Juvenile hormone regulates body size and perturbs insulin signaling in Drosophila

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Edited by Alexander S. Raikhel, University of California, Riverside, CA, and approved April 4, 2014 (received for review July 11, 2013)

The role of juvenile hormone (JH) in regulating the timing and nature of insect molts is well-established. Increasing evidence suggests that JH is also involved in regulating final insect size. We elucidate the developmental mechanism through which JH regulates body size in developing Drosophila larvae by genetically ablating the JH-producing organ, the corpora allata (CA). We found that larvae that lack CA pupariated at smaller sizes than control larvae due to a reduced larval growth rate. Neither the timing of the metamorphic molt nor the duration of larval growth was affected by the loss of JH. Further, we show that the effects of JH on growth rate are dependent on the forkhead box O transcription factor (FOXO), which is negatively regulated by the insulin-signaling pathway. Larvae that lacked the CA had elevated levels of FOXO activity, whereas a loss-of-function mutation of FOXO rescued the effects of CA ablation on final body size. Finally, the effect of JH on growth appears to be mediated, at least in part, via ecdysone synthesis in the prothoracic gland. These results indicate a role of JH in regulating growth rate via the ecdysone- and insulin-signaling pathways.

Significance

Understanding how organisms regulate their body size is a fundamental problem in biology. Body size regulation involves the careful integration of mechanisms that control growth rate with those that control growth duration. In insects, developmental hormones such as juvenile hormone and ecdysone regulate developmental transitions and growth duration. The conserved insulin-signaling pathway regulates growth rates. Our studies reveal an intimate link between the three, whereby juvenile hormone controls body size by regulating ecdysone synthesis, which in turn modifies insulin signaling. In vertebrates, hormones such as androgens and estrogens interact with insulin signaling to influence tumor growth. By studying the developmental context of hormone interactions, our data reveal fundamental features of body size regulation that have important consequences for understanding cancer growth.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1313058111/-/DCSupplemental.
(15, 16). After attainment of critical weight, starvation actually accelerates the time to pupariation. We used this change in the starvation response of pupariation time to explore whether the absence of JH in CAX larvae affects critical weight and its timing.

Fig. 1 shows that ablation of the CA had no effect on critical weight. We used the breakpoint method to assay critical weight in CAX and control larvae (15, 20–22). This method exploits the fact that the relationship between larval weight at starvation and time to pupariation (TTP) changes at critical weight (Fig. 1A). We found that critical weight was not significantly different in CAX and control larvae (Fig. 1B): Critical weight was 0.71 mg in CAX larvae and 0.69 mg in control larvae. Allatectomy did delay when critical weight was attained: Critical weight was attained at 7.5 h after third-instar (L3) ecysis (AL3E) in control larvae but at 15.9 h AL3E in CAX larvae (Fig. 1C).

In Manduca, starvation early in the final instar delays wing disc development to proceed even in the absence of nutrition (23). Similarly, in Drosophila, patterning in the wing disc is delayed in starved precritical weight larvae (24). To test whether JH played a role in the repression of wing disc pattern in Drosophila, we examined whether the expression of Wingless (Wg) and Senseless (Sens) depended on nutrition in the wing imaginal discs of CAX larvae. Starving either CAX or control larvae of protein before critical weight (7 h AL3E) inhibited both Wg and Sens expression in their wing discs (Fig. S1A–D). In contrast, starving CAX and control larvae after critical weight (15 h AL3E) did not (Fig. S1 E–M). When larvae are starved from 9 h AL3E, only 2 out of 15 CAX wing discs up-regulated Sens expression (Fig. S1 E–H) compared with 12 out of 18 control wing discs. This indicates that most CAX larvae have not attained critical weight by 9 h AL3E, whereas most control larvae have. Thus, ablation of the CA, and hence elimination of JH, appears to retard attainment of critical weight but does not affect critical weight itself nor the response of disc patterning to starvation.

Ablation of the Corpora Allata Shortens the Terminal Growth Period. Our data show that JH does not regulate body size by affecting critical weight in Drosophila. However, it is possible that CAX larvae are small because of a reduced TGP between attaining critical weight and the cessation of feeding. We therefore measured the time to pupariation from critical weight (TTPCW) in fed and starved larvae as proxy for the TGP. Allatectomy did not influence the TTPCW in starved larvae but did shorten the TTPCW in fed larvae, albeit by only 6 h (Fig. 1D). Because of the delay in attaining critical weight, the total duration of the third larval instar showed only a slight, albeit significant, increase in CAX larvae (Fig. 1C). The effect of allatectomy on the TTP CW of fed larvae is similar to the effect of starvation: Both starvation and allatectomy shorten the TTPCW (Fig. 1D) (4, 15). Thus, genetic ablation of the CA appears to phenocopy starvation in this respect.

Ablation of the Corpora Allata Slows Growth. Because ablation of the CA (i) had no effect on critical weight itself and (ii) did not appear to substantially reduce the TGP, it follows that CAX adults are smaller primarily due to a reduction in growth rate. To test this hypothesis, we measured larval growth from 0 to 30 h AL3E in CAX and sibling controls (Fig. 2). As expected, the growth rate of the CAX larvae was significantly slower than controls (Fig. 2). Further, CAX larvae were already significantly smaller immediately after molting to L3 (Fig. 2), suggesting that JH also affects either the growth rates or duration of the first and/or second instar.

To confirm that the slow growth of the CAX larvae was due to an absence of JH, we fed them a JH mimic, pyriproxyfen, from 0 h AL3E (Fig. 2). CAX larvae fed the JH mimic grew significantly faster than CAX larvae fed solvent (ethanol) alone, although still significantly slower than controls (Fig. 2). We hypothesized, therefore, that the effect of JH on growth was mediated via the IIS pathway.

To test this hypothesis, we compared the expression of known components of the IIS cascade in CAX and control larvae using quantitative PCR. Suppression of IIS activates the transcription factor FOXO (26–28). FOXO then targets the expression of negative growth factors, such as Thor/4E-binding protein (4E-BP) (28). Also, FOXO drives the expression of Insulin receptor (Irr), which in turn represses FOXO activity, creating a negative feedback loop (28). Consequently, the expression of Irr and 4E-BP can be used as proxies for IIS pathway activity (29).

In control larvae, expression of both Irr and 4E-BP declined slightly at the beginning of the L3, but increased to a peak toward the end (Fig. 3 A and B). Ablation of the CA resulted in a significant increase in the expression of Irr and 4E-BP between 0 and 32 h AL3E (Fig. 3 C and D), suggesting that insulin signaling is suppressed in CAX larvae. This early increase in Irr and 4E-BP expression was not a consequence of a general increase in expression level: Expression of Actin was the same in CAX larvae and controls (Fig. S2C). After 32 h AL3E, 4E-BP expression did not increase in CAX larvae as it did in controls, but Irr increased in both CAX and control larvae.

To further confirm that CAX larvae had suppressed insulin signaling, we used the FOXO response element (FRE)-luc reporter construct to assay FOXO activity in CAX and control larvae. The construct contains the firefly luciferase gene under the transcriptional control of the Herpes simplex minimal promoter and eight direct repeats of the FRE (30). FOXO activity can therefore be assayed by measuring luciferase activity. FOXO activity was significantly higher in CAX larvae compared with controls (Fig. 3E), confirming that CAX larvae have suppressed IIS.
**Fig. 2.** Allatectomized (CAX) larvae grow more slowly than controls. Growth in CAX larvae is significantly slower than in control larvae (ANCOVA<sub>protryp</sub>*<sub>age</sub>, *P* < 0.001). CAX larvae are significantly smaller than controls at ecdisis to the third instar (*t* test, *P* < 0.001). The addition of pyriproxyfen, a JH mimic, to the food increases growth rate in CAX larvae (ANCOVA<sub>treatment</sub>*<sub>age</sub>, *P* = 0.0023), although not to the same rate as controls (ANCOVA<sub>treatment</sub>*<sub>age</sub>, *P* = 0.0103). Pyriproxyfen has no effect on the growth rate of control larvae (ANCOVA<sub>treatment</sub>*<sub>age</sub>, *P* = 0.5972). Error bars are 95% confidence intervals and are obscured by the data points in some cases. Lines are from linear regression. EtOH, ethanol. Sample sizes: ANCOVAs, n = 36 (CAX + EtOH), 42 (CAX + JH), 41 (control + EtOH), and 43 (control + JH); *t* test, *n* = 16 (CAX) and 14 (control).

**FOXO Is Necessary for the Size Reduction in CAX Flies.** To test whether the effect of allatectomy on growth rate is FOXO-dependent, we ablated the CA in flies mutant for FOXO. Allatectomy reduced final body size in animals that were wild-type for FOXO, but did not significantly affect final body size in flies mutant for FOXO (Fig. 3G). Thus, FOXO is necessary for the size reduction in CAX flies, suggesting that the effect of JH on growth depends on the IIS pathway.

**Ablation of the Corpora Allata Elevates Ecdysone Signaling.** In *Drosophila*, elevated ecdysone synthesis by the prothoracic gland (PG) can reduce growth rate by suppressing systemic IIS, without affecting developmental timing (2). Further, there is evidence that JH can suppress ecdysone synthesis by the PG in vitro (31). One hypothesis, therefore, is that loss of JH reduces growth rate by derepressing ecdysone synthesis, elevating the ecdysone titer, and suppressing systemic IIS. To test this, we measured ecdysone titers in control and CAX larvae and found that, consistent with our hypothesis, CAX larvae had significantly elevated ecdysone levels (Fig. 3F). We also examined the expression of the B isoform of the ecdysone-induced protein 74EF (E74B) as an indicator of ecdysone signaling, and found that it was also elevated in CAX larvae relative to controls (Fig. S2 A and B). Overall, there was a positive relationship between Inr<sub>4E-BP</sub> expression and E74B expression throughout larval development when controlling for larval age and phenotype (Fig. S2 D and E), supporting the previous observation that ecdysone signaling negatively regulates IIS.

**Knockdown of Met Systemically and in the PG Alone Reduces Final Body Size.** Like CAX larvae, mutant larvae lacking both of the duplicated, putative JH receptor genes Methoprene-tolerant (Met) and germ cell-expressed (gec) (32) grow slowly (Fig. S3). In this case, feeding these larvae the JH mimic pyriproxyfen did not rescue growth to normal rates. Loss of Met alone caused the formation of small pupae and adults, and these effects on size were rescued by ubiquitous expression of Met (Fig. 4A).

The observation that CAX larvae have elevated ecdysone signaling and reduced IIS is consistent with the hypothesis that JH regulates body size by controlling the synthesis and release of ecdysone. In *Manduca*, JH suppresses ecdysteroidogenesis by inhibiting the synthesis of prothoracotropic hormone (PTTH) in the brain (8). In *Drosophila*, however, JH can act directly on the PG to suppress ecdysone synthesis, at least in vitro (31). To identify the tissue through which JH influences ecdysone synthesis, we disrupted JH signaling in individual tissues by knocking down the expression of *Met* with targeted RNAi. Knockdown of *Met* in the PTTH-producing neurons (*ptth>*Met.RNAi) or the entire nervous system (*elav>*Met.RNAi) had no effect on pupal size, whereas knockdown of *Met* in the PG (*phm>*Met.RNAi) significantly reduced pupal size (Fig. 4B). This reduction in pupal size was correlated with a significant reduction in growth rate from 0 to 25 h AL3 (Fig. 4C). Knocking down *Met* in the PG did not affect the duration of the L3 nor the minimal viable weight, a common proxy for critical weight (4, 15, 33, 34) (Fig. 4 D and E).

**Discussion**

Our results show that JH regulates body size in *Drosophila* not by controlling growth duration, as it does in other insects, but by regulating growth rates. The mechanism for this control is FOXO-dependent and appears to be through the JH regulation of ecdysone synthesis, an antagonist of IIS. Below we discuss our results in the context of what has been previously described for size regulation in *Manduca* and *Drosophila*.

**Critical Weight: Variation in Mechanisms.** For both *Manduca* and *Drosophila* larvae, attaining critical weight means that starvation...
no longer delays metamorphosis (4, 14, 16, 35). However, several differences exist in the critical weight of these two insects. First, after Manduca larvae reach critical weight, starvation has no effect on the time to metamorphosis (35). In contrast, when postcritical weight Drosophila larvae are starved, they accelerate their time to metamorphosis (4, 15). In addition, Manduca larvae reach critical weight ~50% into their final instar (35). Drosophila larvae reach critical weight earlier, ~25% into their final instar (24). These differences suggest that the mechanisms regulating critical weight are not identical in the two species.

The physiology of critical weight supports this notion. Manduca larvae allatectomized immediately after the molt to the final instar, then starved, enter metamorphosis 2–2 d earlier than starved sham-operated controls (8). This occurs because JH cannot no longer suppress the release of PTTH, which stimulates ecdysone release and wandering 1.5 d after larvae reach critical weight (8, 11). Application of JH delays PTTH release and wandering (8). However, infusion of ecysone into precritical weight larvae cannot promote premature metamorphosis in final-instar Manduca larvae (8). These classic experiments suggest that the decline of JH in the final instar of Manduca is the primary response to the developmental transition at critical weight (8, 10, 36).

In Drosophila, our data show that JH does not affect critical weight. Rather, previous work suggests that the critical weight transition occurs as a result of an increase in the ecdysone titer early in the L3 stimulated by IIS/TOR signaling in the PG (2–4,24). Thus, where JH appears to regulate critical weight in Manduca, ecdysone appears to regulate critical weight in Drosophila. The significance of this difference in regulation is not yet clear.

Nevertheless, although JH does not appear to regulate critical weight in Drosophila, our data suggest that it does influence ecdysone synthesis. This presents something of a paradox: If JH regulates ecdysone, and ecdysone regulates critical weight, then loss of JH should also affect critical weight. One solution to this paradox is the observation that a moderate change in ecdysone signaling can affect IIS without affecting developmental timing (2, 22). JH may affect the basal levels of ecdysone synthesized by the PG but not influence the timing of ecdysone peaks that coincide with the attainment of critical weight, larval wandering, and pupariation (22).

**Juvenile Hormone Regulation of Growth Rates.** Our research indicates that JH regulates growth in Drosophila and that this regulation is dependent on FOXO, a key effector of the IIS pathway. These data support and extend previous studies that indicate cross-talk between JH and IIS in several holometabolous insects (37).

In Manduca larvae, JH acts to regulate the growth of the imaginal discs in response to changes in IIS (25). As in Drosophila, starving Manduca larvae before attainment of critical weight suppresses growth and development of their imaginal discs. Ablation of the CA overrides these effects so that disc growth and development continue even in the absence of nutrient (23). Similarly, although imaginal discs continue to develop when cultured in a hormone-free but nutrient-rich medium, development is suppressed when JH is added to the medium (38, 39). The growth-suppressing capacity of JH is overridden when the hormone is also added to the medium (38). Thus, JH appears to regulate growth and development of the imaginal discs by sensitizing them to changes in IIS: In the absence of JH, IIS is not necessary for growth and development, whereas in the presence of JH, it is. The same phenomenon does not appear to be acting in Drosophila. In this case, starvation of precritical weight larvae suppressed imaginal disc growth and development, regardless of whether or not they had the CA and thus JH.

In adult Drosophila, females with hypomorphic mutations of the insulin receptor suppress vitellogenesis (39). The effects of suppressing IIS and JH can be reversed through application of methoprene, suggesting that IIS regulates JH synthesis (39). Subsequent studies have demonstrated that IIS regulates JH in the CA of developing larvae. Suppression of IIS in the CA alone is sufficient to inhibit expression of 3-hydroxy-3-methylglutaryl CoA reductase, an enzyme involved in cholesterol and JH biosynthesis (40). The result is a reduction in final body size, genotyping the effects of genetic allatostasis (12) and the MetW3 null mutation (41, 42). Intriguingly, our data suggest that insulin signaling is also downstream of JH, by demonstrating that the reduction in body size caused by AX in larvae correlates with activation of FOXO and is FOXO-dependent. Thus, JH and IIS appear to interact through a positive feedback loop: A reduction in IIS in the CA suppresses JH synthesis, which in turn reduces systemic IIS.

Positive feedback loops are relatively unusual in physiological systems, in part because they tend to cause system instability. The function of the positive feedback loop between JH and IIS is unclear, but may serve to rapidly reduce the level of circulating JH at a particular point in development by suppressing its IIS-regulated synthesis. This relationship between JH and IIS is in contrast to the relationship between ecdysone and IIS, where the interaction forms a negative feedback loop: IIS in the PG promotes ecysoideroidogenesis (2–4), which in turn suppresses systemic IIS and reduces growth rate (6). The effects of ecdysone on growth rates are bidirectional, such that an increase in ecdysone synthesis decreases growth rate, whereas a decrease in ecdysone synthesis increases growth rate (2, 4). In contrast, the influence of JH on growth rates is unidirectional, such that only loss of JH appears to have an effect, whereas addition of JH does not. This may be because JH is not limiting for IIS under normal physiological conditions, and so feeding larvae pyriproxyfen does not further increase growth rate.

Although our data are consistent with the hypothesis that JH regulates growth via the IIS pathway, it is possible that other mechanisms suppress growth via FOXO, followed by subsequent adjustment in IIS components. For example, FOXO activity is
positively regulated by the stress-inducible kinases Jun N-terminal kinase (JNK) and STE20-like protein kinase 1 (MST1) (43). Consequently, ablation of the CA may suppress growth via activation of the JNK- or MST1/2-signaling pathways. However, our data also implicate edcsyne in the JH regulation of growth, which is a known antagonist of IIS. Ecdysone synthesis has been shown to negatively regulate IIS throughout the body through its action on the fat body (2, 6), and JH can inhibit edcsyne synthesis by the PG in vitro in Drosophila (31). Our data connect these two previously unrelated observations and suggest that JH acts in vivo in Drosophila to regulate systemic IIS and organisal growth by controlling edcsyne synthesis. The significance of the JH regulation of edcsyne and IIS during normal Drosophila development requires further elucidation.

Conclusions
Collectively, our data support the hypothesis that there is an intimate link between the processes that regulate developmental transitions, such as puberty and metamorphosis, and the processes that regulate growth. This link provides a physiological context for the observation that, in humans, developmental hormones such as androgens and estrogens also drive growth of several forms of cancer (44, 45) as well as benign tumors such as vascular malformations (46). The observation that both JH and edcsyne regulate growth in a FOXO- and IIS-dependent manner suggests that IIS may be the nexus at which the hormonal regulation of growth rate, growth duration, and developmental transition meets. Studies of such developmental processes may therefore provide key insights into the growth-regulatory pathways that are targeted in hormone-driven cancers.

Materials and Methods

Fly Stocks and Larval Rearing Conditions. The following flies were used in this study: Aug-21 (41); UAS-grim (48); UAS-reaper (Bloomington Drosophila Stock Center; 5824); FRELuc (30); FOCOXII and FOCOXIII (49); Met27,gce2.5k (42); Met27,gce2.5k (32); UAS-Met and the progenitor stock (41); v; UAS-Met.RNAi (Vienna Drosophila RNAi Center (VDRC) 45852) and the progenitor stock (VDRC; 6000); phm-GAL4 (4); elav-GAL4 (Bloomington Drosophila Stock Center; 8760); and ptthII-GAL4 and ptthII-GAL4 (33). All flies were reared at low density on standard cornmeal molasses fly medium at 29 °C as described previously (4, 24), unless otherwise stated.

Ablation of the Corpora Allata. To genetically ablate the CA, we crossed the w; Aug21;CyO actin-GFP fly line (47) with w; UAS-grim. The CAX larvae (Aug21; UAS-grim) were separated from their sibling controls (CyO actin-GFP; UAS-grim) using the absence of GFP. We combined these alleles with FRELuc to assay FOXO activity in CAX and control larvae, described below. We generated CAX FOXO nulls by crossing w; Aug21;CyO actin-GFP; FOXO25/1TM68 with w; UAS-arraysCyO actin-GFP; FOXO21/7TM68. Unlike Aug21;CyO larval (12), 5% of the Aug21;rp larva eclosed as adults. Out of 389 pupae from a cross between w; Aug21;CyO actin-GFP and UAS-rp, 205 eclosing adults were CyO and 10 were not. Nevertheless, when we dissected 20 Aug21;rp white prepupa none had CA, so these larvae are primarily CAX. Similarly, 17 dissected Aug21;rp FOXO25/1FOXO21/7 white prepupa also did not have CA. FOXO25/1FOXO21 transheterozygotes produce no detectable protein (50) and are assumed to be nulls (49).

Larval Weight and Pharyte Adult Size Measurements. For larval weight, we individually weighed larvae using a Mettler Toledo XP2U Ultra-microbalance [readability (d) 0.1 μg] or XP26 Microbalance (d. 1 μg). To compare growth rates, we collected newly molted L3 larvae every 2 h. These larvae were then returned to food and then weighed at the desired age. For the JH rescue experiments, newly molted larvae were fed a JH mimic by adding 5 μg pyriprooxyfen diluted in 50 μL ethanol to 5 mL fly medium (1 ppm pyriprooxyfen) as described previously (12). Control larvae were fed 50 μL ethanol added to 5 mL fly medium.

We calculated growth rates by regressing larval weight against age, and compared among genotypes and hormone treatments by testing for an interaction between age and genotype or treatment using an analysis of covariance (ANCOVA). We measured pharate adult body size either by weighing the pupae or by measuring their total area in the coronal plane (51).

Critical Weight and Developmental Timing. Individual CAX and control (GFP-positive) L3 larvae were weighed and placed in a 1.5-mL microtube with 10 × 50 mm strip of moist Kimwipe. TTP was recorded by checking larvae every 4 h and, if pupariated, larvae were weighed again 24 h later.

We used the relationship between larval weight and TTP to find the critical weight, using the breakpoint method as described previously (15, 20–22). Briefly, the breakpoint method exploits the fact that the relationship between larval mass at starvation and TTP changes at critical weight, and this change can be identified using a bisegmental linear regression. We repeated the analysis on 1,000 bootstrap samples to generate 95% confidence intervals for the critical weight and the TTP when staggered for CAX and control larvae. We used a permutation test with 1,000 replicates to generate a null distribution of the difference in critical weight and TTPCAX in CAX and control larvae, and used this distribution to estimate a P value for the observed differences.

To measure the mean duration of the L3, we collected 4-h cohorts of edcsyne L3 larvae and checked for pupariation every 8 h. The experiment was replicated four times and the mean duration of the L3 was calculated using a mixed-effect ANOVA, with larval genotype as a fixed effect and replicate as a random effect.

To estimate the TTPCAX for fed larvae, we first calculated the time at which larvae attained critical weight by fitting our value for critical weight for the larval growth curve. We then subtracted this from our calculation of the L3 duration of the CA. We conducted the analysis on 1,000 bootstrap samples to generate 95% confidence intervals for the TGP in CAX and control larvae, and used a permutation test to estimate a P value for the observed differences.

Protein Starvation and Immunocytochemistry. To determine the effects of JH on the patterning of wing discs from protein-starved larvae, we transferred 10–15 larvae at 7, 9, or 15 h AL3E to a 20% (w/vol) sucrose solution. We dissected 10–15 larvae immediately for stage controls and returned 10–15 larvae to fly medium as fed controls. Protein-starved and -fed larvae were dissected 24–25 h later and fixed using 4% (vol/vol) paraformaldehyde in PBS for 30 min at room temperature. Samples were processed for immunocytochemistry as described previously (24) using a 1:100 dilution of mouse anti-β-galactosidase (concentrate from the Developmental Studies Hybridoma Bank) and a 1:1,000 dilution of guinea pig anti-Senseless antibody (from Hugo Bellen, Baylor College of Medicine, Houston). We imaged samples using a Leica LSM 510 or 710 multiphoton microscope.

Quantitative PCR. We used two-step quantitative real-time PCR to assay the expression of Intr, 4E-BP, and E74B during the L3 in CAX and control larvae. Larvae were staged into 4-h cohorts at ec dysis to the L3. We sampled ~25 larvae every 4 h from 8 to 44 h AL3E, and dissected larvae from each time point into five biological replicates, each comprising four or five larvae. RNA was extracted using an RNasea Mini Kit (Qiagen) and reverse-transcribed to cDNA using the GoScript Reverse Transcription System (Promega), and transcript levels were assayed using GoTaq Green Master Mix (Promega) using a standard curve and normalized against expression of 28S. Primers are listed in Table S1. Standard curves were generated using seven serial dilutions of total RNA extracted from two first-instar larvae, two second-instar larvae, two L3 larvae (male), two pupae (male), and two adult flies (male) of OregonR. Differences in gene expression between CAX and control larvae in the first 32 h AL3E were analyzed using an ANOVA (Yi) = u + Gi + Ai + ei, where Yi is expression, u is mean expression, G is genotype, A is age, and e is error), treating both age and genotype as categorical variables.

FRELuciferase Assays. We crossed FRELuc–2LRGlq [, wg Gl–1 Bc1; UAS–Grim/TM68 with Aug21;CyO tub-GFP and compared FOXO activity in FRELuc– Aug21; UAS–gim CAX larvae with FOXO activity in FRELuc–CyO tub-GFP; UAS–grim control larvae. We staged larvae into 2-h cohorts at ec dysis to the L3. They were allowed to feed for an additional 24 h and then removed from the food, washed, and stored at ~80 °C. Larvae were divided into three or four replicates of three larvae, homogenized in 200 μL PBS with protease inhibitor, and centrifuged at 15,900 g for 30 min. Then we tested 10 mL of the supernatant for luciferase activity using the Promega Luciferase Assay System. We measured the protein concentration for each sample using a standard BCA assay (Quantipo BCA Assay Kit, Sigma-Aldrich, St. Louis) and normalized the luciferase activity as activity per milligram. We repeated the experiment over two trials and calculated the mean (log) luciferase activity across trials using an ANOVA.

Edcsyne Quantification. Carefully staged larvae were washed twice in distilled water, weighed, and then flash-frozen on dry ice. Larvae were preserved
in three times their volume of ice-cold methanol and kept at −80 °C. To process the samples, we first homogenized the tissue and centrifuged samples at 15,700 × g at 4 °C. The supernatant was transferred into new tubes and the methanol was evaporated off in a vacuumed centrifuge (Savant SVC-100H with a RH 40-11 rotor). Ecdysone concentration was quantified using the Cayman Chemical 20-Hydroxyecdysone EIA Kit according to the manufacturer’s instructions. Differences in ecdysone concentration between CAX and control larvae in the first 3 h AL3E were analyzed using an ANOVA (Y = u + G × A + e, where Y is ecdysone concentration, u is the mean, G is genotype, A is age, and e is error), treating both age and genotype as categorical variables.

**Mutant Analysis.** We compared pharate adult size of MetW3; Act-Gal4/UAS-Met and MetW3/Act-Gal4/UAS-Met, and v; Act-Gal4 (42) as described above. We knocked down expression of Met in specific tissues using UAS-Met-RNAi (VDCR, 45852) combined with phm-GAL4 (PG), elav-GAL4 (nervous system), and ptth-GAL4 and ptthFL (PTTH-producing neurons). Coisogen control larvae were generated from the RNAi progenitor stock (VDCR, 6000) combined with the GAL4 lines. We used minimal viable weight for pupariation (MVW/P) as a proxy for critical weight for phm-Met-RNAi and controls (4, 15, 33, 33, MVW/P) is defined as the minimal weight at which 50% of larvae survive to pupariation when starved. We used a nominal logistic regression to predict the weight at which 50% of the starved larva survive to pupariation. Larvae were reared at 25 °C.

**Acknowledgments.** We thank Dr. Hugo Bellen for the Senseless antibody. The Wingless antibody was from the Developmental Studies Hybridoma Bank, University of Iowa. We thank Aaron Baumberger for the UAS-Ace-RNAi flies, Jian Wang for the MetRevG, goc324 flies, and Jim Trump for comments on the manuscript. This work was funded by National Science Foundation Grants IOS-0919855 and IOS-0845847 (to A.W.S.), the Howard Hughes Medical Institute (C.K.M. and L.M.R.), the Fundação Calouste Gulbenkian (T.K. and C.K.M.), and the Fundação para a Ciência e a Tecnologia [SRH/Bos/Do Pós-Doutoramento (BPD)/74313/2010; to T.K.].

Fig. S1. Ablation of the corpora allata (CAX) influences when critical weight is attained in Drosophila. Arrowheads point to sensory organ precursors, and asterisks denote chordotonal organs. To starve larvae of protein, we transferred them to a 20% sucrose solution in water for 24 h. Starvation starting at 7 h third-instar ecdysis (AL3E) suppresses the expression of Wingless (Wg) and Senseless (Sens) in both CAX (A–B′) and control (C–D′) wing imaginal discs, indicating that the larvae have not yet attained critical weight. Starvation 15 h AL3E does not suppress the expression of Wg or Sens in CAX (I–J′) and control (L–M′) discs, indicating that all larvae have attained critical weight and up-regulated ecdysteroidogenesis. In contrast, starvation at 9 h AL3E suppresses Wg and Sens expression in CAX wing imaginal discs (E–F′) but not in control wing imaginal discs (G–H′), indicating that the latter have attained critical weight whereas the former have not. In well-fed larvae, allatectomy has no effect on the timing of wing imaginal disc development (compare K and K′ with N and N′). For each sample, we examined wing discs from 10–15 larvae. (Scale bar, 100 μm.)
Fig. S2. Loss of juvenile hormone (JH) up-regulates ecdysone signaling, which correlates with a suppression of insulin/insulin-like growth factor signaling. (A) The expression profile of E74B throughout larval development in CAX and control larvae (n = 5 biological replicates for each data point). (B) Expression of E74B is significantly up-regulated from 0 to 32 h AL3E in CAX larvae relative to control [analysis of covariance (ANCOVA) P < 0.001 for both; n = 35 for CAX and control]. (C) The levels of actin expression are not elevated in CAX larvae relative to controls 24 h AL3E (t test, P = 0.637; n = 5). (D and E) There is a positive correlation between the expression of E74B and (D) Inr and (E) 4E-BP, across both genotypes (CAX and control) and all larval ages. Both relationships are significantly positive when controlling for the effect of genotype and larval age (ANCOVA, F > 37.8, P < 0.001 for both; n = 88). Error bars are 95% confidence intervals.

Fig. S3. JH mimic, pyriproxyfen, does not rescue the reduced growth rate of met,gce mutant larvae. Larvae mutant for met and gce show reduced growth rate (ANCOVA genotype*age, P < 0.001). The addition of pyriproxyfen to the food does not affect the growth rate of either met,gce mutants (ANCOVA treatment*age = 0.5835) or sibling controls (ANCOVA treatment*age = 0.5374). Error bars are 95% confidence intervals and are obscured by the data points in some cases. Lines are from linear regression. EtOH, ethanol. Sample sizes: ANCOVAs: n = 93 (met,gce + EtOH), 88 (met,gce + JH), 122 (control + EtOH), and 139 (control + JH).

Table S1. Primers used for quantitative PCR

<table>
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<th>Gene product</th>
<th>Direction</th>
<th>Primer (5′-3′)</th>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAATGAGGACTCATCCGCC</td>
</tr>
<tr>
<td>Inr</td>
<td>Forward</td>
<td>GACAAGGAGGCTCAAACCC</td>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
<td>Thor4E-BP</td>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
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<td>ATCGGGGGGCTACAGAGG</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGACAGCAATTTGGACATAC</td>
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