Stage-Specific Plasticity in Ovary Size Is Regulated by Insulin/Insulin-Like Growth Factor and Ecdysone Signaling 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 after. We find that this switch in sensitivity results from distinct developmental processes. In precritical 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 postcritical weight larvae, poor nutrition affects only the rate of increase in ovary volume. Our results further indicate that two hormonal pathways, the insulin/insulin-like growth factor and the ecdysone-signaling 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 terminal filament 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.

KEYWORDS critical period for environmental sensitivity; ecdysone signaling; insulin/insulin-like growth factor signaling; larval nutrition; ovary size

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 (Beldade *et al.* 2011; Gotoh *et al.* 2011, 2014; 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

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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 signaling (IIS) pathway. In a well-nourished animal, neurosecretory cells in the brain synthesize and secrete insulin-like peptides (Ikeya *et al.* 2002; Rulifson *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 suppressing IIS in the prothoracic glands, the glands that synthetize ecdysone, regulates the rate of ecdysone synthesis at critical weight (Caldwell *et al.* 2005; Colombani *et al.* 2005; Mirth *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 the EcR/Usp activation function, thereby suppressing all ecdysone signaling (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 knockdown 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). The fact that these manipulations result in opposing phenotypes 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 7–10 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-signaling 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-signaling 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 addresses 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 postcritical 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 signaling 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 *D. melanogaster* founded and maintained as described in Martins *et al.* (2013) and provided by Élio Sucena (Instituto Gulbenkian de Ciência). To genetically manipulate IIS and ecdysone signaling, 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 Lilach Gilboa (Weizmann Institute of Science, Rehovot, Israel). *Traffic jam*-GAL4 was crossed to w^{1118}, obtained from Lynn Riddiford [Janelia Research Campus–Howard Hughes Medical Institute (HHMI)], and the F_1 progeny are 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 Lynn Riddiford (Janelia Research Campus – HHMI). *Hedgehog*-GAL4 and *patched*-GAL4 were provided by Florence Janody (Instituto Gulbenkian de Ciência). *Bric-à-brác*-GAL4 was obtained from Cassandra Extavour (Harvard University). *Nanos*-GAL4 was acquired from Rui Martinho (University of Algarve). Fly stocks were maintained at 22° in bottles on 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). All fly strains are available upon request.

Larval staging and dietary manipulations

Adults were allowed to lay eggs for 2–6 hr on fresh food plates (60- × 15-mm petri dish filled with standard fly food). Egg density was controlled to prevent overcrowding (~200 eggs per plate). Larvae were selected 0–2 hr 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 (2) because it is close to the carbohydrate content of our standard fly medium (~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 20-hydroxyecdysone (20E) feeding experiments, 10–20 precritical weight larvae [after third instar ecdysis (5 hr 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 precritical weight larvae (5 hr 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 25°.

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 min at room temperature. Larvae were then washed three times for 20 min with PBT and blocked in 2% normal donkey serum in PBT for 30 min. We incubated the tissue overnight at 4° in a primary antibody solution containing mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) diluted in 2% normal donkey serum in PBT. After washing three times for 20 min in PBT, larvae were incubated in the dark with goat anti-mouse Alexa 568 (Invitrogen, 1:200) and TRICT-Phalloidin (Sigma, 1:200) diluted in 2% normal donkey serum in PBT overnight at 4°. 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 40× 1.3 numerical aperture 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 1 μm. Images were processed and analyzed using ImageJ (National Institutes of Health) and Adobe Photoshop (Adobe Systems) softwares. For each time point/genotype/food treatment, forming TFs were identified by cell morphology

and Engrailed expression, and the 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 analyzed using linear models and ANCOVAs. When exploring the relative importance of larval age, ecdysone signaling, 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).

Data availability

Data and R scripts for the analysis of TF number and ovary volume can be found on the Dryad digital repository (doi:10.5061/dryad.688nk).

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 to 30 hr 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 hr 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 hr 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 3 days after adult eclosion (Supporting Information, 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 bisegmental 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 hours 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 hr AL3E (95% C.I.: 9.37–13.64 hr AL3E) (Davies 1987; Muggeo 2003, 2007). This estimated breakpoint correlates with critical weight, suggesting that precritical weight ovaries are more sensitive to changes in larval nutrition than postcritical 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 hr starting either at 0 hr AL3E or at 20 hr AL3E and then returned them to standard food until the end of the feeding period. In precritical weight larvae fed first on 20% sucrose food between 0 and 20 hr AL3E and 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 postcritical weight larvae were fed on 20% sucrose food from 20 to 40 hr AL3E and 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 hr AL3E until the end of development (Figure 1, A and B). These observations corroborate a previous study where refeeding precritical 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 *vs.* sucrose food. We first analyzed 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 precritical weight ovaries (from 0 to 10 hr AL3E) (Figure S2A) (Godt and Laski 1995). At 15 hr AL3E, TFCs appeared in the medial side of the ovary, and a few forming TFs were visible (Figure S2, A and B). New TFCs continued to emerge from the surrounding ovarian somatic cells and gradually intercalated

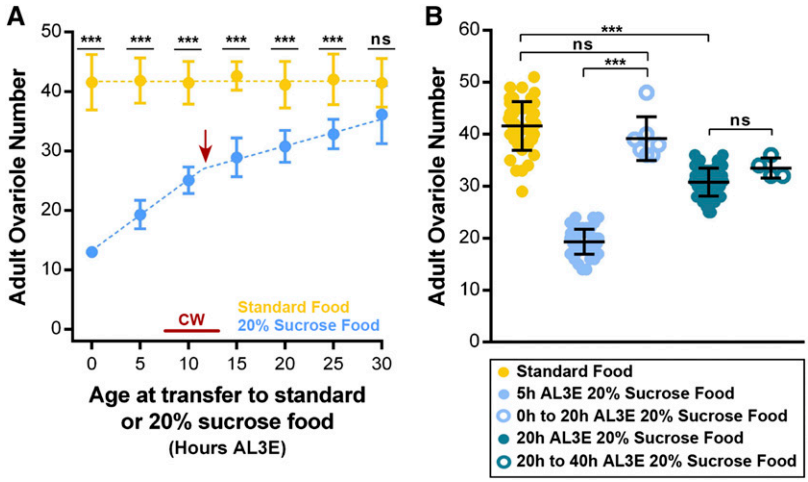

Figure 1 Changes in nutrition during the first phase of sensitivity have greater effects on ovariole number than in the second phase of sensitivity. (A) Adult ovariole number from larvae from an outbred line transferred either to standard food (yellow circles) or to 20% sucrose food (blue circles). Dashed 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 at ~10 hr AL3E (red line). (B) Adult ovariole number from larvae fed on standard food (yellow circles); larvae transferred to 20% sucrose food either at 5 hr AL3E (light blue circles) or at 20 hr AL3E (dark blue circles); and larvae fed on 20% sucrose food for a 20-hr interval either between 0 and 20 hr AL3E (open blue circles) or between 20 and 40 hr AL3E (open dark circles). Plotted values represent means, and error bars show 95% confidence intervals of means. ANOVAs followed by Tukey's HSD test: ***$P < 0.001$. ns, nonsignificant. L3: third instar larvae; AL3E: after L3 ecdysis.

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 ~18–22 TFs had formed (Figure S2, A and 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 responds to changes in nutrition, we fed outbred larvae on 20% sucrose food for 24 hr, starting at 5-hr intervals between 0 and 25 hr AL3E, determined whether TFCs had begun differentiation, quantified the number of TFs, and measured ovary volume for each condition at the end of this 1-day starvation period. When larvae were fed on 20% sucrose food before reaching critical weight (before 10 hr AL3E), we failed to observe any En-positive cells in the ovaries, indicating that the onset of TFC differentiation was delayed (Figure 2, A–D and M). The wing discs and central nervous system of larvae staged before 10 hr 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 hr 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 precritical weight larvae (5 hr AL3E) were fed on 1% sucrose food for 24 hr, the ovaries similarly did not show any TFCs (Figure S3, A, B, and E), and ovary volumes were even smaller than those from larvae fed on 20% sucrose between 5 and 29 hr AL3E (Figure S3F).

In contrast, when larvae were transferred to 20% sucrose food around the time of the critical weight transition (at 10 hr AL3E), most ovaries had a few TFCs (Figure 2, E and 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 hr AL3E) all had forming TFs (Figure 2, G–L). Nevertheless, both TF number and ovary volume were moderately reduced when compared with larvae fed on standard food (Figure 2, M and N). A stronger phenotype was obtained when larvae were transferred to 1% sucrose food after reaching critical weight (15–39 hr AL3E) (Figure S3, C–F); both TF number and ovary volume were reduced when compared to postcritical weight larvae fed on 20% sucrose food during the same period of time (Figure S3, E and F). These data suggest that all three developmental processes are affected by nutrition, but they do not resolve how the dynamics of these processes change over developmental time.

Although ovaries from precritical weight larvae fed on 20% sucrose food for 24 hr did not contain any TFCs (Figure 2, A–D and M), these larvae did eventually give rise to adults with functional ovaries (Figure 1A). Thus, in precritical weight larvae fed on 20% sucrose food, TFC differentiation must eventually occur. We therefore postulated that the ovaries from precritical weight larvae might be more sensitive to nutrition because nutrition affects the timing of the onset of TFC differentiation, as well as the rate of TF addition and/or the rate of increase in ovary volume. In contrast, feeding postcritical 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 S2, B and C). Therefore, to explore how the dynamics of each of these processes change over

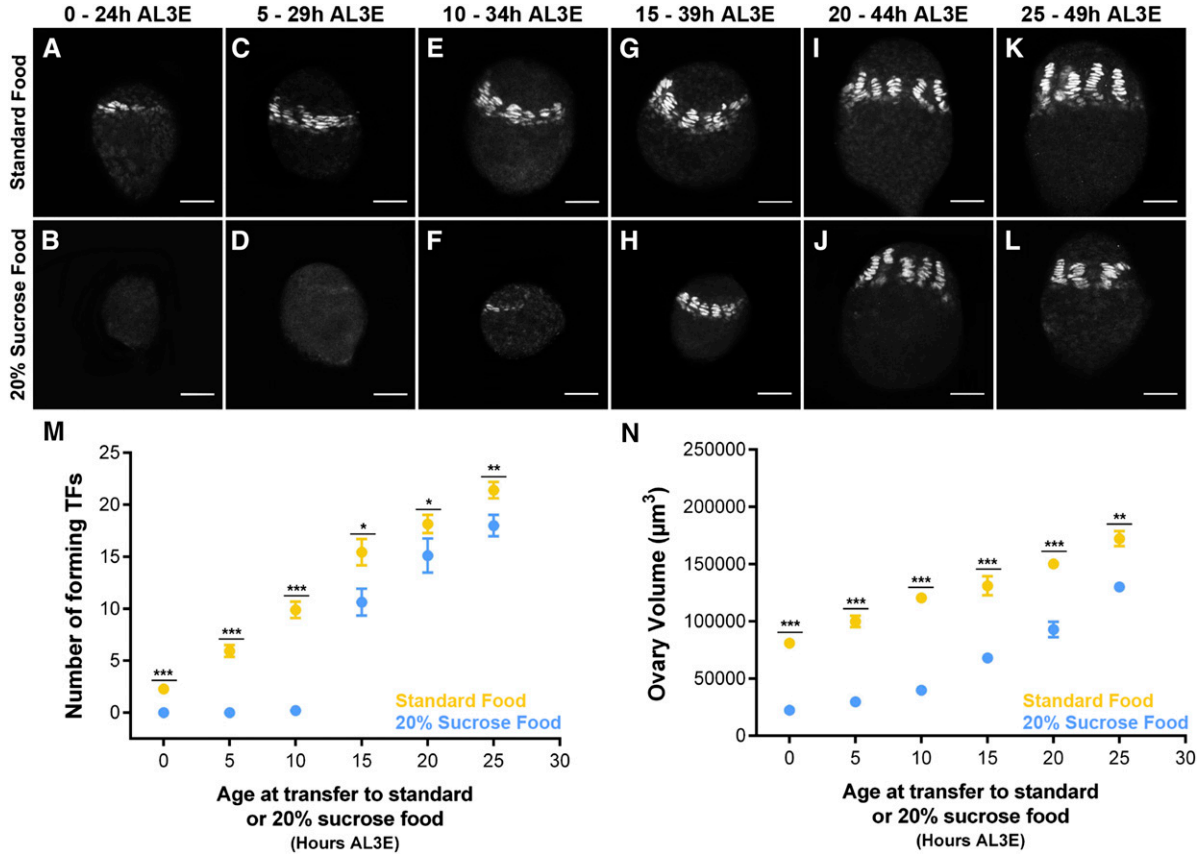

Figure 2 Distinct stage-specific developmental processes during ovary development are regulated by nutrition. (A–L) shows TFs marked with En immunostaining in ovaries from outbred larvae fed on standard food (A, C, E, G, I, and K) or 20% sucrose food (B, D, F, H, J, and L) for 24 hr between 0 and 25 hr AL3E. Bar: 20 μm. (M) Number of forming TFs of ovaries from larvae fed on standard food (yellow points) or 20% sucrose food (blue points). (N) Ovary volume of ovaries from larvae fed on standard food (yellow circles) or 20% sucrose food (blue circles). 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: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. L3: third instar larvae; AL3E: after L3 ecdysis.

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 Analysis of Covariance (ANCOVA) on the $\log_{10}$-transformed data. This allowed 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 meant that the rate of increase differed between treatments for the time interval sampled. By analyzing 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 precritical weight larvae fed on 20% sucrose food (starting at 5 hr AL3E), TFCs and a few forming TFs were observed at 49 hr AL3E (Figure 3, B, B′, and D), and new TFs were still forming at 69 hr AL3E (Figure 3D). Ovaries from precritical 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 postcritical weight larvae fed on 20% sucrose food, ovaries showed significant differences in TF number, but a similar rate of TF addition (Figure 3, A, A′, C, C′, and D and Table S1) when compared to well-fed larvae. Both TF number and the rate of TF addition were higher in ovaries from postcritical weight larvae than in precritical weight larvae fed on 20% sucrose (Table S1).

Similar to what we found for TF number, in precritical weight larvae fed on 20% sucrose both ovary volume and the rate of increase in ovary volume were dramatically reduced with no detectable increase in ovary volume over the time period sampled, when compared to ovaries from well-fed larvae or postcritical 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 postcritical weight

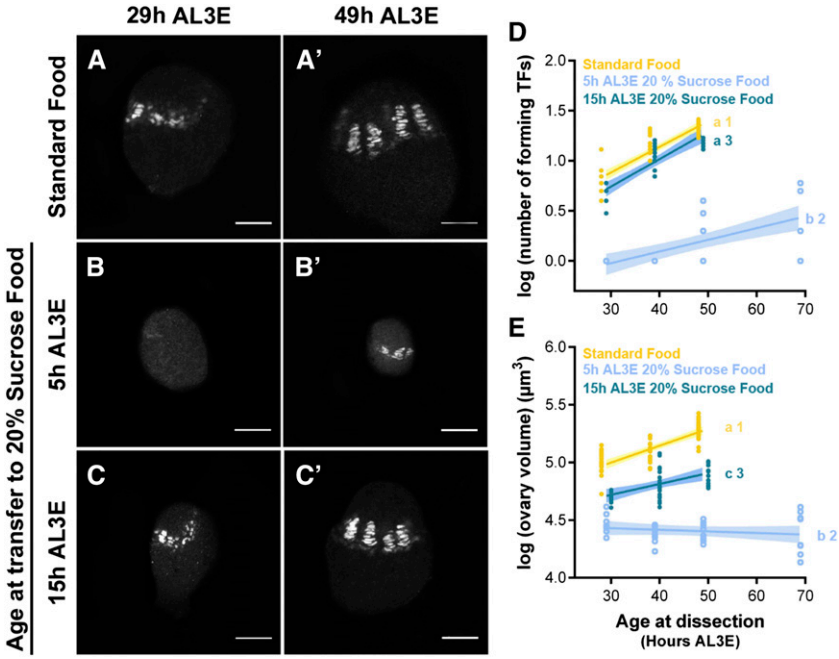

Figure 3 TF number and ovary volume respond differently to pre- and postcritical weight nutrition. (A–C′) shows TFs marked with En immunostaining. (A–A′) Ovaries from outbred larvae reared on standard food. (B–C′) Ovaries from outbred larvae transferred to 20% sucrose food at either (B–B′) 5 hr AL3E or (C–C′′) 15 hr AL3E. Larvae dissected at (A, B, and C) 29 hr AL3E or (A′, B′, and C′) 49 hr AL3E. Bar: 20 μm. (D) Number of forming TFs and (E) ovary volume of ovaries from either larvae fed on standard food (yellow circles); or larvae transferred to 20% sucrose food either at 5 hr AL3E (light blue circles) or at 15 hr AL3E (dark blue points). In D and 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.

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, and thus the 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 precritical weight larvae fed on 20% sucrose food was significantly reduced at 69 hr 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 postcritical weight larvae fed on 20% sucrose, the number of TFCs per TF at 49 hr 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 hr AL3E in postcritical weight larvae, this suggests that the rate of TF addition was transiently reduced between 15 and 29 hr AL3E, but returned to the same rates as fed larvae after 29 hr AL3E. Both ovary volume and the rate of increase in ovary volume were significantly reduced in postcritical weight larvae fed on 20% sucrose food, albeit to a lesser degree than in precritical weight larvae.

Ovariole number is regulated by IIS and ecdysone-signaling pathways

Given the differences in sensitivity to nutrition between pre- and postcritical weight larval ovaries, and in the developmental processes affected at each stage, we next hypothesized that these differences might arise if distinct signaling pathways regulated each process. Previous studies had shown that suppressing IIS or ecdysone signaling 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-signaling pathways in the somatic cells of the larval ovary using the *traffic jam*-GAL4 driver. At 0 hr AL3E, *traffic jam*-GAL4 is expressed in all somatic cells of the larval ovary, but not in germ cells (Figure S4, A–A′′). By 40 hr AL3E, its expression becomes restricted to the posterior part of the ovary (Figure S4, C–C′′). *Traffic jam*-GAL4 is also expressed in the larval brain (Figure S5, A–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 S5, C–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 S6, B and 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 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 S6, B and E), but such reduction in pharate weight was not observed in *elav* > *PTEN* females (Figure S6E). 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 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). Second, by binding to EcR/Usp, ecdysone activates the transcription of target genes (Cherbas 2003; Hu *et al.* 2003). To determine the effects of suppressing ecdysone signaling 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 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 S6, B and 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 analyzed 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 hr AL3E (Figure 4, A and B). In contrast, activating IIS in the ovarian somatic cells did not affect the timing of TFC differentiation in fed larvae (Figure 4, A–C). However, activating IIS in the ovaries in larvae fed on 20% sucrose from 5 hr AL3E onward was sufficient to induce premature onset of TFC differentiation with respect to controls (Figure 4, F, G, and H). Overall, these results suggest that IIS plays a role in regulating the timing of TFC differentiation.

When we analyzed 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 hr AL3E in the majority of the ovaries analyzed (Figure 4, F–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 mean 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). Furthermore, at the time of transfer to 20% sucrose (5 h AL3E),

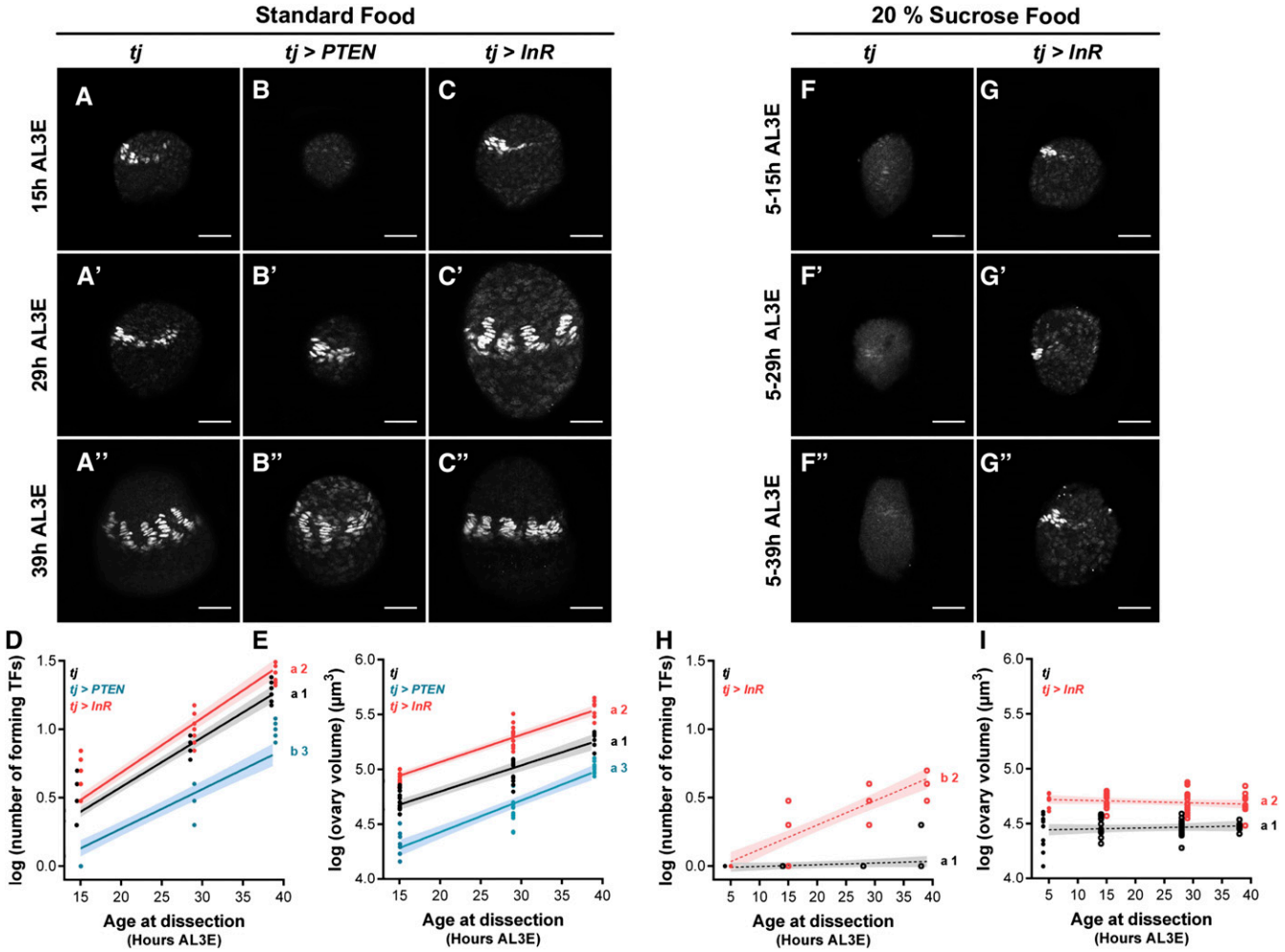

Figure 4 Role of IIS during ovary development. (A–G′′) shows terminal filaments (TFs) marked with En immunostaining. Ovaries from larvae reared on standard food: (A–A′′) *tj*-GAL4 (control), (B–B′′) *tj* > *PTEN* and (C–C′′) *tj* > *InR*. Larvae were dissected at (A, B, and C) 15 hr, (A′, B′, and C′) 29 hr, or (A′′, B′′, and C′′) 39 hr 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 hr AL3E: (F–F′′) *tj*-GAL4 (control) and (G-G′′) *tj* > *InR*. Larvae were dissected at (F and G) 15 hr, (F′ and G′) 29 hr, or (F′′ and G′′) 39 hr 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 and I, closed points represent ovaries from larvae fed on standard food at 5 hr AL3E. In D, E, H, and I, data were $\log_{10}$-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. Bar: 20 µm.

increasing IIS in the somatic cells of the ovary resulted in larger ovary volumes than that of ovaries from control larvae (Figure S7, A, C, and 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 mean ovary volume, but not the rate of increase in ovary volume between 15 and 39 hr AL3E. However, because ovaries from *tj* > *InR* larvae are larger in size at 5 and 15 hr AL3E, IIS is likely to control the rate of increase in ovary volume before larvae reach critical weight.

Role of ecdysone signaling during ovary development

Critical weight itself is regulated by a small nutrition-sensitive ecdysone peak that occurs at ~8–10 hr AL3E (Mirth *et al.* 2005; Warren *et al.* 2006; Koyama *et al.* 2014), which is 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 signaling 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 signaling in the ovary, using the *traffic jam*-GAL4 line, in one of two ways: (1) we repressed ecdysone signaling using UAS-*EcRA.W650A* (*tj* > *EcR-DN*) or (2) we used an RNA interference 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 hr AL3E, control ovaries (*tj*-GAL4) from larvae reared on standard food had TFCs and a few forming TFs (Figure 5, A–A′′ and D). However, we only detected TFCs at 39 hr AL3E when we suppressed ecdysone signaling in the ovaries of well-fed larvae (Figure 5, B–B′′ and D). In well-fed conditions, knocking down EcR in the ovaries did not affect the timing of the onset of TFC differentiation (Figure 5, A and C). But, when we knocked down EcR in the ovaries and fed these larvae on 20% sucrose food starting at 5 hr AL3E, most ovaries already had differentiating TFCs at 15 hr AL3E (Figure 5, G and H). In control larvae fed on 20% sucrose, TFCs were not detected even at 39 hr AL3E (Figure 5, F–F′′ and H). This suggests that, like IIS, ecdysone signaling is important for regulating the timing of the onset of TFC differentiation.

When we examined the effects of ecdysone signaling on TF number, we found that suppressing ecdysone signaling 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 signaling 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 signaling 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 signaling in regulating ovariole number plasticity, we fed wild-type, outbred larvae from 5 to 29 hr AL3E on either standard food or 20% sucrose food supplemented with 0.15 mg/ml of the active ecdysone metabolite 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 6, A, C, and E). However, larvae fed on 20E-supplemented 20% sucrose food initiated TFC differentiation earlier and had significantly more TFs at 29 hr AL3E than larvae fed on 20% sucrose food plus solvent (ethanol) (Figure 6, B, D, and 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 under starved conditions.

Interplay between IIS and ecdysone-signaling 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 signaling 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 signaling regulates its rate of increase throughout development. We next sought to understand how the interaction between these two signaling pathways might result in differences in the ovary's sensitivity to nutrition between pre- and postcritical 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 signaling, we suppressed ecdysone signaling using UAS-*EcR-DN* and induced the derepression function of ecdysone signaling 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 signaling 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 7, A–C, A′ –C′). In contrast, knocking down EcR while upregulating IIS resulted in precocious TFC differentiation, with TFCs appearing as early as 5 hr AL3E (Figure S7D). This onset of TFC differentiation was not only earlier than that of control larvae, but also significantly earlier than the onset of TFC differentiation in *tj* > *InR* and *tj* > *EcR-IR* ovaries (Figure S7A-C; $P < 0.0001$, $\chi^2 = 45$, d.f. = 3, chi-square test). In contrast, we did not note any effects of knocking down EcR while downregulating IIS in the ovary on the timing of the onset of TFC differentiation (Figure 7, A, E, A′, E′). These data suggest that ecdysone signaling 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.

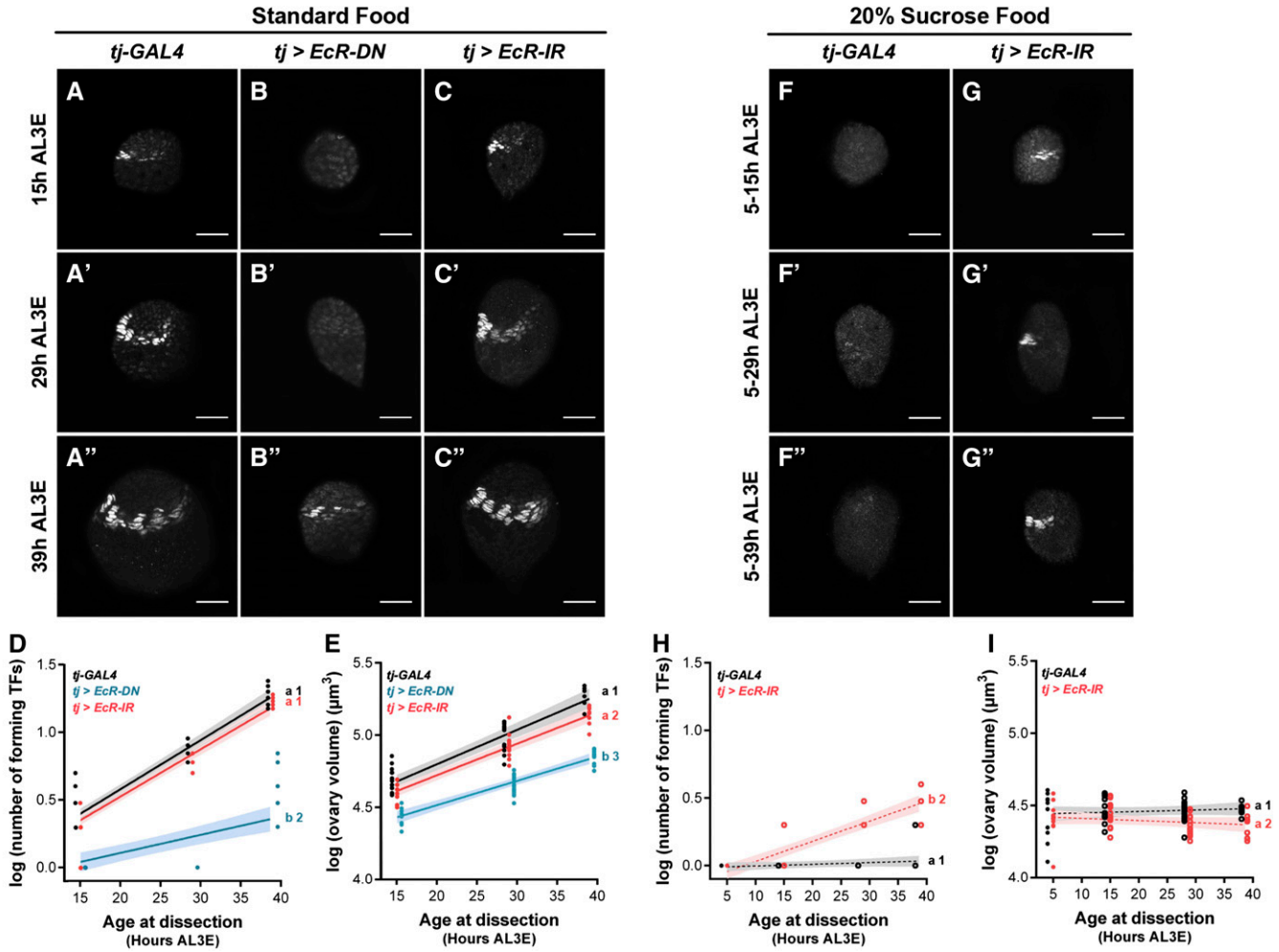

Figure 5 Role of ecdysone signaling during ovary development. (A–G′′) shows TFs marked with En immunostaining. Ovaries from larvae reared on standard food: (A–A′′) *tj*-GAL4 (control), (B–B′′) *tj* > *EcR-DN*, and (C–C′′) *tj* > *EcR-IR*. Larvae were dissected at (A, B, and C) 15 hr, (A′, B′, and C′) 29 hr, or (A′′, B′′, and C′′) 39 hr 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 hr AL3E: (F-F′′) *tj*-GAL4 (control) and (G-G′′) *tj* > *EcR-IR*. Larvae were dissected at (F and G) 15 h, (F′ and G′) 29 h or (F′′ and 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 and I, closed points represent ovaries from larvae fed on standard food at 5 h AL3E. In D, E, H, and I, data were $\log_{10}$-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. Bar: 20 μm.

Variation in larval age, ecdysone signaling, IIS, and the interaction between ecdysone signaling 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 signaling, IIS, and the interaction between both pathways showed that they contributed to 33–54, 31-50, 8–21, and 1–6% of the total R^2, respectively. Thus, ecdysone signaling 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 signaling and IIS resulted in the lowest TF numbers and lowest rates of addition, with very few TFs forming between 15 and 39 hr AL3E (Figure 7F and Table S6). Repressing ecdysone signaling 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

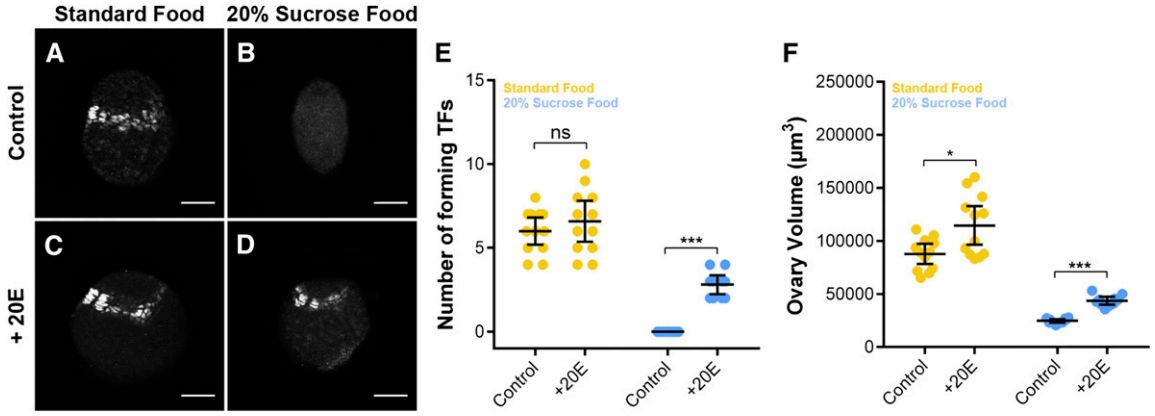

Figure 6 Feeding wild-type larvae with 20E-supplemented food increases TF number and ovary volume. (A–D) shows TFs marked with En immunostaining. Ovaries from outbred larvae reared on standard food: (A) plus ethanol (control) or (C) plus 20E (+20E). Ovaries from larvae reared on 20% sucrose food: (B) plus ethanol (control) or (D) plus 20E (+20E). Larvae were dissected at 29 hr AL3E. Bar: 20 μm. (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: $*P < 0.05$ and $***P < 0.001$; ns, nonsignificant.

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 signaling, 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%; see Table S4, Table S6, and Table S7). Larval age, ecdysone signaling, and the interaction between the two pathways contributed to explaining 28-38, 3-12, and 0.4-2% of the total R^2, respectively.

Ovary volume under fed conditions was significantly different between all genotypes, with the smallest ovary volumes resulting from reducing the signaling activity of both pathways and the largest ovary volumes generated by increasing both ecdysone signaling and IIS (Figure 7G and Table S6). We only observed a difference in the rate of increase in ovary volume when both ecdysone signaling and IIS were simultaneously reduced in the ovary (Figure 7G and Table S6). Upregulating IIS while downregulating ecdysone signaling 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 hr AL3E. Ecdysone signaling regulates the rate of increase in ovary volume after 15 hr AL3E; however, increasing IIS can compensate for reduced ecdysone signaling.

Activating both IIS and ecdysone-signaling pathways in ovarian somatic cells of well-fed larvae induced an earlier onset of TFC differentiation (Figure S7, A–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 signaling 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 hr AL3E, TF number and rate of addition was significantly higher than that of control ovaries (Figure 8D and Table S7). When we knocked down EcR while suppressing IIS in the ovarian somatic cells and fed these larvae on 20% sucrose, we observed a slight delay in the onset of TFC differentiation. We did not observe any TFCs in these larvae at 15 hr AL3E (Figure 8C), although some TFCs were detected at 29 and 39 hr AL3E in half of the ovaries analyzed (Figure 8, C′, C′′, and D). In addition, TF number and the rate of TF addition were suppressed to the same level as control larvae fed on 20% sucrose (Figure 8D and Table S7). This indicates that increasing both signaling pathways in the ovarian somatic cells can overcome some of the effects of poor nutrition on TF number. Nevertheless, even if ecdysone signaling is sufficient to induce precocious TFC differentiation in larvae fed on 20% sucrose, the rate of TF addition increases only when IIS is sufficiently high.

Knocking down EcR while increasing IIS in the ovaries resulted in larger ovary volumes at 5 hr AL3E when compared to controls (Figure S7, A, D, and E). However, we did not observe any further increase in ovary volume after transferring these larvae to 20% sucrose (Figure 8E and Table S7). In contrast, knocking down EcR while suppressing IIS in the ovarian somatic tissue resulted in dramatic reductions in ovary volume at 5 hr AL3E (Figure 8E and Table S7). Interestingly, after transferring these larvae to 20% sucrose, their ovaries showed a significant decrease in volume compared to similarly treated controls. Together, these results corroborate our previous experiment demonstrating that IIS is the primary determinant of ovary volume, but also show that increasing IIS

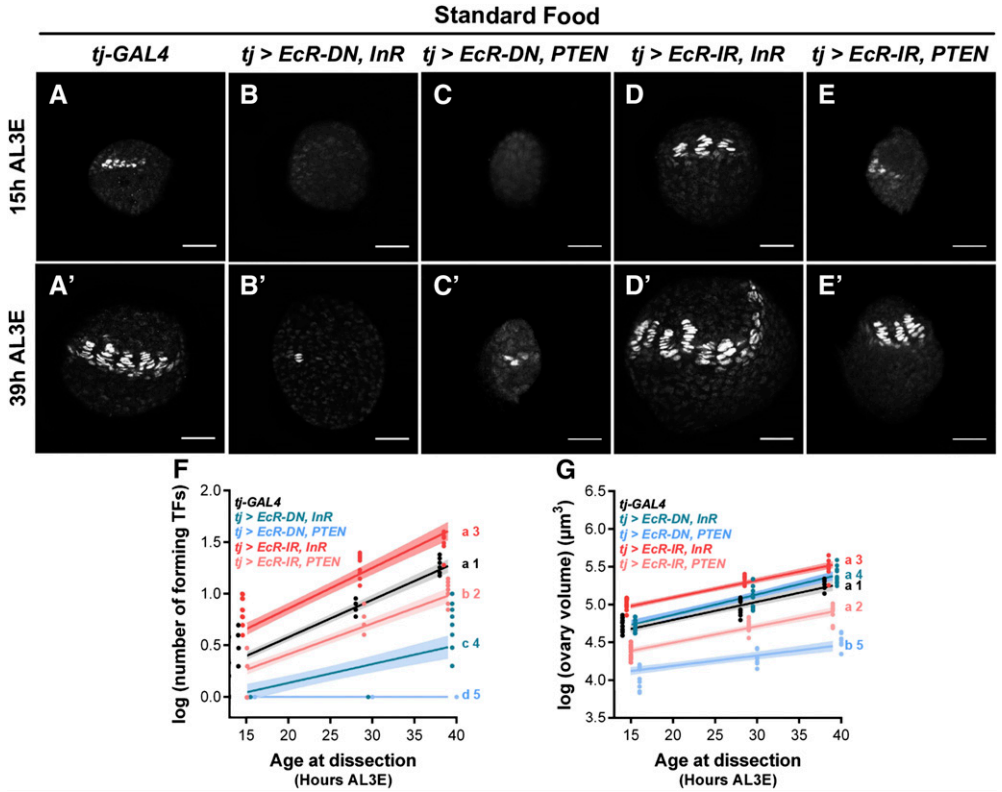

Figure 7 The complex interaction between IIS and ecdysone-signaling pathways in well-fed larvae. (A–E′) shows TFs marked with En immunostaining. Ovaries from larvae reared on standard food: (A-A′) *tj*-GAL4 (control), (B-B′) *tj* > *EcR-DN*, *InR*, (C-C′) *tj* > *EcR-DN*, *PTEN*, (D-D′) *tj* > *EcR-IR*, *InR*, and (E-E′) *tj* > *EcR-IR*, *PTEN*. Larvae were dissected at (A, B, C, D, and E) 15 hr and (A′, B′, C′, D′ and E′) 39 hr AL3E. Bar: 20 μm. (F) Number of forming TFs and (G) 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 F and G data were $\log_{10}$-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.

and ecdysone signaling in the ovary cannot counteract the effects of poor nutrition.

Discussion

Environmental conditions can direct the development of organs along distinct trajectories for growth and differentiation, a phenomenon known as "developmental plasticity." The sensitivity to these conditions typically changes with developmental time, with some stages showing higher sensitivity than others. Here we explored the stage-specific mechanisms controlling nutritional plasticity in ovariole number as a method to address the physiological underpinnings that cause organs to alter their 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-signaling pathways regulate ovariole number by controlling different developmental processes; while IIS primarily regulates ovary size (Green and Extavour 2012; Gancz and Gilboa 2013), ecdysone signaling 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 signaling 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. Precritical 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 ~10–15 hr 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 precritical 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 precritical weight larvae; ovaries from precritical 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

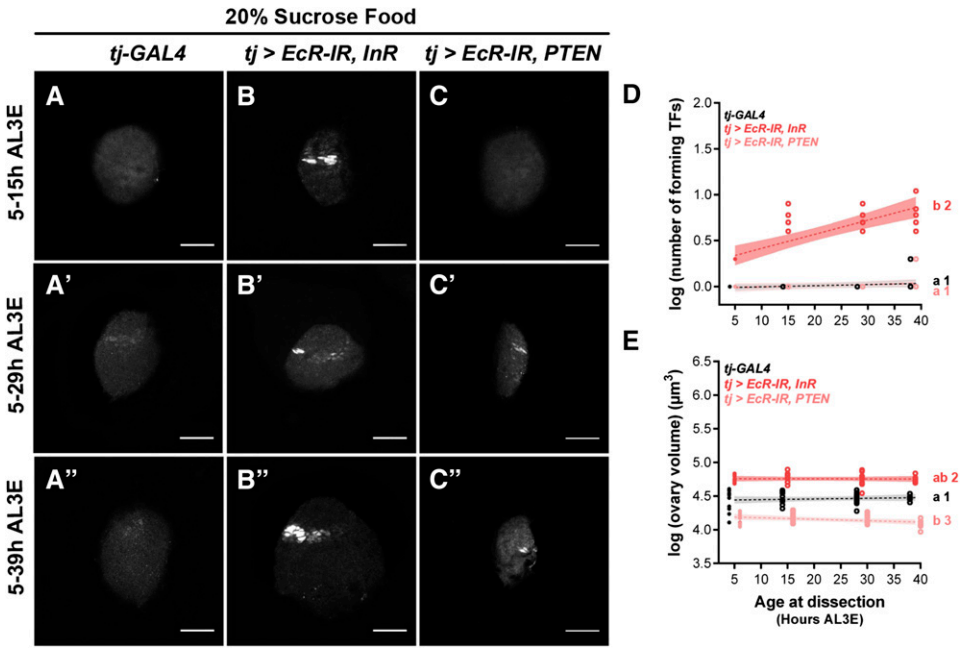

Figure 8 Simultaneously activating both IIS and ecdysone signaling in 20% sucrose food promotes precocious onset of TFC differentiation, increases the rate of TF addition, but does not increase ovary volume. (A-C′′) shows TFs marked with En immunostaining. Ovaries from larvae transferred to 20% sucrose food at 5 hr AL3E: (A–A′′) *tj*-GAL4 (control), (B–B′′) *tj* > *EcR-IR*, *InR*, and (C and C′′) *tj* > *EcR-DN*, *PTEN*. Larvae were dissected at (A, B, and C) 15 hr, (A′, B′, and C′) 29 hr, or (A′′, B′′, and C′′) 39 hr AL3E. Bar: 20 μm. (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 and E, data were $\log_{10}$-transformed, and closed points represent ovaries from larvae fed on standard food at 5 hr 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.

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

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

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

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

This change in the regulation of TF addition rate is most likely due to the relative effects of ecdysone signaling and IIS on this process. Suppressing either ecdysone signaling and/or IIS delayed the onset of TFC differentiation and reduced both TF number and addition rate. Ecdysone signaling 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 precritical weight larvae, our results show that events occurring postcritical 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 signaling 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 precritical weight phase. On the other hand, IIS is likely to be the principal regulator of TF number during the postcritical weight phase. We propose that the change in sensitivity to nutrition that occurs after critical weight results in part due to the change in the regulation of TF number, with ecdysone signaling 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 postcritical weight larvae. In this case, nutrition affected ovary volume and the rate of increase in ovary volume in larvae of both stages. Under poor nutritional conditions, ovaries from precritical 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 to the ovaries (Shingleton *et al.* 2008; Mirth *et al.* 2009; Lanet *et al.* 2013). Starving larvae before they reach critical weight arrests growth of the wing discs. Once larvae surpass critical weight, the progression of growth continues under starvation conditions, albeit at a reduced rate (Shingleton *et al.* 2008; Mirth *et al.* 2009).

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

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

Ecdysone signaling also played a clear role in regulating ovary volume. Manipulating ecdysone signaling in the ovarian somatic cells altered the rate of increase in ovary volume in well-fed, postcritical weight larvae. In addition, feeding larvae 20E was the only treatment that increased ovary volume under 20% sucrose food conditions, although it was insufficient to restore ovary volume to fed conditions. Because both the control and 20E fed larvae were from the same cohort of wild-type, outbred flies, ovary volumes were almost certainly indistinguishable between treatments at the beginning of the experiment. This means that changes in ovary volume over the 24 hr time period are necessarily due to changes in the rate of increase in ovary volume. Taken together, our data show that ecdysone contributes to regulating the rate of increase in ovary volume principally in the postcritical weight phase. We propose that the change in the sensitivity of ovary volume across development stages results from changes in regulation of its rate of increase. While IIS signaling regulates the rate of increase in ovary volume during the more sensitive precritical weight stage, ecdysone signaling regulates this process after critical weight.

The effects of ecdysone signaling and IIS in ovary volume parallel those found in the wing imaginal discs of other insects. In both *Manduca sexta* and the butterfly *Precis coenia*, IIS and ecdysone signaling 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. Furthermore, we require more dedicated studies to understand the nature of the molecular interactions between ecdysone signaling 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 signaling pathway that contributed the most to variation in the trait acted primarily in the earlier, more sensitive precritical 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, such as male genital size in *D. melanogaster* (Tang *et al.* 2011), and traits that respond more plastically to changes in nutrition, such as the size of male horns in rhinocerous beetles (Emlen *et al.* 2012), do so by altering the level of signaling 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.

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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Author's contributions: Both authors contributed to conceiving the project, designing the experiments, analyzing the data, and writing and revising the manuscript. C.C.M. performed all the experimental work under the supervision of C.K.M.

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Stage-Specific Plasticity in Ovary Size Is Regulated by Insulin/Insulin-Like Growth Factor and Ecdysone Signaling in *Drosophila*

Cláudia C. Mendes and Christen K. Mirth

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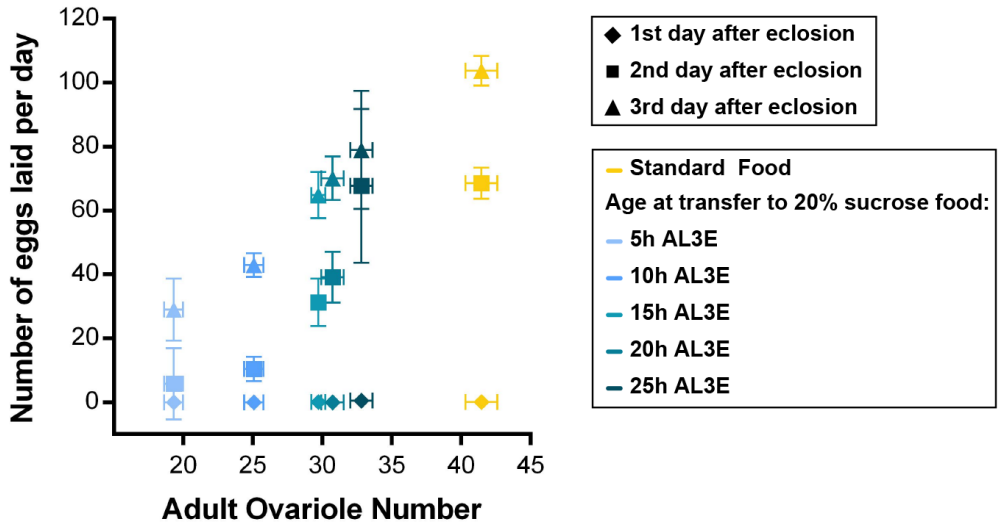
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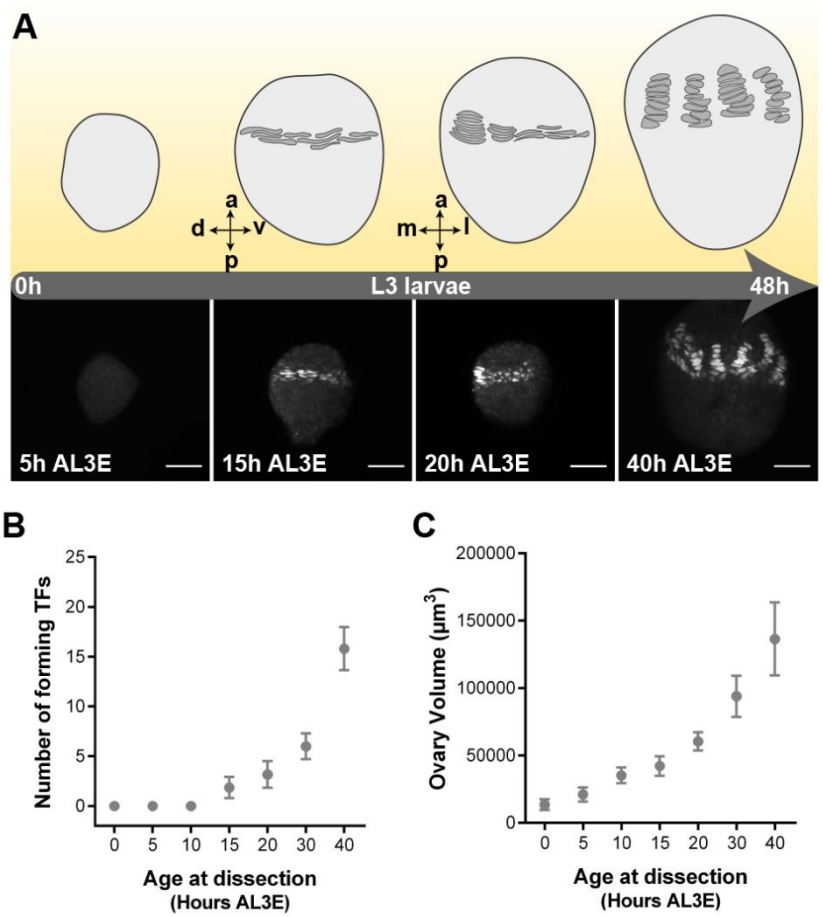
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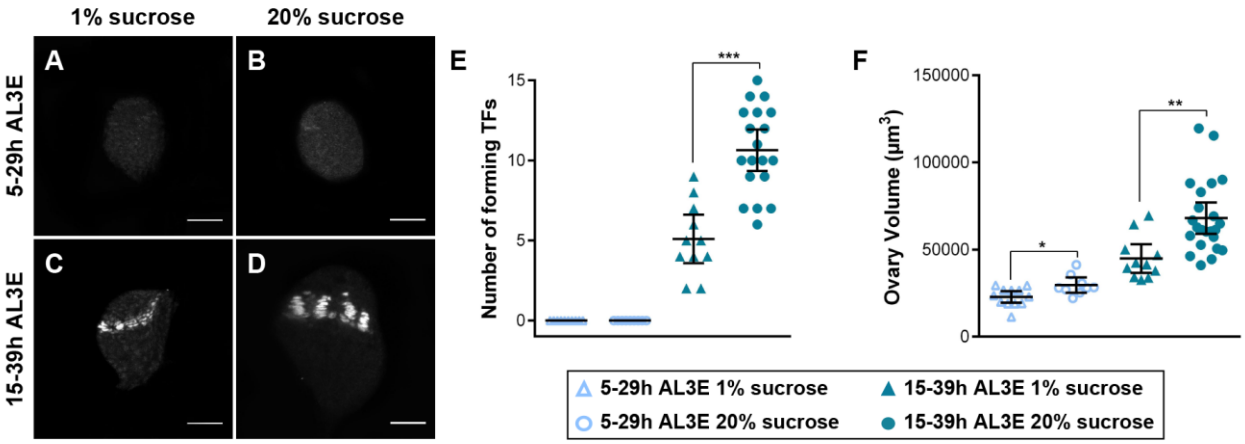
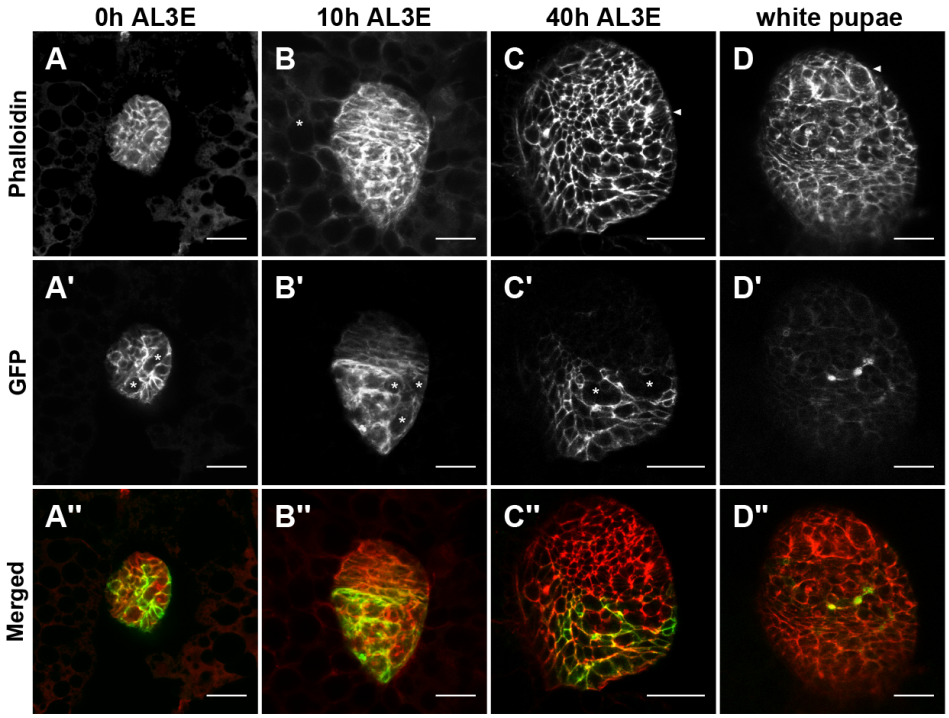
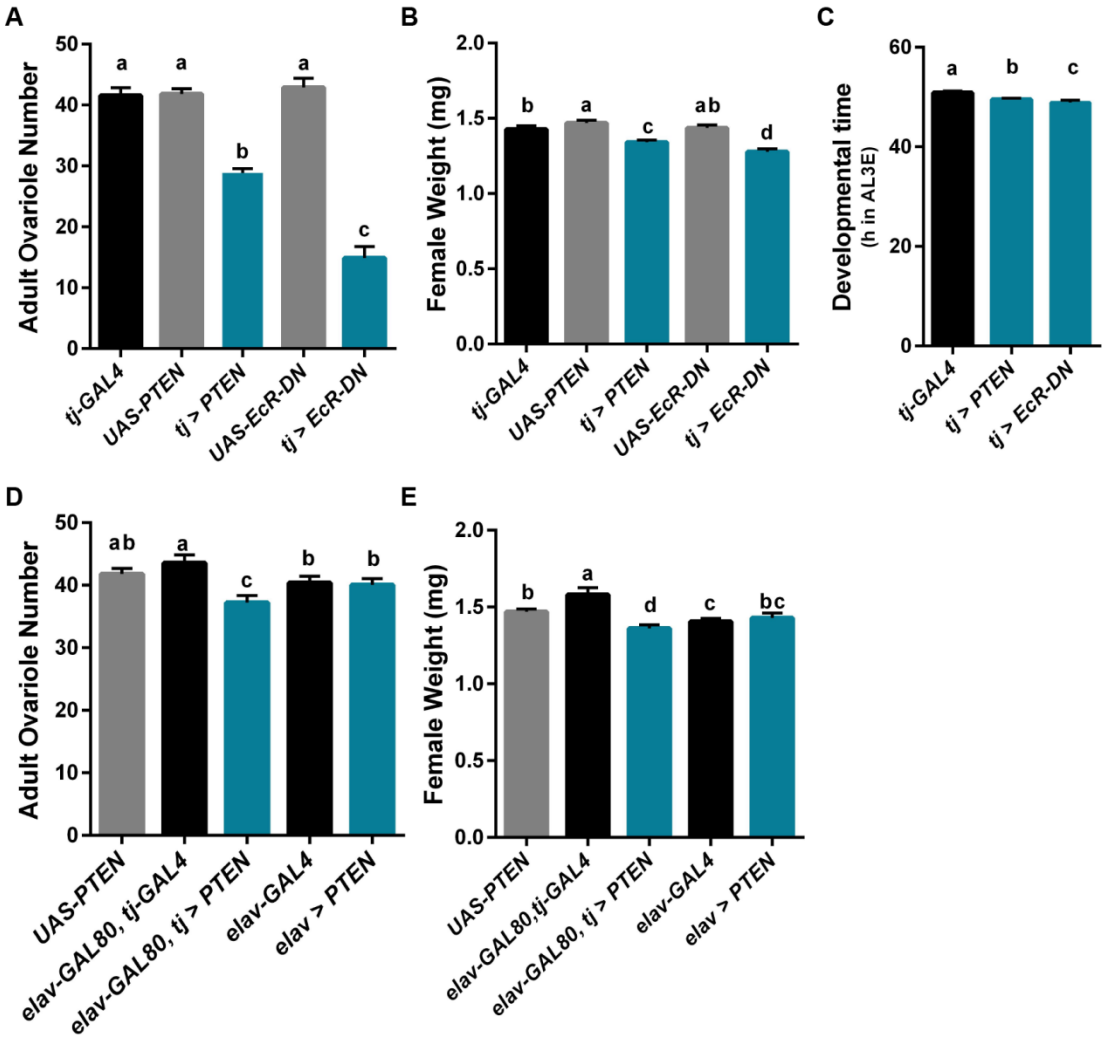
20
30
40
Age at dissection
(Hours AL3E)


1% sucrose
20% sucrose
A
B
C
D
5-29h AL3E
15-39h AL3E
E
Number of forming TFs
15
10
5
0
***
F
Ovary Volume (µm3)
150000
100000
50000
0
*
**
5-29h AL3E 1% sucrose
15-39h AL3E 1% sucrose
5-29h AL3E 20% sucrose
15-39h AL3E 20% sucrose


0h AL3E
10h AL3E
40h AL3E
white pupae
Phalloidin
GFP
Merged
A
B
C
D
A'
B'
C'
D'
A''
B''
C''
D''


A
Adult Ovariole Number
50
40
30
20
10
0
a
a
b
a
c
tj-GAL4
UAS-PTEN
tj > PTEN
UAS-EcR-DN
tj > EcR-DN
B
Female Weight (mg)
2.0
1.5
1.0
0.5
0.0
b
a
c
ab
d
tj-GAL4
UAS-PTEN
tj > PTEN
UAS-EcR-DN
tj > EcR-DN
C
Developmental time
(h in AL3E)
60
40
20
0
a
b
c
tj-GAL4
tj > PTEN
tj > EcR-DN
D
Adult Ovariole Number
50
40
30
20
10
0
ab
a
c
b
b
UAS-PTEN
elav-GAL80, tj-GAL4
elav-GAL80, tj > PTEN
elav-GAL4
elav > PTEN
E
Female Weight (mg)
2.0
1.5
1.0
0.5
0.0
b
a
d
c
bc
UAS-PTEN
elav-GAL80,tj-GAL4
elav-GAL80, tj > PTEN
elav-GAL4
elav > PTEN

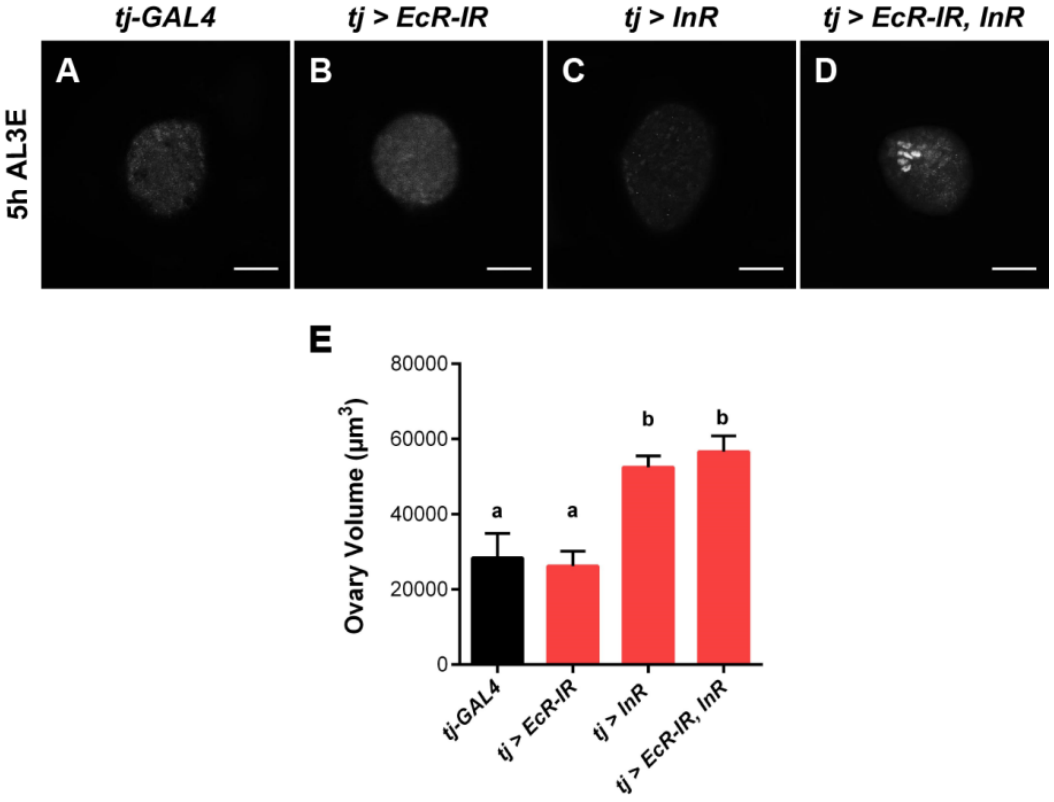

tj-GAL4
tj > EcR-IR
tj > InR
tj > EcR-IR, InR
A
B
C
D
5h AL3E
E
Ovary Volume (µm3)
80000
60000
40000
20000
0
a
a
b
b
tj-GAL4
tj > EcR-IR
tj > InR
tj > EcR-IR, InR

Table S1. Rate of TF addition and of increase in ovary volume in outbred larvae fed on standard food or 20% sucrose food from 5 h AL3E or 15 h AL3E. The number of TFs and ovary volume present in Figures 3D and 3E were log_{10}-transformed followed by ANOVAs to compare TF number, ovary volume, rates of TF addition, and rates of increase in ovary volume. Significant codes: values not sharing the same letter (least square trends) or number (least square means) are significantly different (ANCOVAs with Holm's correction; $p < 0.05$).

	Treatment	**Transfer to 20% sucrose at:**	**Least Square Trends (value ± s.e.)**	**Sign. Code**	**Least Square Means (value ± s.e.)**	**Sign. Code**
Rate of TF addition	Standard Food	n/a	0.026 ± 0.002	a	1.170 ± 0.018	1
	20% sucrose	5 h AL3E	0.012 ± 0.002	b	0.109 ± 0.024	2
		15 h AL3E	0.027 ± 0.004	a	1.049 ± 0.026	3
Rate of increase in ovary volume	Standard Food	n/a	0.0196 ± 0.0009	a	5.115± 0.012	1
	20% sucrose	5 h AL3E	-0.0014 ± 0.0012	b	4.417± 0.019	2
		15 h AL3E	0.0094 ± 0.0022	c	4.804± 0.016	3

Table S2. TFC number per TF is reduced in ovaries from pre-critical weight larvae fed on 20% sucrose food. Terminal filaments (TFs) were labelled with Engrailed. Five ovaries from outbred larvae were analysed for each environmental condition and the number of terminal filament cells (TFCs) were counted in five forming TFs, which were randomly selected. Significant codes: values not sharing the same letter are significantly different (ANOVA followed by Tukey's HSD test; $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis

Treatment	**Transfer to 20% sucrose at:**	**Time point analysed**	**TFCs per TF (mean ± s.d.)**	**n**	**Sign. Code**
Standard Food	n/a	49 h AL3E	7.80 ± 1.08	25	a
20% sucrose	5 h AL3E	69 h AL3E	2.83 ± 0.71	18	b
	15 h AL3E	49 h AL3E	7.32 ± 0.90	25	a

Table S3. Adult ovariole number of females in which IIS was inhibited in different cell types in the larval ovary. Phosphatase and tensin homolog (*UAS-PTEN*) was overexpressed under the control of four different GAL4 driver lines: *hedgehog-GAL4* (*hh-GAL4*), *bric-á-brác*-GAL4 (*bab-GAL4*), *patched-GAL4* (*ptc-GAL4*) and *nanos-GAL4* (*nos-GAL4*). Significant codes: values not sharing the same letter are significantly different (ANOVAs followed by Tukey's HSD test; $p < 0.05$).

Expression Pattern	**Genotype**	**Adult Ovariole Number (mean ± s.d.)**	**n**	**Sign. Code**
Terminal filament cells	*hh-GAL4*	36.22 ± 2.05	9	a
	UAS-PTEN	41.81 ± 3.80	59	b
	hh>PTEN	38.00 ± 2.16	4	ab
Somatic cells and terminal filament cells	*bab-GAL4*	45.79 ± 5.28	52	a
	UAS-PTEN	41.81 ± 3.80	59	b
	bab>PTEN	20.21 ± 3.53	19	c
Anterior somatic cells	*ptc-GAL4*	33.70 ± 2.40	27	a
	UAS-PTEN	41.81 ± 3.80	59	b
	ptc>PTEN	35.29 ± 2.61	28	a
Germ cells	*nos-GAL4*	38.44828	29	a
	UAS-PTEN	41.81 ± 3.80	59	b
	nos>PTEN	37.42500	40	a

Table S4. Rates of TF addition and of increase in ovary volume when IIS was manipulated. The number of TFs and ovary volume presented in Figures 4D, E, H and I were log_{10}-transformed followed by ANCOVAs to compare differences in the mean number of TFs and ovary volume (least square means) and changes in the rates of TF addition and of increase in ovary volume (least square trends). Significant codes: values not sharing the same letter (least square trends) or number (least square means) are significantly different (ANCOVAs with Holm's correction; $p < 0.05$).

	Treatment	**Genotype**	**Least Square Trends (value ± s.e.)**	**Sign. Code**	**Least Square Means (value ± s.e.)**	**Sign. Code**
Rate of TF addition	Standard Food	*tj-GAL4*	0.0364 ± 0.0018	a	0.606 ± 0.022	1
		tj>InR	0.0400 ± 0.0015	a	0.717 ± 0.019	2
		tj>PTEN	0.0288 ± 0.0015	b	0.298 ± 0.019	3
	20% sucrose	*tj-GAL4*	0.0012 ± 0.0015	a	0.010 ± 0.017	1
		tj>InR	0.0178 ± 0.0012	b	0.296 ± 0.015	2
Rate of increase in ovary volume	Standard Food	*tj-GAL4*	0.0238 ± 0.0013	a	4.870 ± 0.015	1
		tj>InR	0.0249 ± 0.0011	a	5.141 ± 0.014	2
		tj>PTEN	0.0289 ± 0.0017	a	4.515 ± 0.019	3
	20%	*tj-GAL4*	0.0010	a	4.462	1

	sucrose		± 0.0010		± 0.012	
		tj>InR	-0.0012 ± 0.0009	a	4.698 ± 0.012	2

Table S5. Rates of TF addition and of increase in ovary volume when ecdysone signalling was manipulated. The number of TFs and ovary volume presented in Figures 5D, E, H and I were log_{10}-transformed followed by ANCOVAs to compare differences in the mean number of TFs and ovary volume (least square means) and changes in the rates of TF addition and of increase in ovary volume (least square trends). Significant codes: values not sharing the same letter (least square trends) or number (least square means) are significantly different (ANCOVAs with Holm's correction; $p < 0.05$).

	Treatment	**Genotype**	**Least Square Trends (value ± s.e.)**	**Sign. Code**	**Least Square Means (value ± s.e.)**	**Sign. Code**
Rate of TF addition	Standard Food	*tj-GAL4*	0.0363 ± 0.0018	a	0.609 ± 0.022	1
		tj>EcR-IR	0.0350 ± 0.0016	a	0.556 ± 0.021	1
		tj>EcR-DN	0.0134 ± 0.0015	b	0.120 ± 0.020	2
	20% sucrose	*tj-GAL4*	0.0012 ± 0.0011	a	0.009 ± 0.013	1
		tj>EcR-IR	0.0149 ± 0.0009	b	0.171 ± 0.012	2
Rate of increase in ovary volume	Standard Food	*tj-GAL4*	0.0238 ± 0.0011	a	4.886 ± 0.014	1
		tj>EcR-IR	0.0219 ± 0.0010	a	4.083 ± 0.013	2
		tj>EcR-DN	0.0168 ± 0.0016	b	4.577 ± 0.016	3

	20% sucrose	*tj-GAL4*	0.0010 ± 0.0011	a	4.461 ± 0.013	1
		tj>EcR-IR	-0.0015 ± 0.0011	a	4.393 ± 0.014	2

Table S6. Rates of TF addition and of increase in ovary volume when both IIS and ecdysone signalling were manipulated in larvae reared in standard food. The number of TFs and ovary volume presented in Figures 7D, E, H and I were log_{10}-transformed followed by ANCOVAs to compare differences in the mean number of TFs and ovary volume (least square means) and changes in the rates of TF addition and of increase in ovary volume (least square trends). Significant codes: values not sharing the same letter (least square trends) or number (least square means) are significantly different (ANCOVAs with Holm's correction; $p < 0.05$).

	Genotype	**Least Square Trends (value ± s.e.)**	**Sign. Code**	**Least Square Means (value ± s.e.)**	**Sign. Code**
Rate of TF addition	*tj-GAL4*	3.64e-02 ± 0.0019	a	6.05e-01 ±0.024	1
	tj>EcR-IR, PTEN	2.94e-02 ± 0.0015	b	4.42e-01 ± 0.024	2
	tj>EcR-IR, InR	3.95e-02 ± 0.0017	a	8.87e-01 ±0.022	3
	tj>EcR-DN, InR	1.82e-02 ± 0.0016	c	1.50e-01 ± 0.021	4
	tj>EcR-DN, PTEN	-6.94e-18 ± 0.0018	d	1.61e-15 ± 0.023	5
Rate of increase in ovary volume	*tj-GAL4*	0.0238 ± 0.0013	a	4.86 ± 0.015	1
	tj>EcR-IR, PTEN	0.0216 ± 0.0012	a	4.56 ± 0.015	2
	tj>EcR-IR, InR	0.0227 ± 0.0012	a	5.16 ± 0.016	3

	tj>EcR-DN, InR	0.0269 ± 0.0018	a	4.94 ± 0.020	4
	tj>EcR-DN, PTEN	0.0137 ± 0.0013	b	4.23 ± 0.016	5

Table S7. Rate of TF addition and of increase in ovary volume when both IIS and ecdysone signalling were manipulated in larvae fed on 20% sucrose food. The number of TFs and ovary volume presented in Figures 8D and E were log_{10}-transformed followed by ANCOVAs to compare differences in the mean number of TFs and ovary volume (least square means) and changes in slope of the rates of TF addition and of increase in ovary volume (least square trends). Significant codes: values not connected by the same letter (least square trends) or number (least square means) are significantly different (ANCOVAs with Holm's correction; $p < 0.05$).

	Genotype	**Least Square Trends (value ± s.e.)**	**Sign. Code**	**Least Square Means (value ± s.e.)**	**Sign. Code**
Rate of TF addition	*tj-GAL4*	0.0012 ± 0.0018	a	0.011 ± 0.021	1
	tj>EcR-IR, InR	0.0154 ± 0.0015	b	0.584 ± 0.020	2
	tj>EcR-IR, PTEN	0.0013 ± 0.0015	a	0.011 ± 0.019	1
Rate of increase in ovary volume	*tj-GAL4*	0.0010 ± 0.0009	a	4.461 ± 0.011	1
	tj>EcR-IR, InR	-0.0001 ± 0.0009	ab	4.757 ± 0.012	2
	tj>EcR-IR, PTEN	-0.0022 ± 0.0009	b	4.154 ± 0.012	3

Supplementary Information

Supplementary Figure Legends

Figure S1. Ovariole number is positively correlated with early female fecundity. Number of eggs laid was counted in the first three days after eclosion (diamond: 1st day after eclosion; square: 2nd day after eclosion; circle: 3rd day after eclosion) from outbred females fed on standard food as larvae (yellow symbols) and outbred females fed on 20% sucrose food as larvae at timed intervals starting between 5 h to 25 h AL3E (symbols with different shades of blue) until the end of the feeding period. Plotted values represent means and error bars show 95% confidence intervals of means.

Figure S2. Ovary development during L3 larval stages under optimal nutritional conditions. (A) Schematic drawings representing ovary development in L3 larvae reared in standard food. Terminal filaments (TFs) are represented as dark grey symbols. Axis are presented as A-P, anterior-posterior; D-V, dorsal-ventral; M-L, medial-lateral. Pictures show developing ovaries from outbred larvae during L3 larval stages under standard food. Engrailed (grey) marks terminal filament cells (TFCs). Scale bar: 20µm. (B) Number of forming terminal filaments (TFs). (C) Ovary volume. Plotted values represent means and error bars show 95% confidence intervals of means. L3: third instar larvae; AL3E: after L3 ecdysis.

Figure S3. Similar results were obtained when larvae were fed on either 1% or 20% sucrose food. (A-D) shows terminal filaments (TFs) marked with En immunostaining. Ovaries from outbred larvae reared on 1% sucrose food between: (A) 5-29 h AL3E or (C) 15-39 h AL3E. Ovaries from larvae reared on 20% sucrose food between: (B) 5-29 h AL3E or (D) 15-39 h AL3E. Scale bar: 20µm. (E) Number of forming terminal filaments (TFs) and (F) ovary volume of ovaries from larvae fed on 1% (triangles) or 20% (points) sucrose food; larvae were transferred to 20% sucrose food either at 5 h AL3E (light blue circles) or at 15 h AL3E (dark blue points). Error bars show 95% confidence intervals of means. Wilcoxon rank test: *$p<0.05$, ***$p<0.01$, ***$p<0.001$. L3: third instar larvae; AL3E: after L3 ecdysis.

Figure S4. *Traffic jam*-GAL4 is expressed in ovarian somatic cells during L3 larval stages. (A, B, C, D and A'', B'', C'', D'') Phalloidin marks F-actin to outline cell membranes (grey, red). (A', B', C', D' and A'', B'', C'', D'') GFP reporter line under the control of *traffic jam*-GAL4 driver line (grey, green). Scale bar: 20µm. In (C, D), white arrowheads denote forming terminal filaments. In (A', B' and D'), asterisks denote germ cells. L3: third instar larvae; AL3E: after L3 ecdysis.

Figure S5. *Traffic jam*-GAL4 expression patterns in the larval brain during larval stages. (A, B, C, D and A'', B'', C'', D'') Phalloidin marks F-actin to outline cell membranes (grey, red). (A', B' and A'', B'') GFP reporter line under the control of *traffic jam*-GAL4 driver line (grey, green). (C', D' and C'', D'') GFP reporter line in the *elav*GAL80, *traffic jam*-GAL4 driver line (grey, green). Scale bar: 20µm. L2: second instar larvae. L3: third instar larvae.

Figure S6. Manipulating IIS or ecdysone signalling in the larval ovary reduces adult ovariole number and female weight. (A) Adult ovariole number, (B) female pharate weight and (C) developmental times represented in hours after third instar ecdysis (h AL3E) to pupariation of individuals with disruption of IIS or ecdysone signalling under the control of *traffic jam*-GAL4 driver line (*tj > PTEN* and *tj > EcR-DN*, respectively; blue bars). (D) Adult ovariole number and (E) pharate weight of females with disruption of IIS specifically in ovarian somatic cells under the control of *traffic jam*-GAL4 (*elav-GAL80, tj > PTEN*) or in neuroblasts and neurons of the larval brain using the *elav*-GAL4 driver (*elav > PTEN*) (blue bars). Controls are either driver (*elav*-GAL80, *tj*-GAL4; *elav*-GAL4 and *tj*-GAL4; black bars) or reporter (UAS-*PTEN* and UAS-*EcR-DN*; grey bars) lines. Error bars show 95% confidence intervals of means. ANOVAs followed by Tukey's HSD test: values not sharing the same letter are significantly different ($p < 0.05$).

Figure S7. Activating IIS and/or ecdysone signalling results in different ovary volumes at 5 h AL3E. Ovaries from larvae reared on standard food: (A) *tj*-GAL4 (control), (B) *tj > EcR-IR*, (C) *tj > InR* and (D) *tj >EcR-IR, InR*. Scale bar: 20µm. (E) Ovary volume of ovaries from *tj*-GAL4 control larvae (black bar), *tj > EcR-IR, tj > InR* and *tj >EcR-IR, InR* larvae (red bars). Larvae were dissected at 5 h AL3E. Kruskall-wallis followed by Wilcoxon rank test: values not sharing the same letter are

significantly different (Holm's correction $p < 0.05$). L3: third instar larvae; AL3E: after L3 ecdysis.