The Temporal Requirements for Insulin Signaling During Development in *Drosophila*

Alexander W. Shingleton1*, Jayatri Das1, Lucio Vinicius2, David L. Stern1

1 Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey, United States of America, 2 Leverhulme Centre for Human Evolutionary Studies, University of Cambridge, Cambridge, United Kingdom

Recent studies have indicated that the insulin-signaling pathway controls body and organ size in *Drosophila*, and most metazoans, by signaling nutritional conditions to the growing organs. The temporal requirements for insulin signaling during development are, however, unknown. Using a temperature-sensitive insulin receptor (Inr) mutation in *Drosophila*, we show that the developmental requirements for Inr activity are organ specific and vary in time. Early in development, before larvae reach the “critical size” (the size at which they commit to metamorphosis and can complete development without further feeding), Inr activity influences total development time but not final body and organ size. After critical size, Inr activity no longer affects total development time but does influence final body and organ size. Final body size is affected by Inr activity from critical size until pupariation, whereas final organ size is sensitive to Inr activity from critical size until early pupal development. In addition, different organs show different sensitivities to changes in Inr activity for different periods of development, implicating the insulin pathway in the control of organ allometry. The reduction in Inr activity is accompanied by a two-fold increase in free-sugar levels, similar to the effect of reduced insulin signaling in mammals. Finally, we find that varying the magnitude of Inr activity has different effects on cell size and cell number in the fly wing, providing a potential linkage between the mode of action of insulin signaling and the distinct downstream controls of cell size and number. We present a model that incorporates the effects of the insulin-signaling pathway into the *Drosophila* life cycle. We hypothesize that the insulin-signaling pathway controls such diverse effects as total developmental time, total body size and organ size through its effects on the rate of cell growth, and proliferation in different organs.


**Introduction**

Development in multicellular animals is a process that involves both tight control and flexibility in the regulation of cell size and cell number. Tight control is necessary to produce an animal in which each organ is of an appropriate size relative to the whole body. Flexibility is necessary to produce an animal in which the whole body is of an appropriate size relative to environmental conditions. One key environmental factor shown to influence size is nutrition. In animals as diverse as humans and flies, malnutrition delays development and reduces adult body and organ size. Recent studies indicate that the insulin-signaling pathway (Gene Ontology ID GO:0008286) coordinates growth with nutritional condition in most metazoans, and is remarkably conserved. In *Drosophila*, expression of insulin-like peptides is nutritionally regulated [1], as is expression of IGF1 (insulin-like growth factor 1) in developing rats [2]. Flies carrying mutations of the insulin receptor (Inr) and mice carrying mutations of the IGF1 receptor show delayed development and growth deficiency with a reduction in body and organ size [3–6]. These and similar “knock out” experiments have demonstrated the gross effects of the insulin-signaling pathway on adult phenotype. Nevertheless, little is known of how the pathway acts during development to affect changes in the adult. In mice, such elucidation is hampered by the inaccessibility of the developing fetus enclosed in the uterus. In flies, however, developing larvae can be easily studied and manipulated.

Here we explore the temporal requirement for insulin signaling in developing *Drosophila melanogaster*. In *Drosophila*, development proceeds through three larval instars to pupariation, pupation, and finally adult eclosion. Feeding is restricted to the first (L1), second (L2), and most of the third (L3) instar. Early in the third instar, the larvae reach a “critical size” at which point they have acquired sufficient nutrients to complete development in the absence of food [7–10]. After critical size is attained, larvae stop feeding, leave the food, and search for a pupariation site. There is, however, a delay between the time critical size is reached and the time larvae stop feeding and reach their maximum body size. Both critical size and the time between reaching critical size and the cessation of feeding are unaffected by nutrition [8,10]. Consequently, nutrition ostensibly affects final adult size through the amount of feeding in the fixed period between critical size and pupariation [10].

Because feeding affects final size only in the period between critical size and pupariation [8,10], one may predict that insulin signaling affects size only during the same period. However, organ growth continues well after pupariation [11,12] and is autonomously modulated by the insulin-
signaling pathway [5,13]. Insulin signaling may therefore continue to influence size after pupariation. Details of the temporal requirements for insulin signaling are unknown in Drosophila, but a recent study on the Drosophila forkhead transcription factor (dFOXO) suggests that they vary during development [14]. Unphosphorylated dFOXO negatively regulates growth, but activation of the insulin receptor signaling pathway induces dFOXO phosphorylation, excluding it from the nucleus and inhibiting its activity [15,16]. Constitutive activation of dFOXO in the first and second instars causes developmental arrest, whereas dFOXO activation in the third instar causes a reduction in adult size [14]. Work on Caenorhabditis elegans also indicates that the timing requirements for insulin signaling might vary during the lifecycle. In C. elegans, the pathway acts during early development to regulate diapause, and during adulthood to influence ageing [17]. Resolving the links between larval size, nutrition, and developmental progression on the one hand, and the time-dependence of insulin-pathway gene effects on the other, requires a more precise understanding of the temporal requirements for insulin signaling during development.

We have used a temperature-sensitive mutation of the insulin receptor to investigate the role of the insulin-signaling pathway during different stages of development in Drosophila. By reducing insulin receptor activity at different points in development, we identify the periods of development during which insulin signaling affects adult phenotype. We show that total developmental time is affected by changes in insulin signaling only if those changes occur early in development, before a larva has reached critical size. In contrast, final body and organ size are affected by changes in insulin signaling only if those changes occur late in development, once a larva has passed critical size. Insulin signaling continues to influence organ size, but not body size, after larvae have stopped feeding and throughout much of pupal development. Not all organs respond equally to changes in insulin signaling, however. We find that the genitals show a limited response to suppression of insulin signaling in Inr

Results

Temperature-Sensitive Suppression of the Insulin Receptor

During a study of the interaction of environmental factors and the insulin-signaling pathway, we discovered that flies trans-heteroallelic for insulin receptor mutations Inr

However, Inr

Figure 1. Temperature Sensitivity in Inr

However, Inr

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when the larvae are moved to 25°C for 12 h (Figure 2E and 2F). Localization does not appear to be lost at 25°C in InrE19 or InrGC25/TM6B-Tb controls (Figure 2G and 2H). The phenotypic effects of temperature on InrE19/InrGC25 flies do, therefore, appear to be a consequence of temperature-sensitive suppression of the insulin-signaling pathway. However, we do not know the molecular basis for the temperature sensitivity of the InrE19/InrGC25 transheterozygotes. It may arise from temperature-sensitive expression of one or both of the mutant alleles or from decreased function of the mutant receptor at higher temperatures.

Due to the low viability of InrE19/InrGC25 flies reared at 25°C, all subsequent experiments involved comparisons of flies reared at 17°C with flies reared at 24°C. Further, we used the InrE19/TM3 siblings reared under identical conditions as controls. InrE19/TM3 flies show only a moderate reduction in Inr activity and have a slight reduction in body size relative to wild-type [4]. Because temperature affects overall body-size in

Figure 2. Increasing the Temperature of InrE19/InrGC25 Flies Suppresses the Insulin-Signaling Pathway
The dFOXO panel shows localization of dFOXO protein in the fat body, the propidium iodide panel shows the position of the nuclei, and the merge panel clarifies the degree of dFOXO localization to the nuclei.
(A) Endogenous dFOXO in the fat body of InrE19/InrGC25 third instar larvae has weak nuclear localization at 17°C.
(B) Increase in rearing temperature causes a decrease in cytoplasmic distribution and an increase in nuclear localization of dFOXO, consistent with a decrease in the level of insulin signaling.
(C and D) Temperature has no detectable effect on dFOXO localization in InrE19/TM3 control flies.
(E) GPH membrane localization reveals high levels of insulin signaling in the fat body of InrE19/InrGC25 second instar larvae reared at 15°C. GPH is in green, DNA is stained blue.
(F) This localization is lost when the larvae are moved to 25°C for 12 h, consistent with a decrease in the level of insulin signaling.
(G and H) Temperature has no detectable effect on GPH membrane localization in InrE19/TM3 control flies.
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wild-type flies [19], it is necessary to distinguish between changes in \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) phenotype that are a consequence of changes in the level of \( \text{Inr} \) expression from those resulting from changes in rearing temperature. To do this we report the phenotype of \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies as a percentage of the phenotype of \( \text{Inr}^{E19/\text{TM3}} \) control flies that have undergone temperature shifts at the same developmental time.

The Changing Role of Insulin Signaling during Development

We used the temperature-sensitive \( \text{Inr} \) mutants to investigate the role of the insulin pathway during \( Drosophila \) development. We reduced \( \text{Inr} \) activity during different periods of development by transferring \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies from a permissive 17 °C to a restrictive 24 °C at different points in development. After the switch, the flies were left to complete development at 24 °C. We were able to identify temperature-sensitive periods (TSPs) during which increasingly earlier switches from 17 °C to 24 °C resulted in increasingly abnormal phenotypes.

Total developmental time is sensitive to \( \text{Inr} \) activity only before the middle of the third larval instar (Figure 3A). Switching \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies from 17 °C to 24 °C changes the time to adult eclosion during the first 9 d of development; the earlier the switch, the greater the delay in eclosion. After the ninth day of development at 17 °C, when the flies are approximately 40% through the third instar (Figure 3B), a shift to the restrictive temperature does not delay adult eclosion. At 17 °C, \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies show a slight delay in eclosion relative to \( \text{Inr}^{E19/\text{TM3}} \) flies, suggesting that \( \text{Inr} \) activity is still a little impaired at this temperature. The delay in \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies reared at both 17 °C and 24 °C relative to the \( \text{Inr}^{E19/\text{TM3}} \) controls occurs predominantly through extension of the third instar (Figure 4).

In contrast, adult wing area is sensitive to \( \text{Inr} \) activity only during late third instar and early pupation (see Figure 3A). Switching \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies from 17 °C to 24 °C changes adult wing size between day 9 and 20; the earlier the shift, the smaller the wings. The precise TSP of female \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies for wing area may be smaller than implied by Figure 3A because the data are based on population rather than individual measures. The wings may be insensitive to changes in \( \text{Inr} \) activity as early as day 17. Before day 9, however, shifting the flies to the restrictive temperature earlier in development has no additional effect on adult wing size. At 17 °C, the wings of \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies are slightly smaller than in \( \text{Inr}^{E19/\text{TM3}} \) control flies, again suggesting that \( \text{Inr} \) activity is marginally reduced at this temperature.

We tested whether total body mass was sensitive to \( \text{Inr} \) activity over the same period as wing size. We compared the dry mass of adult \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) males reared under several thermal conditions: 24 °C, 17 °C, and a series of samples switched from 17 °C to 24 °C on days 9 through 16 (pupariation is at approximately day 13 at 17 °C). Adult body mass is sensitive to reduction in \( \text{Inr} \) activity between day 9 and day 13 at 17 °C (see Figure 3C). Shifting \( \text{Inr}^{E19/\text{Inr}^{GC25}} \) flies to the restrictive temperature after pupariation, and therefore after larvae have stopped feeding, has no influence on adult mass. Shifting the flies earlier than day 9 has no additional effect on adult body mass.

\( \text{Inr} \) activity therefore influences total development time, adult wing size, and adult mass for different periods of

\[ \text{Inr}^{E19/\text{Inr}^{GC25}} \]
development. We tested whether the time at which down-regulation of the insulin pathway switches from delaying development to reducing the size of the resulting flies coincides with attainment of critical size. In practice, the critical size is determined as the size at which 50% of larvae proceed to pupariation in the absence of food [8]. We measured the timing of critical size in Inr E19/Inr GC25 flies reared at 17 °C under our experimental conditions and found that it is attained at approximately day 9 (see Figure 3D), coinciding with the time at which Inr activity switches from affecting developmental time to body size. Therefore, larval critical size coincides with the shift in Inr function.

A Reduction in Inr Activity Changes Body Chemistry

To better understand why reduction of Inr activity after pupariation results in an allometric change in body size, we measured the content of protein, lipid, glycogen, and sugar of flies reared at 17 °C (Table 1). There is no similar response in protein, glycogen, or lipid levels. Lipid levels are, however, elevated in Inr E19/Inr GC25 flies reared at 17 °C up to pupariation, then either switched to 24 °C or maintained at 17 °C.

Reducing Inr activity after pupariation results in an approximate doubling of free-sugar concentration in Inr E19/Inr GC25 flies (Table 1). There is no similar response in protein, glycogen, or lipid levels. Lipid levels are, however, elevated in both Inr E19/Inr GC25 flies maintained at 17 °C and those switched to 24 °C at pupariation relative to Inr E19/ TM3 flies (Table 1). Inr E19/Inr GC25 flies grown at a restrictive 24 °C for their entire development also have elevated lipid levels (138% ± 13.3 of Inr E19/TM3 flies). This suggests that the slight deficiency in insulin signaling at 17 °C may account for the entire effect on lipid levels.

A Reduction in Inr Activity Affects Different Organs Differently

Different organs typically grow at different rates in developing animals, a phenomenon called “allometry.” Patterns of allometry in populations can also result from organs growing at the same rate but starting and stopping growth at different times in development [20]. We investigated whether the insulin-signaling pathway might be involved in the regulation of allometry by examining whether different organs respond differently to reductions in Inr activity induced by a temperature shift of Inr E19/Inr GC25 flies from 17 °C to 24 °C. We examined organs located at the anterior, median, and posterior of males: the maxillary palp, wing, and genital arch. We chose the maxillary palp rather than another anterior organ because it is similar in size to the genital arch, allowing us to control for absolute size in this comparison.

Figure 5 shows the relative areas of wings, genital arch, and maxillary palps of male Inr E19/Inr GC25 and male Inr E19/TM3 flies reared at either 17 °C or 24 °C. At 17 °C, all three organs are smaller in Inr E19/Inr GC25 males than Inr E19/TM3 males. At 24 °C, the wing and maxillary palps are further reduced in Inr E19/Inr GC25 males, consistent with a further reduction in the level of Inr activity. In contrast, there is no difference in the size of the genital arches, relative to Inr E19/TM3 males, in flies reared at 17 °C and 24 °C.

This suggests that the size of the genital arches may not be regulated by Inr activity. However, the arches are smaller in Inr E19/Inr GC25 males than in Inr E19/TM3 males at both rearing temperatures. It is possible that this size difference is a consequence of genetic background, unrelated to differences in Inr activity in the two genotypes. Alternatively, the genital arches may show a limited response to changes in Inr activity. They may be sensitive to a mild reduction in Inr activity experienced by Inr E19/Inr GC25 flies reared at 17 °C, but be insensitive to a further reduction experienced by Inr E19/Inr GC25 flies reared at 24 °C.

To distinguish between these two hypotheses, we generated large Minute clones homozygous for chico 1. Homozygous chico 1 clones produce phenotypes identical to mutant Inr clones [13] and autonomously cause a dramatic size reduction in fly wings and eyes, whereas the heterozygous chico 1 cells behave as wild-type [5]. We identified males that were homozygous for chico 1 throughout one side of the genitals and heterozygous for chico 1 on the other. If genital size is independent of the insulin-signaling pathway, then the genitals from either compartment should be identical in size. Each comparison was made within a single male, automatically controlling for total body size. We found that genital arches consisting of mutant chico 1 clones were 16% smaller than paired genital arches on the same male (genital arch area: chico 1 mutant = 2,840 ± 60 μm², chico 1 wild-type = 3,230 ± 110 μm², paired-sample t test: n = 6, t = 1.67, p = 0.0214). This reduction of 16% is consistent with the 16% reduction observed in Inr E19/ Inr GC25 males relative to controls (Figure 5). In contrast, maxillary palps consisting of mutant chico 1 clones were 45% smaller than paired palps on the same male (maxillary palp area: chico 1 mutant = 4,820 ± 100 μm², chico 1 wild-type = 8,640 ± 170 μm², n = 7). The genital arches do, therefore, show a limited response to changes in insulin signaling. They are sensitive to a mild reduction in Inr activity, such as observed in Inr E19/Inr GC25 flies reared at 17 °C. Further reduction in Inr activity, such as observed in Inr E19/Inr GC25 flies reared at 24 °C, has no additional effect on genital arch size but does have an effect on maxillary palp and wing size.
A Reduction in Inr Activity Affects Cell Size and Cell NumberIndependently

To investigate the cellular basis for the effect of reduction of Inr activity on size, we compared the size and number of epidermal wing cells in InrE19/InrGC25 flies with InrE19/TM3 flies, reared at 17°C and 24°C. The wing-size difference between InrE19/InrGC25 and InrE19/TM3 flies reared at 17°C is due to smaller cells but not fewer cells in the wings of InrE19/InrGC25 males (compare relative sizes at 17°C, Figure 6, Table S2). In InrE19/InrGC25 females reared at 17°C, cell size is also smaller than in InrE19/TM3 females, but there are also slightly fewer cells. In both males and females, the additional difference in wing-size observed in InrE19/InrGC25 relative to InrE19/TM3 flies reared at 24°C is due to fewer, but not smaller, cells: Cell size in InrE19/InrGC25 wings relative to InrE19/TM3 wings is the same at both 17°C and 24°C (Figure

Table 1. Protein, Sugar, Glycogen, and Lipid Content of InrE19/InrGC25 and InrE19/TM3 Flies Reared at 17°C until Pupariation and then Switched to 24°C or Maintained at 17°C

<table>
<thead>
<tr>
<th>Content</th>
<th>Pupation Temperature</th>
<th>Genotype</th>
<th>Relative Levels in InrE19/InrGC25a</th>
<th>F-Test</th>
<th>Genotype × Temperatureb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>17°C</td>
<td>InrE19/TM3</td>
<td>13.8 ± 0.282</td>
<td>14.3 ± 0.512</td>
<td>104% ± 4.24</td>
</tr>
<tr>
<td></td>
<td>24°C</td>
<td>InrE19/InrGC25</td>
<td>12.3 ± 0.530</td>
<td>13.6 ± 0.693</td>
<td>111% ± 7.08</td>
</tr>
<tr>
<td>Sugars</td>
<td>17°C</td>
<td>InrE19/InrGC25</td>
<td>0.0395 ± 0.00630</td>
<td>0.0294 ± 0.00548</td>
<td>74% ± 21.2</td>
</tr>
<tr>
<td></td>
<td>24°C</td>
<td>InrE19/InrGC25</td>
<td>0.0394 ± 0.00627</td>
<td>0.0282 ± 0.0103</td>
<td>210% ± 30.6</td>
</tr>
<tr>
<td>Glycogen</td>
<td>17°C</td>
<td>InrE19/InrGC25</td>
<td>0.0842 ± 0.0287</td>
<td>0.138 ± 0.0244</td>
<td>164% ± 44.8</td>
</tr>
<tr>
<td></td>
<td>24°C</td>
<td>InrE19/InrGC25</td>
<td>0.0849 ± 0.0127</td>
<td>0.0946 ± 0.0142</td>
<td>111% ± 22.4</td>
</tr>
<tr>
<td>Lipids</td>
<td>17°C</td>
<td>InrE19/InrGC25</td>
<td>0.242 ± 0.0138</td>
<td>0.336 ± 0.0268</td>
<td>139% ± 12.5</td>
</tr>
<tr>
<td></td>
<td>24°C</td>
<td>InrE19/InrGC25</td>
<td>0.264 ± 0.0146</td>
<td>0.315 ± 0.0185</td>
<td>119% ± 8.92</td>
</tr>
</tbody>
</table>

*Expressed as percent of InrE19/TM3 control.
*Tests whether protein, sugar, glycogen, or lipid levels differ between InrE19/InrGC25 and InrE19/TM3 independent of temperature.
*Test whether the effect of temperature on body chemistry differs between InrE19/InrGC25 and InrE19/TM3 flies, that is, whether a reduction in Inr activity in temperature-sensitive InrE19/InrGC25 flies influences body chemistry.
*All concentrations are milligrams per milligram of total dry mass.
*p < 0.01, **p < 0.001.

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Figure 5. Different Organs Respond Differently to Suppression of Inr Activity
Bars show organ area in InrE19/InrGC25 males as a percentage of area in InrE19/TM3 males, to control for temperature effects. Bars with different letters indicate organs that differ: A, B, and C are significantly different at α = 0.05 (Tukey-Kramer pairwise comparison). Mean areas of all organs given in Table S1. s.e., standard error.
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addition, varying the magnitude of Inr activity has different effects on cell size and cell number.

**Insulin Signaling, Critical Size, and Developmental Delay**

We found that a reduction in Inr activity after critical size does not delay development (see Figure 3A). Therefore reducing Inr activity affects total developmental time by delaying the time at which larvae reach critical size. Critical size has been identified as the key stage in insect maturation that determines the point at which, in holometabolous insects, a larva becomes committed to metamorphosis [21]. In Drosophila it is also the minimal viable weight necessary to survive pupation. Consequently, because larvae can complete development without any additional feeding after reaching its critical size, the critical size sets the lower limit of final adult size.

How insects measure critical size is largely unknown. Our results indicate that a reduction in Inr activity delays the point at which Drosophila larvae reach critical size. We hypothesize that critical size measurement involves a specific organ or organs, and that it is the slow growth of this organ or organs that delays development in Inr mutants. Possible candidates include the imaginal discs and the fat body. Regeneration of damaged imaginal discs delays pupariation in Drosophila [22,23], indicating that some or all of the imaginal discs need to grow to a particular size before a larva can pupariate. Complete removal of the discs does not, however, delay pupariation [23]. Another organ must therefore measure critical size and initiate pupariation, with the immature imaginal discs inhibiting pupariation. Slow growth of either the imaginal discs or a “critical-size organ” could delay development in Inr mutants. Recent studies suggest this “critical-size organ” could be the fat body, which functions as a nutrient sensor and is involved in the coordination of organismal growth [24]. Suppressing Inr/PI3K signaling in the fat body alone is sufficient to inhibit larval growth and mimics the effects of starvation [18].

**Insulin Signaling and Final Body and Organ Size**

A reduction in Inr activity after critical size reduces final adult size and organ size (see Figure 3A and 3C). Inr activity influences adult body size and wing size for different periods of development. As expected, adult body size is influenced by Inr activity only between critical size and pupariation, after which the larva does not feed and becomes a “closed system,” neither gaining nor losing mass. However, insulin signaling continues to influence the final size of adult organs well into the pupal stage. Inr E19/Inr GC25 flies switched from 17°C to 24°C at pupariation have the same mass as Inr E19/Inr GC25 flies maintained at 17°C, but have reduced wings. At the same time, the temperature-shifted flies have a much higher free-sugar concentration as adults. These two findings appear to be linked. Considerable cell proliferation in the imaginal discs occurs after the larva has stopped feeding [11,12,25], and this proliferation relies entirely on stored nutrients as an energy source. Nutrient storage occurs predominantly in the fat body cells, which accumulate reserves of proteins, lipids, and glycogen (the major carbohydrate storage compound) during the third larval instar [18]. Both starvation and suppression of the insulin pathway cause these nutrients to be mobilized for use by growing cells [18]. The finding that Inr E19/Inr GC25 mutants reared at 24°C have elevated free-
sugar levels, but do not have elevated glycogen levels, indicates that they are able to mobilize their carbohydrate reserves, but that the free-sugars are not taken up by growing organs, and remain in the haemolymph.

Insulin Signaling and Cell Size and Cell Number

Any mechanism that influences organ size does so by changing cell size, cell number, or both. Although mutations of Inr, PI3K, and chico reduce both cell size and cell number [4,5,13,26], downstream components of the insulin-signaling pathway can affect cell size and cell number independently. The RPS6-p70-protein kinase (S6K) branch of the pathway appears to influence cell size but not cell number [27], whereas the dFOXO branch appears to influence cell number but not cell size [15,16]. Because these signaling branches diverge downstream of the insulin receptor, it has not been clear how insulin signaling could affect cell size and number differentially. These changes are ultimately a consequence of changes in the relative rates of cell growth and division [28]. For example, the reduction in cell size, but not number, in S6K mutants implies a reduction in the rate of cell growth but not of cell division. Conversely, the reduction in cell number, but not cell size, when dFOXO is over-expressed implies that the rates of cell growth and division are reduced equally. Finally, a reduction in both cell size and cell number will result if both the rates of cell growth and division are reduced, if the former is reduced to a greater extent than the latter.

Our results support the hypothesis that insulin signaling differentially affects cell size and cell number via different levels of insulin receptor activity. Slightly reduced levels of activity, as in Inr^{E19/Inr GC25} flies raised at 17 °C, reduce cell size, possibly through a reduction in the rate of cell growth but not of cell division. Further reductions in the levels of activity, as in Inr^{E19/Inr GC25} flies raised at 24 °C, reduces cell number only, possibly through a subsequent balanced reduction in both the rate of cell growth and cell division. These data are consistent with a result from Bohni et al. [5]. They showed that the wings of chico^2 homozygotes are smaller due to a reduction of both cell size (17%) and cell number (27%). However, a further reduction in insulin signaling through the removal of a single copy of Inr enhances the small-size phenotype exclusively through a reduction in cell number but not cell size. Although we cannot exclude the possibility that the differences in cell size between Inr^{E19/Inr GC25} and Inr^{E19/TM3} flies are a consequence of genetic background unrelated to differences in Inr activity, the reduction in cell number alone is clearly due to reduction in insulin signaling (see Figure 5).

Insulin Signaling and Allometry

Variation in insulin signaling appears to affect the allometric relationship between organs. For example, at 17 °C, male Inr^{E19/Inr GC25} flies have body size, wings, maxillary palps, and genital arches approximately 85% of wild-type. Increasing the rearing temperature of Inr^{E19/Inr GC25} flies from 17 °C to 24 °C, however, causes a further reduction in wing, maxillary palp, and overall body size but does not affect the size of the genital arches (Figure 5). Consequently, flies reared at 24 °C have larger genitals relative to their body and wings compared to flies reared at 17 °C. The apparently restricted response of the genitals to changes in insulin signaling is not a consequence of the particular alleles used in this study: chico-mutant clones also have much less of an effect on size when they are in the genital arches than when they are in the maxillary palps.

The mechanism by which different organs respond differently to changes in insulin signaling is unclear. Organs may vary in their expression of the insulin receptor gene or may limit the activity of certain downstream components of the insulin-signaling pathway. In 17 °C Inr^{E19/Inr GC25} males, the genital arches, wings, and maxillary palps are all reduced by approximately the same amount relative to 17 °C Inr^{E19/TM3} males. In the wing, this reduction in area is a consequence of a reduction in cell size. A further decrease in Inr activity (through an increase in rearing temperature to 24 °C) reduces wing area through a reduction in cell number alone. If the genital arches are like the wing, then their response to insulin signaling may be restricted to changes in cell size and not cell number. The cells of the genital arches may therefore be deficient in components of the insulin-signaling pathway that regulate cell number but not components that regulate cell size.

A Model of the Insulin-Signaling Regulation of Growth and Development

The insulin-signaling pathway appears to play a different role after critical size than before. Similarly, a temperature-sensitive lethal mutation, l(1)ts-1126, which reduces the rate of cell proliferation in Drosophila, delays pupariation when larvae are moved to a restrictive temperature before the third instar, but reduces adult size when larvae are moved to a restrictive temperature late in the third instar [29]. The two effects of reduced Inr activity may therefore result from the same process: a reduction in the rate of cell growth and proliferation. We have developed a model of the insulin-signaling regulation of growth and development in Drosophila (Figure 7). (A similar model has recently been developed by Davidowitz and Nijhout [30] to explain variation in body size in response to temperature in the tobacco hornworm, Manduca sexta.) A reduction in Inr activity prior to critical size slows cell growth and proliferation and delays the time at which the larvae reaches critical size. Critical size is not substantially influenced by nutritional conditions [8] or insulin signaling in Drosophila, although this does not seem to be the case for all insects, for example, M. sexta [21]. Once critical size is reached, the time to pupariation and adult eclosion is fixed, as are the remaining periods of growth prior to adult differentiation of individual imaginal discs. The duration of these intervals is un influenced by nutritional conditions or insulin signaling. A reduction in Inr activity during these periods also slows cell growth and proliferation, but now reduces the amount of growth attained before differentiation, resulting in smaller organs and a smaller fly. Because different structures grow for different periods, they are sensitive to Inr activity at different times. For example, wing size begins to be insensitive to changes in Inr activity at approximately the same time as cell proliferation ceases, around 25% into the pupal stage. Adult body mass becomes insensitive to changes in Inr activity just before pupariation, when the larvae stops feeding and final body size is fixed.

A key component of this model is that after critical size, the remaining periods of growth of individual imaginal discs and of the body as a whole are fixed and uninfluenced by insulin
signaling. Our data show that the length of the period between critical size and pupariation, the remaining period of growth of the body as a whole, is not substantially affected by a reduction in Inr activity. In Drosophila and other insects, this interval is controlled by endocrine events. In Drosophila, a small peak in the ecdysteroid titer coincides with attainment of critical size [31], followed by a second peak 12 h later, just before the larvae leave the food [32]. In M. sexta this second peak acts directly on the nervous system to initiate wandering behavior [33], and the same is likely true for Drosophila [34]. The period in which Inr activity can influence final body size is therefore terminated by hormones. Importantly, hormones other than insulin also control the period of cell proliferation in the imaginal discs. For example, in M. sexta, ecdysteroids govern the phases of eye development during metamorphosis [35, 36]. When the ecdysteroid titer rises above a minimum threshold just before pupariation, it stimulates a wave of cell proliferation to pass across the eye primordium. This proliferation is sustained until the ecdysteroid titer rises above a maximum level in the middle of pupal development, whereupon cell proliferation ceases and maturation of the ommatidia begin. These and similar data in other insects [37–39], including Drosophila [40–43], suggest that cell proliferation in imaginal discs may be temporally regulated by thresholds of sensitivities to fluctuating levels of ecdysteroids and juvenile hormone (JH) [44]. Different organs have different thresholds of sensitivity and hence grow for different periods of time. Insulin signaling may therefore control body and organ size by regulating the amount of growth attained during these periods of cell proliferation.

This model requires that changes in ecdysteroid and JH levels are unaffected by the insulin-signaling pathway. It is known that adult Inr and chico mutant flies have reduced levels of JH and impaired ovarian ecdysone synthesis [45, 46]. However, the same hormone fluctuations that putatively control the period of cell proliferation also initiate pupariation [34] and, because the timing of pupariation is unaffected by Inr activity, it seems likely that the temporal dynamics of the hormonal cascade in the larvae are also unaffected by Inr activity. We predict, therefore, that the insulin-signaling pathway regulates cell proliferation in imaginal discs but that the duration of proliferative phases is controlled by other endocrine cues, such as JH and ecdysteroids, that are themselves unaffected by the insulin-signaling pathway.

This model demonstrates how the pleiotropic effects of insulin signaling on developmental time and final body and organ size can be separated, and may be available for independent evolutionary modification. For example, changes in the relative size of an organ may occur by organ-specific modifications in its growth response to insulin signaling, through organ-specific changes in the expression of Inr, adjustments in Inr activity, or adjustments in the expression and activity of downstream components of the insulin-signaling pathway. Alternatively, changes in the period of an organ's growth, through alterations in its sensitivity to other endocrine cues, may have a similar effect [44]. In metazoans in general, each organ has a unique timetable for cellular events in tissue development. Our model, and the data upon which it is based, indicate that in order to understand the effects of insulin signaling on adult phenotype it is necessary to understand how temporal changes in insulin signaling interact with this timetable.

Materials and Methods

Mutant stocks. Inr<sup>GC25</sup> is a chromosomal inversion with a breakpoint upstream of the Inr. Inr<sup>F17</sup> is an uncharacterized mutation induced by ethyl methanosulfonate. Both were described in Chen et al. [4] and obtained from the Bloomington Stock Center. The chico<sup>2</sup> allele is a P-element insertion allele whose phenotype is similar to the null chico<sup>1</sup> allele [5] and was kindly provided by Ernst Hafen. The tub-GFP-PH flies were kindly supplied by Bruce Edgar. The flies were maintained as chico<sup>1</sup>CyO [5], Inr<sup>E19</sup> and Inr<sup>GC25</sup> [4] balanced over
TM6B-Tb, T3M, T3M-pAcd-GFP, and tub-GFP-PH; Inr E19/TM3 [18] at 17 °C on standard yeast cornmeal agar medium.

Immunolocalization. We crossed Inr E19/TM6B-Tb with Inr GC25/TM6B-Tb flies and reared them at either 17 °C or 24 °C. When the larvae reached third instar, we genotyped them as either insulin receptor mutants (Inr E19/TM6B-Tb) or Inr GC25/TM6B-Tb. The fat bodies were dissected out in PBS, fixed in 4% paraformaldehyde for 10 min, and stored in absolute methanol at −20 °C. They were permeabilized with PBT (0.3% Triton X in PBS) for 30 min, washed in BBT (0.5% bovine serum albumin in PBT) for 30 min, then blocked in NGS/BBT (5% normal goat serum at room temp for 30 min. They were then treated with anti-dFOXO 2095 [16] (kindly provided by O. Puig) (1:1,000 in PBT) and fluorescein anti-rabbit (1:500 in PBT) (Vector Laboratories). The fat bodies were stained with propidium iodide (1:1,000 in PBS with 1 μg/mL of RNase A). We mounted the fat bodies in Vectashield (Vector Laboratories, Burlingame, California, United States). DNA was stained with propidium iodide (1:1,000 in PBS with 1 μg/mL of RNase A) and mounted in Vectashield. We then observed the fat bodies with a confocal microscope (Perkin Elmer UltraVIEW RS3; PerkinElmer Life Sciences).

Immunolocalization. We fixed the fat bodies with PBT (0.3% Triton X in PBS) for 30 min, washed in BBT (0.5% bovine serum albumin in PBT) for 30 min, then blocked in NGS/BBT (5% normal goat serum at room temp for 30 min. They were then treated with anti-dFOXO 2095 [16] (kindly provided by O. Puig) (1:1,000 in PBT) and fluorescein anti-rabbit (1:500 in PBT) (Vector Laboratories, Burlingame, California, United States). DNA was stained with propidium iodide (1:1,000 in PBS with 1 μg/mL of RNase A) and mounted in Vectashield. We then observed the fat bodies with a confocal microscope (PerkinElmer UltraVIEW RS3; PerkinElmer Life Sciences).

We then dissected the larvae in 100% methanol kept at 15 °C for 24 h. We then dissected the larvae in 100% methanol, fixed them in methanol, and stored their fat bodies without additional fixing in 100% methanol at −20 °C. We quickly washed the fat bodies in 50% methanol in PBS, and then 100% PBS before mounting them in Vectashield with Hoechst 33342 (2:1,000) to stain DNA. We observed the fat bodies with a confocal microscope (Zeiss LSM 510; Zeiss, Gena, Germany).

Ten flies were then transferred to fresh food and reared them at 15 °C and 24 °C. We reared flies at a number of temperature regimes. They were either maintained at 17 °C, 18 °C, 24 °C, or 25 °C or switched from 17 °C to 24 °C on day 1 to day 27 of development. We set up four bottles for each temperature regime, each containing standard yeast cornmeal agar medium. Two were the product of male Inr E19/TM6B-Tb flies and female Inr GC25/TM3 and two were the product of female Inr E19/TM6B-Tb and male Inr GC25/TM3. Each bottle contained between 100 and 200 eggs laid over a 4-h period. Temperature shifts were performed according to the median age of flies in each bottle. Larvae were reared on standard yeast cornmeal agar medium. Total developmental time for each fly was converted into degree-days (DD) to control for the effects of temperature on growth rates. DD is a measure of the heat accumulation above a minimal degree-days (DDs) to control for the effects of temperature on growth rates. DD is a measure of the heat accumulation above a minimal degree-days (DDs) to control for the effects of temperature on growth rates.

Developmental staging of Inr E19/TM6B-Tb and Inr GC25/TM3 at 17 °C and 24 °C. We reared flies as controls because they were transferred to moistened Kimiwipes ( Kimberly-Clark, Neenah, Wisconsin, United States) in Petri dishes. Preparations of each genotype were split between further development at 17 °C and 24 °C. We collected adults within 8 h of eclosion, after their cuticle had hardened, and immediately froze them in liquid nitrogen for later analysis. Wet and dry masses of individual flies were measured with an analytical balance (Mettler Toledo AX26 DeltaRange). All metabolic assays were performed on individual dried males (n = 10 for each assay). Glycogen and sugar content were measured using a protocol of van Handel [48]. Lipid content was quantified using another protocol of van Handel [49]. To determine protein concentration, flies were homogenized in 100 μL 0.1 M Na2HPO4. Ten μL of each sample was combined with the Bio-Rad protein assay dye reagent to calculate the fitted values.

Supporting Information

Figure S1. Fitted Values for Wing Area of Inr E19/TM3 Control Flies.

Wing area of Inr E19/TM3 flies in Figure 2 is expressed as percentage of wing area of Inr E19/TM3 flies kept under the same temperature regime, using the fitted values shown on the plot. Temperature affects wing area differently before and after critical size. Consequently, fitted values were calculated by regressing wing area of Inr E19/TM3 flies against transfer age before critical size (35% development) and after critical size. A similar method was used to determine the dry mass of Inr E19/TM3 males, except a single regression analysis was used to calculate the fitted values.

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Table S1. Wing, Genital Arch, and Maxillary Palp Area of Inr E19/TM3 and Inr GC25/TM3 Males Reared at 17 °C and 24 °C.

Found at DOI: 10.1371/journal.pbio.0030289.s001 (57 KB DOC).

Table S2. Total Area, Cell Area, and Cell Number of the Wings of Inr E19/TM3 and Inr GC25/TM3 Flies Reared at 17 °C and 24 °C.

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Accession Numbers

The FlyBase (http://flybase.bio.indiana.edu/search) accession numbers for the genes and gene products discussed in this paper are Chico (Fbg00024248), chico1 (FBAL0003103), dFOXO (FBgn0038197), Inr (FBgn0013984), InrE19 (FBal0009421), IncGC25 (FBgl0010755), PI3K (FBgn0015279), and S6K (FBgn0015806). The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) accession number for IGF1 receptor is NM_015013.

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Competing interests. The authors have declared that no competing interests exist.

Author contributions. JD, LV, DSL, and AWS conceived, designed, and performed the experiments. AWS analyzed the data and wrote the paper.

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