable from each other (Figure 7F and Table S6). Finally, knocking down EcR and upregulating IIS resulted in ovaries with the highest TF number (Figure 7F and Table S6). Taken together, these data suggest that both pathways contribute to TF number and addition rate, even though they differ in their relative contributions to variation in TF number.

Similarly, we used linear models to explore the relative importance of larval age, ecdysone signalling, IIS and the interaction between the two pathways on ovary volume. Variation in all four variables explains 94% of the observed variance in ovary volume. IIS contributed the greatest proportion of this variance (54-64%, and see Table S4, S6, S7). Larval age, ecdysone signalling, and the interaction between the two pathways contributed to explaining 28-39%, 3-11%, and 0.3-2% of the total R^2 respectively.

Ovary volume in fed conditions was significantly different between all genotypes, with the smallest ovary volumes resulting from reducing the signalling activity of both pathways and the largest ovary volumes generated by increasing both ecdysone signalling and IIS (Figure 7G and Table S6). We only observed a difference in the rate of increase in ovary volume when both ecdysone signalling and IIS were simultaneously reduced in the ovary (Figure 7G and Table S6). Upregulating IIS while downregulating ecdysone signalling in the ovaries of well-fed larvae rescued the ovary volume to values higher than control larvae, and restored the rate of ovary volume to levels indistinguishable from the controls (Figure 7G and Table S6). On the other hand, knocking down EcR while downregulating IIS resulted in ovary volumes smaller than controls, but with the same rate of increase. Thus, it appears IIS plays a primary role in determining ovary volume and regulating rate of increase in ovary volume before 15 h AL3E. Ecdysone signalling regulates the rate of increase in ovary volume after 15 h AL3E, however increasing IIS can compensate for reduced ecdysone signalling.

Activating both IIS and ecdysone signalling pathways in ovarian somatic cells of well-fed larvae induced an earlier onset of TFC differentiation (Figure S7A-D), and promoted a greater increase in TF number than all previous genetic manipulations in well-fed larvae (Figure 7F and Table S6). This led us to hypothesize that activating both signalling pathways may overcome most of the effects of poor nutrition. When we activated both pathways in the ovarian somatic cells (*tj* > *EcR-IR, InR*) and fed these larvae 20% sucrose food between 5 and 15 h AL3E, TF number and rate of addition was

728 significantly higher than that of control ovaries (Figure 8D and Table S7). When we
729 knocked down EcR while supressing IIS in the ovarian somatic cells and fed these
730 larvae on 20% sucrose, we observed a slight delay in the onset of TFC differentiation.
731 We did not observe any TFCs in these larvae at 15 h AL3E (Figure 8C), although some
732 TFCs were detected at 29 and 39 h AL3E in half of the ovaries analysed (Figure 8C',
733 C'', D). In addition, TF number and the rate of TF addition were suppressed to the same
734 level as control larvae fed on 20% sucrose (Figure 8D and Table S7). This indicates that
735 increasing both signalling pathways in the ovarian somatic cells can overcome some of
736 the effects of poor nutrition on TF number. Nevertheless, even if ecdysone signalling is
737 sufficient to induce precocious TFC differentiation in larvae fed on 20% sucrose, the
738 rate of TF addition only increases when IIS is sufficiently high.
739 Knocking down EcR while increasing IIS in the ovaries resulted in larger ovary
740 volumes at 5 h AL3E when compared to controls (Figure S8A, D, E). However, we did
741 not observe any further increase in ovary volume after transferring these larvae to 20%
742 sucrose (Figure 8E and Table S7). In contrast, knocking down EcR while suppressing
743 IIS in the ovarian somatic tissue resulted in dramatic reductions in ovary volume at 5 h
744 AL3E (Figure 8E and Table S7). Interestingly, after transferring these larvae to 20%
745 sucrose their ovaries showed a significant decrease in volume compared to similarly-
746 treated controls. Together, these results corroborate our previous experiment
747 demonstrating that IIS is the primary determinant of ovary volume, but also show that
748 increasing IIS and ecdysone signalling in the ovary cannot counteract the effects of poor
749 nutrition.
750

751 # Discussion

752

753 Environmental conditions can direct the development of organs along distinct
754 trajectories for growth and differentiation, a phenomenon known as developmental
755 plasticity. The sensitivity to these conditions typically changes with developmental
756 time, with some stages showing higher sensitivity than others. Here we explored the
757 stage-specific mechanisms controlling nutritional plasticity in ovariole number as a
758 method to address the physiological underpinnings that cause organs to alter their
759 sensitivity throughout their development.

Previous studies of the developmental effects of nutrition on ovariole number had shown that diluting the food on which larvae were raised altered ovariole number by changing the total number of TFCs (Sarikaya *et al.* 2012) or the rate of TF addition in late L3 larvae (Hodin and Riddiford 2000). Yet, it remained unclear whether the developing ovaries changed their sensitivity to nutrition with developmental time. In addition, several authors reported that both IIS and ecdysone signalling pathways regulate ovariole number by controlling different developmental processes; while IIS primarily regulates ovary size (Green and Extavour 2012; Gancz and Gilboa 2013), ecdysone signalling is required to induce the onset of TFC differentiation (Hodin and Riddiford 2000; Gancz *et al.* 2011). Nonetheless, these studies did not address whether the phenotypes induced by manipulating IIS and/or ecdysone signalling phenocopied a nutrition-dependent developmental response, whether the ovary showed phases of sensitivity for nutrition, or how these pathways controlled the rates of developmental processes.

In this study, we identified two phases of sensitivity in the developing ovary, separated by the developmental checkpoint known as critical weight. Pre-critical weight larvae reared under poor nutritional conditions show severe reductions in ovariole number. Once critical weight has been reached, larvae show a more moderate reduction in ovariole number in response to changes in nutrition. These differences in sensitivity to nutrition result from differences in the developmental processes that occur during the two developmental stages: the onset of TFC differentiation, the rate of TF formation, and the rate of increase in ovary volume.

The onset of TFC differentiation begins approximately 10-15 h AL3E (Godt and Laski 1995) around the time of critical weight (Shingleton *et al.* 2005; Mirth *et al.* 2005, 2009; Koyama *et al.* 2014). A small peak of ecdysone induces the developmental transition at critical weight (Mirth *et al.* 2005; Warren *et al.* 2006; Koyama *et al.* 2014). In the wing imaginal discs, this peak switches patterning from the nutrition-sensitive pre-critical weight phase to a nutrition-insensitive phase of development (Mirth *et al.* 2009). This led us to hypothesize that the peak of ecdysone that induces critical weight might also initiate the onset of TFC differentiation.

We found that the onset of TFC differentiation is highly sensitive to nutrition in pre-critical weight larvae; ovaries from pre-critical weight larvae fed on sucrose alone showed strong delays in the onset of TFC differentiation. Similar to patterning in the wing discs (Mirth *et al.* 2009), we found that the timing of the onset of TFC

794 differentiation was regulated by ecdysone signalling. These data support our hypothesis
795 that the nutrition-sensitive peak of ecdysone at critical weight acts to induce the onset of
796 TFC differentiation.

797 Although previous studies suggested that ecdysone signalling, but not IIS,
798 regulated the timing of TFC differentiation (Gancz and Gilboa 2013), our data shows
799 that both pathways play a role. Suppressing either IIS and/or ecdysone signalling in the
800 developing ovary delayed the timing of the onset of TFC differentiation. The
801 discrepancy between these datasets is almost certainly due to differences in the temporal
802 resolution between the studies; previous studies did not employ the same rigorous
803 staging methods, causing them to miss the more subtle differences in developmental
804 timing.

805 IIS exerts its effects on the onset of TFC differentiation in an ecdysone-
806 dependent manner. Ovaries in which IIS was upregulated while ecdysone signalling was
807 repressed delayed the onset of TFC differentiation as much as ovaries in which only
808 ecdysone signalling was repressed. In addition, partially inducing ecdysone signalling in
809 ovarian somatic cells, by knocking down EcR, can overcome the defects in the onset of
810 TFC differentiation arising from inhibiting IIS. Nevertheless, the two pathways
811 appeared to interact; upregulating both pathways in the ovary resulted in earlier onset of
812 TFC differentiation than upregulating either pathway on its own. Potentially, these data
813 could indicate that nutrition, via IIS, modifies the sensitivity of the ovary to ecdysone
814 signalling. Under high levels of IIS, the ovary may require lower levels of ecdysone
815 signalling to induce the onset of TFC differentiation, resulting in earlier onset.
816 Additional studies are required to fully understand the nature of the link between IIS
817 and ecdysone signalling in this developmental process.

818 Poorly fed pre-critical weight larvae show changes both in TF number and the
819 rate of TF addition, whereas similar treatment of post-critical weight larvae only
820 affected their TF number. This suggests two things about the regulation of TF addition
821 rate. First, the timing of TFC differentiation determines most of the variation in the rate
822 of TF addition. Second, although changes in nutrition during post-critical weight phase
823 did not alter the rate of TF addition, the total number of TFs was significantly reduced
824 relative to standard food controls. This means that TF addition rate must be transiently
825 delayed upon transfer to 20% sucrose, before recovering to normal rates. Thus, the
826 effect of poor nutrition on TF addition rate switches from continuous to transient
827 suppression at critical weight.

This change in the regulation of TF addition rate is most likely due to the relative effects of ecdysone signalling and IIS on this process. Supressing either ecdysone signalling and/or IIS delayed the onset of TFC differentiation and reduced both TF number and addition rate. Ecdysone signalling appears to contribute more to determining TF number because it has a stronger effect on the timing of the onset of TFC differentiation than IIS.

Although TF number was more affected in the ovaries of poorly fed pre-critical weight larvae, our results show that events occurring post-critical weight are also important. When we knocked down EcR, but suppressed IIS, in the ovarian somatic cells and fed these larvae on 20% sucrose, TFCs began differentiating, but TFs failed to form over the time period sampled. This could occur if IIS either controlled the available pool of TFC precursors that differentiate by regulating ovary volume, or mediated the intercalation of TFCs into TFs. Our knowledge of when and how the precursors of TFCs are produced, and the processes that lead to TFC intercalation into TFs, have thus far been limited (Sahut-Barnola *et al.* 1996; Lengil *et al.* 2015). Future work on identifying additional TFC markers may help us understand whether nutrition affects proliferation of TFC precursors and how this may influence the rate of TF addition and TF number.

Taken together, our results indicate that whereas ecdysone signalling contributes more to determining TF number, this appears to be due to its effects in the timing of the onset of TFC differentiation in the pre-critical weight phase. On the other hand, IIS is likely to be the principal regulator of TF number during the post-critical weight phase. We propose that the change in sensitivity to nutrition that occurs after critical weight occurs partly because of this change in the regulation of TF number, with ecdysone signalling playing the primary role before critical weight, and IIS contributing after this developmental transition.

The effects of nutrition on ovary volume also changed between pre- and post-critical weight larvae. In this case, nutrition affected ovary volume and the rate of increase in ovary volume in larvae of both stages. In poor nutritional conditions, ovaries from pre-critical weight larvae do not show any additional increase in ovary volume. However, once critical weight is reached, poor nutrition significantly reduces but does not preclude the rate of increase in ovary volume. Critical weight regulates the nutrition-sensitive growth of several other tissues in a similar manner as the ovaries (Shingleton *et al.* 2008; Mirth *et al.* 2009; Lanet *et al.* 2013). Starving larvae before they reach

862 critical weight arrests growth of the wing discs. Once larvae surpass critical weight, the
863 progression of growth continues under starvation conditions, albeit at a reduced rate
864 (Shingleton *et al.* 2008; Mirth *et al.* 2009).

865 Variation in IIS signalling explained the greatest proportion of the variation in
866 ovary volume. Interestingly, most of these effects appeared to be due to the effects of
867 IIS in regulating ovary volume in pre-critical weight stages. While IIS did not
868 contribute to regulating the rate of increase in ovary volume in well-fed, post-critical
869 weight larvae, increasing IIS in the ovary led to larger ovary volumes in pre-critical
870 weight larvae. This suggests that IIS regulates the rate of increase in ovary volume
871 before the critical weight transition. It is worth noting that this need not be limited to
872 changes in the rate of increase in the third instar, but could also affect rates of increase
873 in ovary volume in the first and second instar.

874 Despite this, activation of IIS failed to promote further increases in ovary
875 volume in larvae fed on sucrose alone. A second nutrient-sensitive pathway, the target
876 of rapamycin (TOR) pathway, responds directly to intracellular concentrations of amino
877 acids to promote growth (Gao *et al.* 2002). Inactivating components of the TOR
878 signalling pathway leads to a reduction in ovary size (Gancz and Gilboa 2013) and thus,
879 its activation might be sufficient to induce an increase in ovary volume in larvae fed on
880 20% sucrose food. This differs from growth in polyploidy tissues. In early larval stages
881 before the attainment of critical weight, activation of either IIS or TOR signalling
882 bypasses the requirement of dietary protein for growth in larval polyploid tissues
883 (Britton and Edgar 1998; Britton *et al.* 2002; Saucedo *et al.* 2003).

884 Ecdysone signalling also played a clear role in regulating ovary volume.
885 Manipulating ecdysone signalling in the ovarian somatic cells altered the rate of
886 increase in ovary volume in well-fed, post-critical weight larvae. In addition, feeding
887 larvae 20E was the only treatment that increased ovary volume in 20% sucrose food
888 conditions, although it was insufficient to restore ovary volume to fed conditions.
889 Because both the control and 20E fed larvae were from the same cohort of wild-type,
890 outbred flies, ovary volumes were almost certainly indistinguishable between treatments
891 at the beginning of the experiment. This means that changes in ovary volume over the
892 24 h time period are necessarily due to changes in the rate of increase in ovary volume.
893 Taken together, our data show that ecdysone contributes to regulating the rate of
894 increase in ovary volume principally in the post-critical weight phase. We propose that
895 the change in the sensitivity of ovary volume across development stages results from

changes in regulation of its rate of increase. While IIS signalling regulates the rate of increase in ovary volume during the more sensitive pre-critical weight stage, ecdysone signalling regulates this process after critical weight.

The effects of ecdysone signalling and IIS in ovary volume parallel those found in the wing imaginal discs of other insects. In both *M. sexta* and the butterfly *Precis coenia,* IIS and ecdysone signalling act synergistically to promote wing disc growth in culture (Nijhout and Grunert 2002; Nijhout *et al.* 2007). More recent studies have shown that ecdysone regulates growth in the wing disc of *D. melanogaster* by controlling the expression of a component of IIS, *Thor/4E-BP* (Herboso *et al.* 2015). Whether this interaction between pathways contributes to the synergistic effects on wing disc growth observed in other studies remains unclear. Further, we require further dedicated studies to understand the nature of the molecular interactions between ecdysone signalling and IIS in regulating ovary volume.

In broader terms, our work has highlighted a previously unappreciated mechanism underlying change in sensitivity to nutrition with developmental stage. In the regulation of both TF number and ovary volume, the signalling pathway that contributed the most to variation in the trait acted primarily in the earlier, more sensitive pre-critical weight stage. With the critical weight transition, both TF number and ovary volume came under the regulation of the pathway that contributed less to their variation. Previous studies on differences in plasticity between organs have shown that traits that show reduced plasticity in response to nutrition, like male genital size in *D. melanogaster* (Tang *et al.* 2011), and traits that responded more plastically to changes in nutrition, like the size of male horns in rhinocerous beetles (Emlen *et al.* 2012), do so by altering the level of signalling of a single pathway, the IIS pathway. Our data highlight the possibility that the mechanisms that regulate changes in plasticity with developmental time within an organ might differ fundamentally from those that regulate differences in plasticity between organs. Further dedicated experiments are required to determine if this is true for other traits.

Conclusions

In summary, our findings underscore the importance of hormonal pathways in coordinating stage-specific developmental processes with environmental conditions,

and specifically suggest that changes in the hormonal pathways that regulate trait development may induce differences in plastic responses with developmental stage. The powerful developmental approach employed here will lend insight into how developmental processes respond to environmental variation for other traits and other organisms.

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Competing interests

We declare that none of the authors in this manuscript have any competing interests that could interfere with our presentation or interpretation of the data.

Author's Contributions

Both authors contributed to conceiving the project, designing the experiments, analysing the data, and writing and revising the manuscript. CCM performed all the experimental work under the supervision of CKM.

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Figure Legends

Figure 1. Changes in nutrition during the first phase of sensitivity have greater effects on ovariole number than in the second phase of sensitivity. (A) Adult ovariole number from larvae from an outbred line transferred either to standard food (yellow circles) or to 20% sucrose food (blue circles). Dashed lines show the best fit lines from the segmental regression analyses. The red arrow denotes change in slope around a single breakpoint. Critical weight (CW) is attained around 10 h AL3E (red line). (B) Adult ovariole number from larvae fed on standard food (yellow circles); larvae transferred to 20% sucrose food either at 5 h AL3E (light blue circles) or at 20 h AL3E (dark blue circles) and larvae fed on 20% sucrose food for a 20 h interval either between 0 h to 20 h AL3E (open blue circles) or between 20 h to 40 h AL3E (open dark circles). Plotted values represent means and error bars show 95% confidence intervals of means. ANOVAs followed by Tukey's HSD test: *$p<0.05$, ***$p<0.001$, ns non-significant. L3: third instar larvae; AL3E: after L3 ecdysis.

Figure 2. Distinct stage-specific developmental processes during ovary development are regulated by nutrition. (A-L) shows terminal filaments (TFs) marked with Engrailed immunostaining in ovaries from outbred larvae fed on standard

food (A, C, E, G, I, K) or 20% sucrose food (B, D, F, G, H, I) for 24 h starting between 0 h to 25 h AL3E. Scale bar: 20µm. (N) Number of forming terminal filaments (TFs) of ovaries from larvae fed on standard food (yellow circles) or 20% sucrose food (blue circles). (M) Ovary volume of ovaries from larvae fed on standard food (yellow points) or 20% sucrose food (blue points). Plotted values represent means and error bars show 95% confidence intervals of means. In some cases, error bars are too small to be seen. ANOVAs followed by Tukey's HSD test:*p<0.05, **p<0.01, ***p<0.001. L3: third instar larvae; AL3E: after L3 ecdysis.

Figure 3. TF number and ovary volume respond differently to pre- and post-critical weight nutrition. (A-C') shows terminal filaments (TFs) marked with En immunostaining. (A-A') Ovaries from outbred larvae reared on standard food. (B-C') Ovaries from outbred larvae transferred to 20% sucrose food from: (B-B') 5 h or (C-C'') 15 h AL3E. Larvae dissected at (A, B, C) 29 h or (A´, B', C') 49 h AL3E. Scale bar: 20µm. (D) Number of forming terminal filaments (TFs) and (E) ovary volume of ovaries from larvae fed on standard food (yellow circles); larvae were transferred to 20% sucrose food either at 5 h AL3E (light blue circles) or at 15 h AL3E (dark blue points). In (D, E), regression lines and 95% confidence intervals of means are shown. ANCOVAs: values that do not share the same letter (slopes) or number (means) are significantly different (Holm's correction; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis.

Figure 4. Role of IIS during ovary development. (A-G'') shows terminal filaments (TFs) marked with En immunostaining. Ovaries from larvae reared on standard food: (A-A'', F) *tj*-GAL4 (control), (B-B'') *tj* > *PTEN* and (C-C'', G) *tj* > *InR*. Larvae were dissected at (A, B, C) 15 h, (A', B', C') 29 h or (A'', B'', C'') 39 h AL3E. (D) Number of forming TFs and (E) ovary volume of ovaries from *tj*-GAL4 larvae (black points), *tj* > *PTEN* larvae (blue points) and *tj* > *InR* larvae (red points) fed on standard food. Ovaries from larvae transferred to 20% sucrose food at 5 h AL3E: (F-F'') *tj*-GAL4 (control) and *tj* > *InR*. (G-G''). Larvae were dissected at (F, G) 15 h, (F', G') 29 h or (F'', G'') 39 h AL3E. (H) Number of forming TFs and (I) ovary volume of ovaries from *tj*-GAL4 control larvae (open black points) and *tj* > *InR* larvae (open red points) fed on 20% sucrose food. In (H, I), full points represent ovaries from larvae fed on standard food at 5 h AL3E. In (D, E, H, I), data was $\log_{10}$-transformed and regression lines and

95% confidence intervals of means are shown. ANCOVAs: values that do not share the same letter (slopes) or number (means) are significantly different (Holm's correction; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis. Scale bar: 20µm.

Figure 5: Role of ecdysone signalling during ovary development. (A-G'') shows terminal filaments (TFs) marked with En immunostaining. Ovaries from larvae reared on standard food: (A-A'') *tj*-GAL4 (control), (B-B'') *tj* > *EcR-DN* and (C-C'') *tj* > *EcR-IR*. Larvae were dissected at (A, B, C) 15 h, (A', B', C') 29 h or (A'', B'', C'') 39 h AL3E. (D) Number of forming TFs and (E) ovary volume of ovaries from *tj*-GAL4 larvae (black points), *tj* > *EcR-DN* larvae (blue points) and *tj* > *EcR-IR* larvae (red points) fed on standard food. Ovaries from larvae transferred to 20% sucrose food at 5 h AL3E: (F'-F'') *tj*-GAL4 (control) and *tj* > *EcR-IR*. (F'-F''). Larvae were dissected at (F, G) 15 h, (F', G') 29 h or (F'', G'') 39 h AL3E. (H) Number of forming TFs and (I) ovary volume of ovaries from *tj*-GAL4 control larvae (open black points) and *tj* > *EcR-IR* larvae (open red points) fed on 20% sucrose food. In (H, I), full points represent ovaries from larvae fed on standard food at 5 h AL3E. In (D, E, H, I), data was $\log_{10}$-transformed and regression lines and 95% confidence intervals of means are shown. ANCOVAs: values that do not share the same letter (slopes) or number (means) are significantly different (Holm's correction; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis. Scale bar: 20µm.

Figure 6. Feeding wild-type larvae with 20E-supplemented 20% sucrose food increases TF number and ovary volume. (A-D) shows terminal filaments (TFs) marked with En immunostaining. Ovaries from outbred larvae reared on standard food: (A) plus ethanol (control) or (C) plus 20E (+20E). Ovaries from larvae reared on 20% sucrose food: (B) plus ethanol (control) or (D) plus 20E (+20E). Larvae were dissected at 29 h AL3E. Scale bar: 20µm. (E) Number of forming TFs and (F) ovary volume of ovaries from larvae fed either on standard food plus ethanol (control) or on 20E-supplemented standard food (+20E) (yellow points) and larvae fed either on sucrose alone plus ethanol (control) or on 20E-supplemented 20% sucrose food (+20E) (blue points). Error bars show 95% confidence intervals of means. Welch Two sample t-test: *p<0.1, ***p<0.001, ns non-significant.

Figure 7. The complex interaction between IIS and ecdysone signalling pathways in well-fed larvae. (A-E') shows terminal filaments (TFs) marked with En immunostaining. Ovaries from larvae reared on standard food: (A-A'') *tj*-GAL4 (control), (B-B'') *tj* > *EcR-DN, InR*, (C-C'') *tj* > *EcR-DN, PTEN*, (D-D'') *tj* > *EcR-IR, InR* and (E-E'') *tj* > *EcR-IR, PTEN*. Larvae were dissected at (A, B, C, D, E) 15 h and (A', B', C', E') 39 h AL3E. Scale bar: 20μm. (D) Number of forming TFs and (E) ovary volume of ovaries from *tj*-GAL4 control larvae (black points), *tj* > *EcR-DN, InR* larvae (dark blue points), *tj* > *EcR-DN, PTEN* larvae (light blue points), *tj* > *EcR-IR, InR* larvae (red points) and *tj* > *EcR-IR, PTEN* larvae (pink points). In (D, E, H, I), data was $\log_{10}$-transformed and regression lines and 95% confidence intervals of means are shown. ANCOVAs: values that do not share the same letter (slopes) or number (means) are significantly different (Holm's correction; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis.

Figure 8. Simultaneously activating both IIS and ecdysone signalling in 20% sucrose food promotes precocious onset of TFC differentiation, increases the rate of TF addition, but not of increase in ovary volume. (A-C') shows terminal filaments (TFs) marked with En immunostaining. Ovaries from larvae transferred to 20% sucrose food at 5 h AL3E: (A-A'') *tj*-GAL4 (control), (B-B'') *tj* > *EcR-IR, InR* and (C, C'') *tj* > *EcR-DN, PTEN*. Larvae were dissected at (A, B, C) 15 h, (A', B', C') 29 h or (A'', B'', C'') 39 h AL3E. Scale bar: 20μm. (D) Number of forming TFs and (E) ovary volume of ovaries from *tj*-GAL4 control larvae (open black points), *tj* > *EcR-IR, InR* larvae (open red points) and *tj* > *EcR-DN, PTEN* (pink points). In (D, E), data was $\log_{10}$-transformed and full points represent ovaries from larvae fed on standard food at 5 h AL3E. Regression lines and 95% confidence intervals of means are shown. ANCOVAs: values that do not share the same letter (slopes) or number (means) are significantly different (Holm's correction; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis.

Figure 1

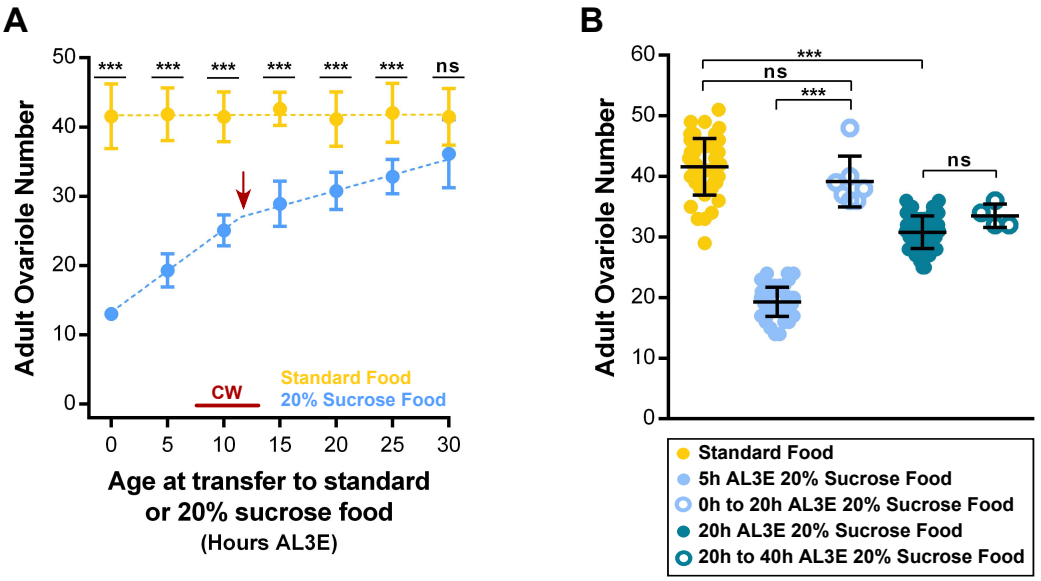

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Figure 2

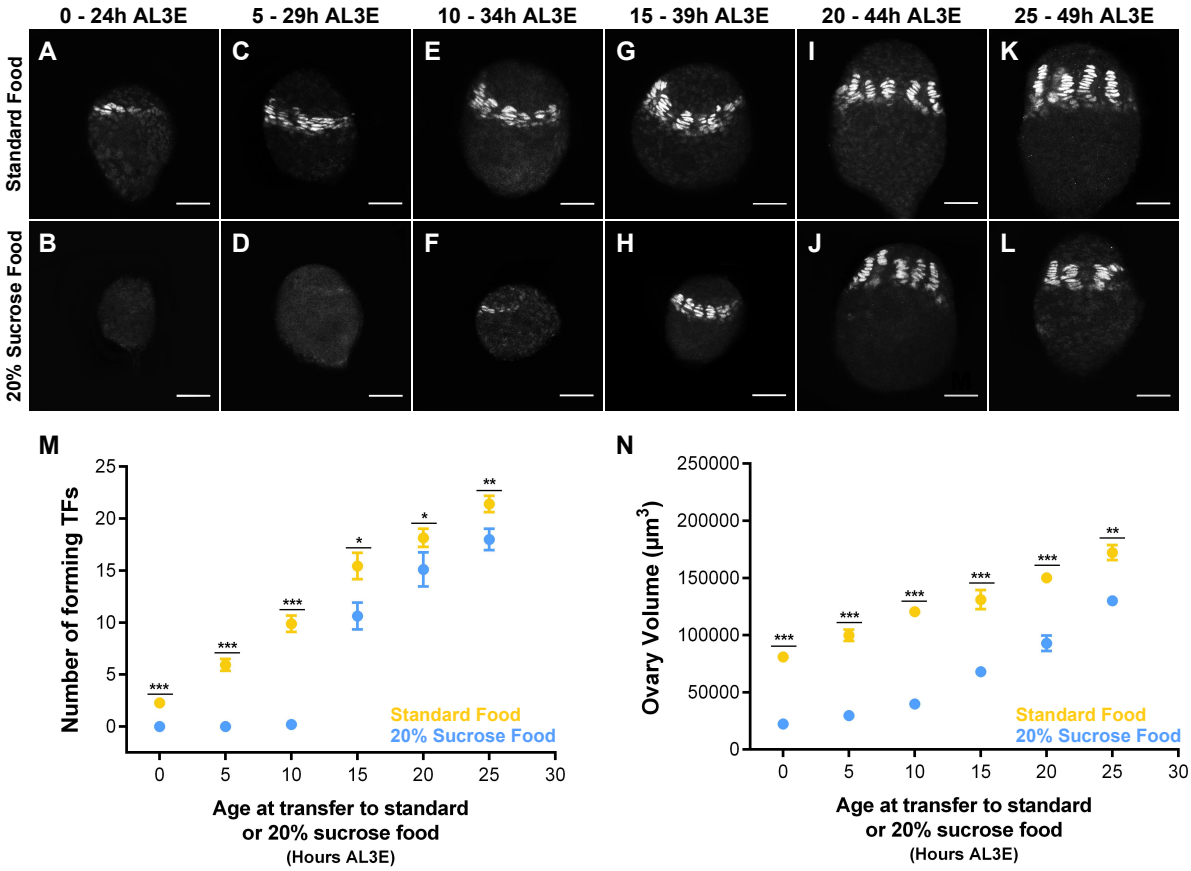

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Figure 3

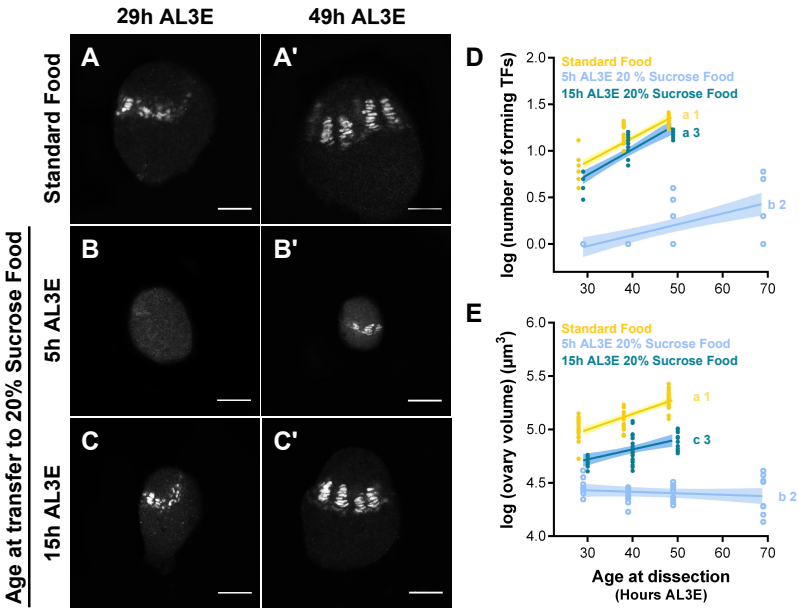

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Figure 4

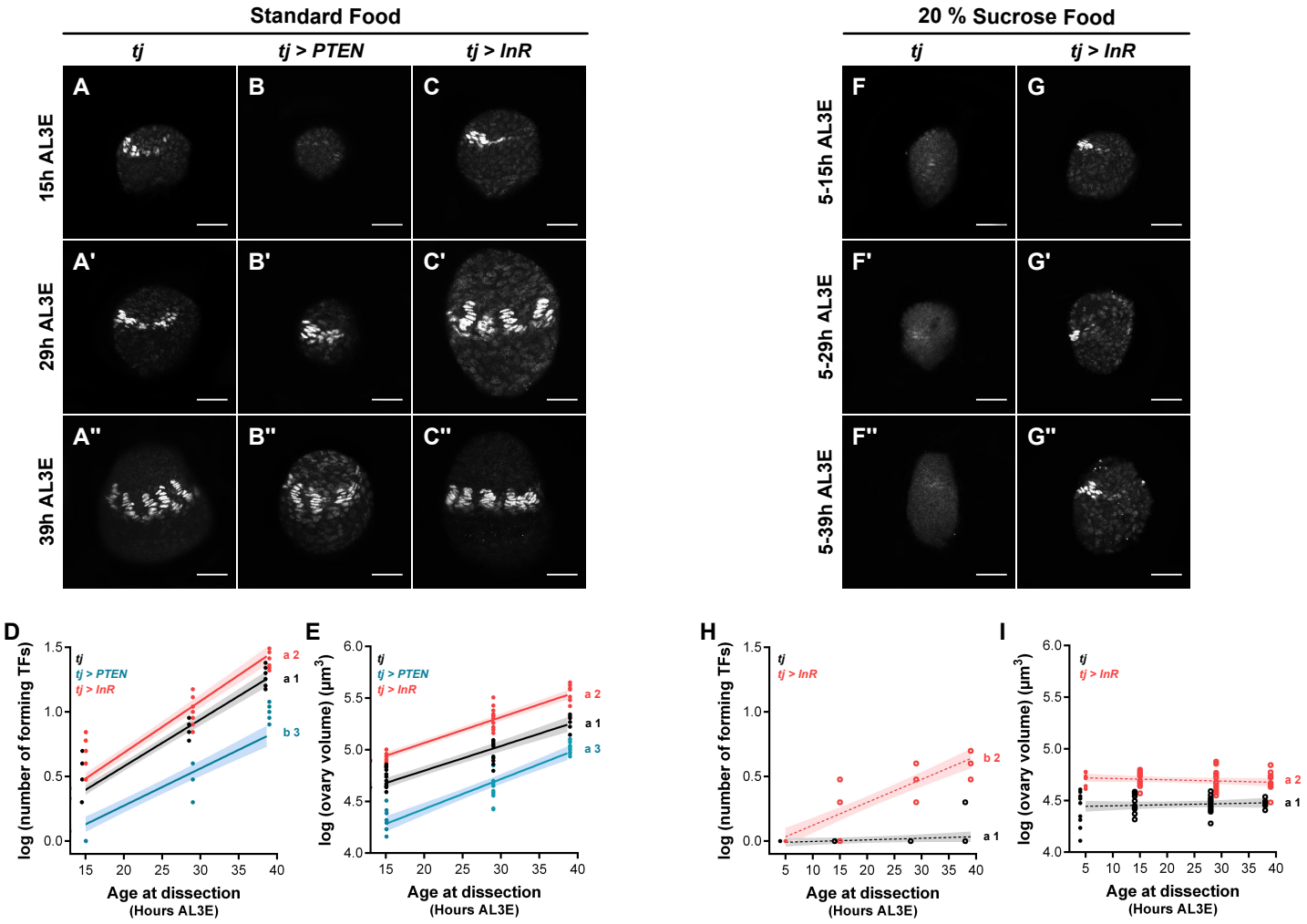

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Figure 5

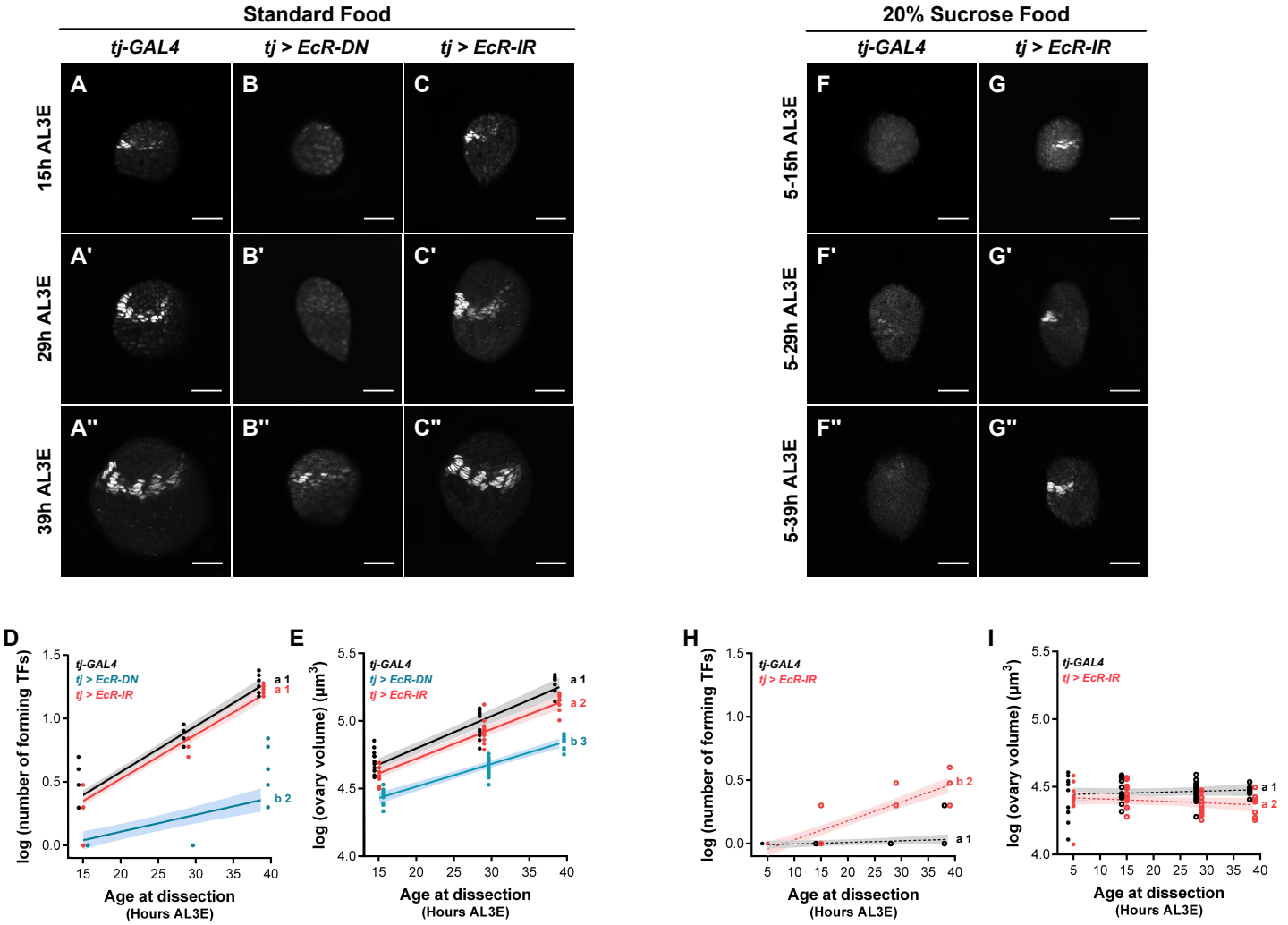

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Figure 6

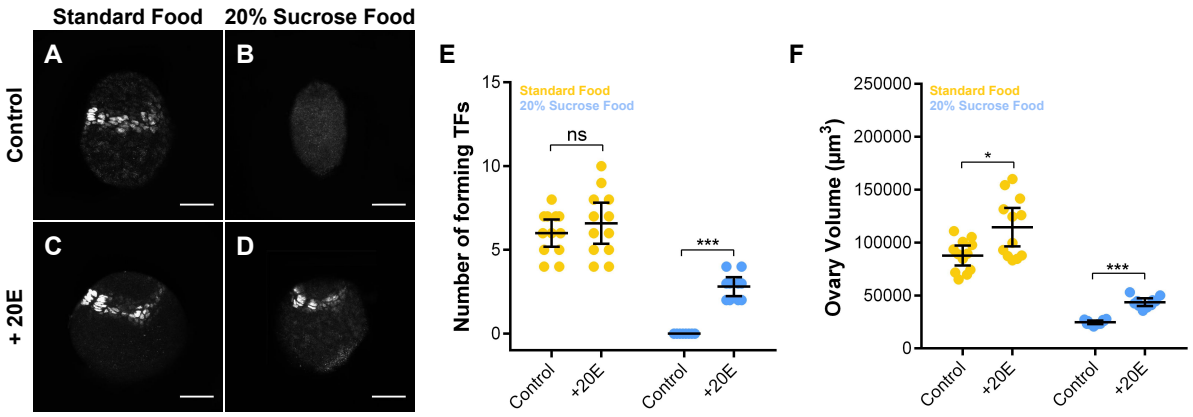

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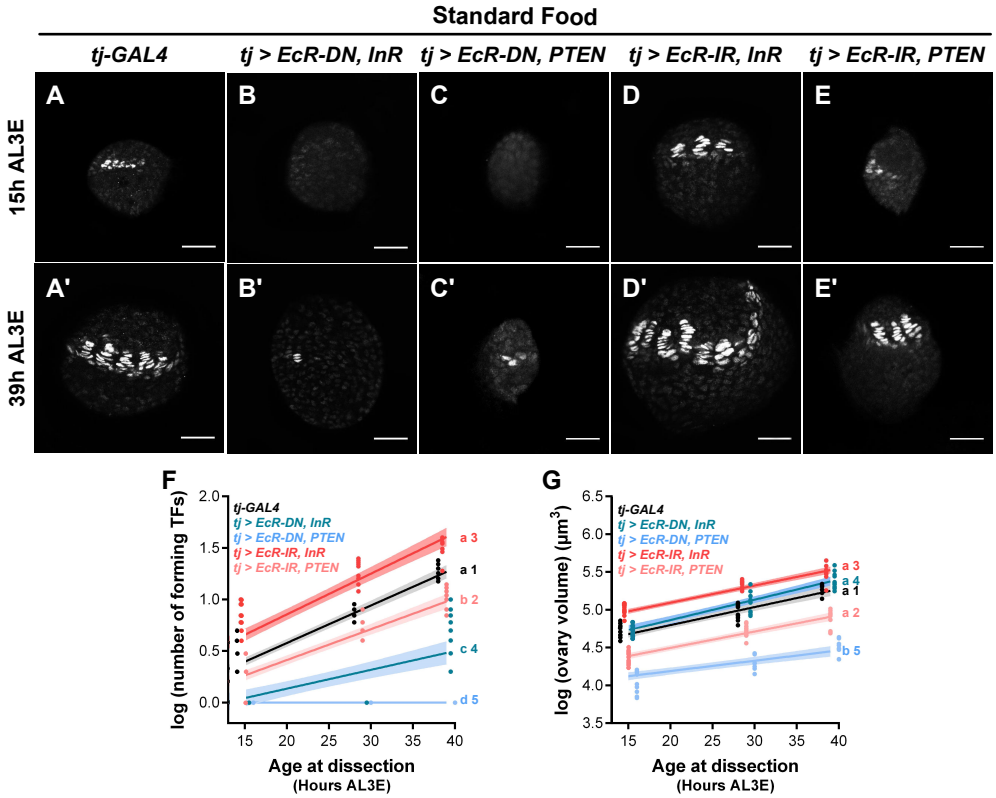
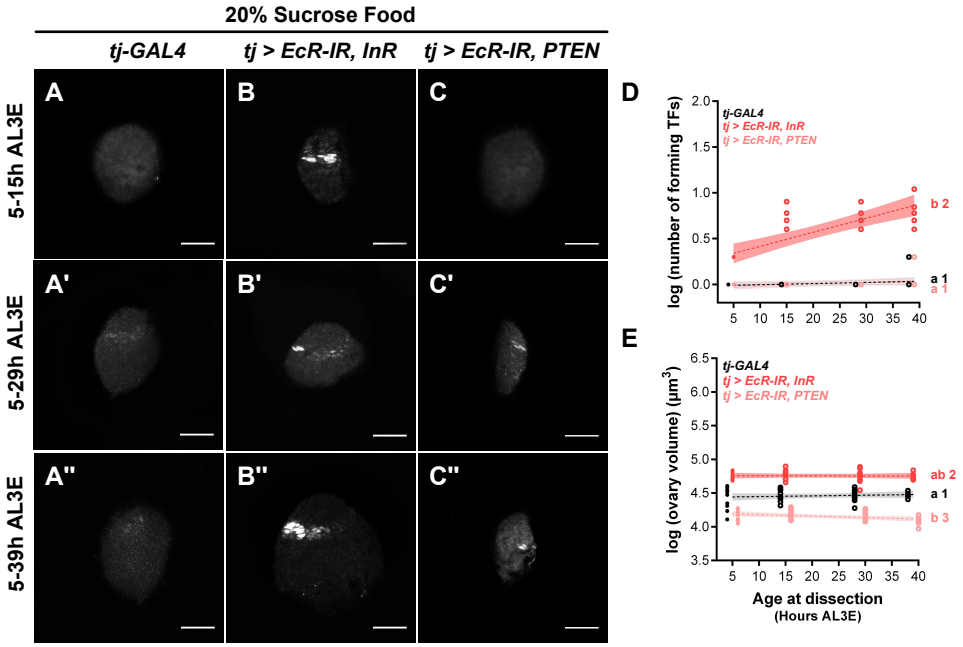
Figure 7



Mendes, C. & Mirth, C.

Figure 8



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