Tissue Damage Disrupts Developmental Progression and Ecdysteroid Biosynthesis in Drosophila

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Abstract

In humans, chronic inflammation, severe injury, infection and disease can result in changes in steroid hormone titers and delayed onset of puberty; however the pathway by which this occurs remains largely unknown. Similarly, in insects injury to specific tissues can result in a global developmental delay (e.g. prolonged larval/pupal stages) often associated with decreased levels of ecdysone – a steroid hormone that regulates developmental transitions in insects. We use Drosophila melanogaster as a model to examine the pathway by which tissue injury disrupts developmental progression. Imaginal disc damage inflicted early in larval development triggers developmental delays while the effects are minimized in older larvae. We found that the switch in injury response (e.g. delay/no delay) is coincident with the mid-3rd instar transition – a developmental time-point that is characterized by widespread changes in gene expression and marks the initial steps of metamorphosis. Finally, we show that developmental delays induced by tissue damage are associated with decreased expression of genes involved in ecdysteroid synthesis and signaling.

Introduction

Insects proceed through a series of precisely timed developmental transitions during their life cycle. In Drosophila melanogaster, after egg hatching, larvae progress through three instars that are separated by a molt during which the cuticle is shed and resynthesized to accommodate continued growth [1,2]. The third and final instar is followed by pupariation, the onset of the larval-pupal transition which is characterized by eversion of anterior spiracles, contraction of the larval body and tanning of the larval cuticle to form the puparium [3,4]. It is within this protective casing that metamorphosis to the adult occurs [3,4,5]. The timing of these developmental transitions is influenced by nutritional and environmental cues and is regulated by systemic signals such as steroid hormones that direct coordinated developmental responses throughout the insect [6,7,8].

In insects, localized tissue damage is frequently associated with a systemic injury response resulting in delayed development (e.g. prolonged larval or pupal stages) [9,10,11,12,13,14,15,16,17,18,19,20,21]. In Drosophila, damage to imaginal (adult precursor) tissues via irradiation, induction of cell death clones, or localized activation of apoptosis causes a prolonged third larval instar [10,11,13,14,20,21]. The mechanism by which localized tissue damage disrupts developmental progression is poorly understood but appears to involve a reduced systemic hemolymph ecdysteroid titer.

In Drosophila, as in most arthropods, the timing of developmental transitions is coordinated by a transient rise in the titer of the steroid hormone ecdysone (E) [22,23]. Production and release of ecdysteroids is regulated by a small secreted neuropeptide known as prothoracicotropic hormone (Pth) [24,25,26,27,28,29,30]. Pth stimulates ecdysone synthesis, at least in part, by regulating transcription of a number of Halloween genes, a family of genes encoding cytochrome P450 enzymes that are required for ecdysone synthesis in the prothoracic gland cells of the ring gland [24,25,26,27,28,29]. Ecdysone is released from the ring gland into the hemolymph and transported to peripheral tissues where it is converted to its active form, 20-hydroxyecdysone (20E), which binds to its receptor comprised of the Ecdysone receptor (EcR) and Ultraspireacle (Usp) [31,32,33]. EcR/Usp heterodimers bind to DNA and regulate transcription of target genes such as Ecdysone-inducible proteins −71CD (Eip71CD), −74EF (Eip74EF), −75B (Eip75B), −78C (Eip78C), and Br (Broad) leading to widespread physiological changes and developmental progression [34,35,36,37,38,39,40,41,42]. It has been suggested that imaginal disc damage triggers developmental delays, possibly by preventing the synthesis or release of ecdysone [17,20,21,43]. The mechanism by which injury leads to decreased hemolymph ecdysteroid titers remains unclear but appears to involve delayed release of Pth [9,10,20,44,45,46]. The effects of tissue damage on other components required for ecdysone synthesis and signaling are less clear.

The effects of injury on developmental progression are dependent upon the developmental stage of the animal at the...
time injury is sustained [9,10,11,13,19]. Imaginal tissue damage induced by irradiation or genetic cell ablation only appears to retard pupariation when induced at or before an Injury Response Checkpoint (IRC) which is reached sometime during the second half of the third larval instar [10,11,13,17,20,21,47]. The exact time that the IRC is reached has not been clearly defined, however a number of studies in Drosophila and Lepidoptera (Ephestia kuhniella and Lymantria dispar) have demonstrated that tissue damage induced early in the last larval instar retards development while injury inflicted closer to pupariation time no longer affects developmental timing [10,11,13,19,20,21,48,49]. In Drosophila, there are two additional critical developmental time points that are known to occur during the third larval instar. One of these critical periods is Critical Weight (CW), a size-assessment checkpoint reached early in the third instar, after which starvation no longer influences the time to pupariation [50]. The second critical period is the Mid-third Instar Transition (MIT), a developmental time point which marks the initial steps of metamorphosis, is associated with widespread changes in gene expression, and occurs during the middle of the third larval instar [51]. The possibility that the IRC corresponds with another critical developmental checkpoint (e.g. CW, MIT) has not been explored.

Here we examine the timing of the IRC and the mechanism by which localized tissue damage triggers developmental delays. We find that imaginal disc damage leads to delayed onset of the MIT, pupariation and adult eclosion. The effects of injury on developmental timing are minimized or absent closer to pupariation time and the switch from retardation to no response is coincident with the MIT. In addition, we find that tissue damage is associated with (1) reduced ecdysteroid titers, (2) decreased expression of most genes involved in ecdysteroid synthesis and signaling and (3) increased expression of Ecdysone oxidase (Eo), a gene involved in ecdysone catabolism. Together our data suggest that systemic injury response signals act on multiple targets to regulate ecdysteroid titers and ecdysone signaling pathway components.

Results

Timing of Developmental Transitions at 18°C

To induce tissue damage, we utilized flies containing a nGAL4 enhancer trap [52], a UAS-eiger transgene, and a temperature sensitive GAL80 variant driven by a tubulin promoter (tubGAL80°), all recombined onto a single third chromosome (Figure 1A) [21]. The nGAL4 driver is expressed throughout the third larval instar in the wing pouch, the peripodial epithelium overlying the wing pouch, the haltere disc and a ring in the leg discs [21]. In addition, we observed low but detectable levels of nGAL4 expression in 1–3 cells in each salivary gland throughout the third larval instar. Eiger (egr) encodes the Drosophila ortholog of tumor necrosis factor-alpha (TNFα) and induces cell death via downstream activation of c-Jun N-terminal kinase (JNK) [53,54]. The temperature sensitive variant of GAL80 represses GAL4 function at 18°C but not at 30°C [55] and was used to regulate egr expression. As shown in Figure 1A, Males of the genotype w^+;nGAL4, UAS-egr, tubGAL80°/TM6 Tb°, tubGAL80° crossed to w^+;+; (not shown) or w^+; P[Sgs3-GFP] females to produce w^+;+;nGAL4, UAS-egr, tubGAL80°/P[Sgs3-GFP] (referred to as Ablating Genotype) and w^+;+;TM6 Tb°, tubGAL80°/P[Sgs3-GFP] (referred to as Non-Ablating Genotype).

The timing of developmental transitions is known to be influenced by temperature as well as genetic background [8,56,57]. Our first set of experiments was therefore designed to determine the timing of a number of developmental transitions for each of the genotypes (Ablating and Non-Ablating) when maintained at a constant temperature of 18°C, a temperature at which GAL80° inhibits GAL4 thereby preventing eiger-induced cell death. The molt from 2nd to 3rd larval instar (L3) was determined by examination of larval mouth hooks in animals reared at 18°C (Figure 2A). Larvae of the Ablating (n = 59) and Non-Ablating (n = 63) genotype molted to L3 at a similar time, approximately 130 hours after egg laying (AEL; Figure 2A, Figure 3).

The Mid-third Instar Transition (MIT) is a developmental time point associated with a low titer ecdysteroid pulse and is characterized by widespread changes in gene expression including induction of a glue gene – Salivary gland secretion 3 (Sgs3) [51,58]. The timing of the MIT was determined by examination of an Sgs3GFP reporter that is expressed in salivary glands beginning at the MIT [58,59]. As shown in Figure 2B, there was no significant difference in the timing of onset of Sgs3GFP expression between Ablating (n = 250) and Non-Ablating (n = 192) genotypes when maintained at 18°C. Based on visualization of GFP in the salivary glands of whole larvae, the MIT appears to occur between 185 and 195 hours AEL (Figure 2B, Figure 3).

Larvae were maintained at 18°C and checked at intervals of 24 hours for completion of pupariation formation (Figure 2C) and adult eclosion (Figure 2D). Pupariation time (time from midpoint of the egg-laying period to completion of pupariation formation) was approximately 235 hours AEL for both Ablating (n = 42) and Non-Ablating (n = 44) genotypes (Figure 2C, Figure 3). Similarly, we found no significant difference in time to adult eclosion between Ablating and Non-Ablating animals. Adult eclosion occurred at approximately 420 and 425 hours AEL for Ablating (n = 37) and Non-Ablating (n = 42) genotypes, respectively (Figure 2D, Figure 3).

Critical Weight (CW) is the weight at which starvation no longer delays time to pupariation [50]. A second size assessment checkpoint is Minimum Viable Weight (MVW) which represents the weight at which larvae have enough nutritional stores in the form of fat body to survive the next developmental transition [50]. In Drosophila, CW and MVW occur at approximately the same time, early in the third larval instar [60,61]. To identify the time that these checkpoints are reached in Ablating and Non-Ablating genotypes when maintained at 18°C, we determined the minimum weight needed for larvae to pupariate following starvation (Minimum Weight to Pupariate, MWV; Figure 2F). Third instar larvae of known weights were starved and the proportion of larvae that successfully pupariated was measured. Larvae of both the Ablating and Non-Ablating genotypes exhibited a 50% threshold for pupariation after starvation at approximately 0.9 mg/larva. Based on the growth rate observed during L3 (Figure 2F), larvae of both the Ablating (n = 199) and Non-Ablating (n = 200) genotypes are predicted to reach the CW/MVW checkpoint at approximately 142 hours AEL (MWV, Figure 3).

Effects of Tissue Damage on the Mid-Third Instar Transition

To examine how localized tissue damage influences the timing of the MIT, we induced cell ablation in the wing imaginal discs (Figure 1B) at 172 hours AEL and examined larvae for expression of the Sgs3GFP reporter in salivary glands (Figure 4). At 164 hours AEL, before the induction of cell ablation, Sgs3GFP expression was observed in 3.6% of Non-Ablating larvae (n = 28; Figure 4A) and 2.6% of Ablating larvae (n = 38; Figure 4D). Following the heat-treatment to induce cell death via eiger expression in the wing discs,
most (77.3%; n = 22) larvae of the Non-Ablating genotype expressed Sgs3GFP by 197 hours AEL (Figure 4B). Sgs3GFP expression was maintained at high levels throughout the remainder of the third larval instar and was detected in 91.7% of larvae at 215 hours AEL (n = 26; Figure 4C) and in 100% of larvae at 236 hours AEL (n = 19; data not shown) in Non-Ablating animals. In contrast, following induction of cell death, only 10.0% (n = 30) of Ablating larvae displayed any Sgs3GFP expression by 197 hours AEL and expression was consistently lower in Ablating larvae compared to Non-Ablating controls at this time point (compare figure 4E to 4B). High levels of Sgs3GFP expression were detected in only 53.9% (n = 26) of Ablating larvae by 215 hours AEL (Figure 4F) and in only 75.0% (n = 29) of larvae by 236 hours AEL (data not shown). We detected no obvious morphological defects in salivary glands following cell ablation and no signs of cell death within salivary glands at any time following cell ablation suggesting that delayed onset of Sgs3GFP expression is a result of imaginal disc cell ablation (data not shown).

Influence of Tissue Damage on Time to Pupariation and Eclosion

Delay of pupariation was measured as the difference between mean pupariation time of Ablating larvae and Non-Ablating larvae housed in the same vial. To examine how localized tissue damage influences the timing of pupariation we induced cell ablation in the wing imaginal discs in larvae of various ages. Cell ablation in the wing disc at 173 hours AEL delayed pupariation and adult eclosion by 59 and 64 hours, respectively (Figure 5A, B). Similar results were obtained when cell ablation was induced at 150, 162 or 184 hours AEL (Figure 3). Wing disc cell ablation induced at 198 hours AEL delayed mean pupariation and adult eclosion times by 49 hours (p<0.0001) and 79 hours (p<0.0001), respectively (Figure 5C, D). Similar results were obtained when cell ablation was induced at 190 hrs AEL (Figure 3). Injury induced between 190–198 hrs AEL resulted in two groups of Ablating larvae – those that delayed development in response to tissue damage and those that eclosed at the same time as Non-Ablating controls (See Figure 5D). Larvae that delayed development in response to wing disc ablation typically eclosed as adults with regenerated wings while those that eclosed at the same time as Non-Ablating controls emerged as wingless adults (Figure S1). Similarly, adult eclosion was delayed by 64 hours (p<0.0001; Figure 5B) following the induction of cell death in imaginal discs at 173 hrs AEL.

Influence of Larval Age on the Systemic Injury Response

To assess the effects of larval age on the systemic injury response we induced tissue damage in the wing imaginal discs in larvae of various ages. Cell ablation in the wing disc at 173 hours AEL delayed pupariation and adult eclosion by 59 and 64 hours, respectively (Figure 5A, B). Similar results were obtained when cell ablation was induced at 150, 162 or 184 hours AEL (Figure 3). Wing disc cell ablation induced at 198 hours AEL delayed mean pupariation and adult eclosion times by 49 hours (p<0.0001) and 79 hours (p<0.0001), respectively (Figure 5C, D). Similar results were obtained when cell ablation was induced at 190 hrs AEL (Figure 3). Injury induced between 190–198 hrs AEL resulted in two groups of Ablating larvae – those that delayed development in response to tissue damage and those that developed at the same time as Non-Ablating controls (See Figure 5D). Larvae that delayed development in response to wing disc ablation typically eclosed as adults with regenerated wings while those that eclosed at the same time as Non-Ablating controls emerged as wingless adults (Figure S1).

Wing disc cell ablation induced between 213–223 hours AEL resulted in no significant difference in the mean time to pupariation or adult eclosion in Ablating animals compared to Non-Ablating controls (Figure 5E, 5F; Figure 3). None of the

Figure 1. Cell Ablation Strategy. (A) Strategy used to produce Ablating and Non-Ablating larvae. w*; P[Sgs3-GFP]3 females were crossed to w*;+;UAS-eGr,tubGAL80®/TM6B, Tb¹, tubGAL80 males to give rise to the Ablating genotype (w*;+;UAS-eGr,tubGAL80®/Sgs3GFP) and the control Non-Ablating genotype (w*;+;TM6B, tubGAL80/Sgs3GFP). (B) Strategy to induce cell ablation. Embryos of the Ablating and Non-Ablating genotypes were collected at room temperature in four hour intervals and transferred to 18°C. First-instar larvae (48 hours AEL) vials were transferred to 30°C for 40 hours, returned to 18°C and monitored daily to document the time to Sgs3GFP expression, pupariation or eclosion. RNA for qPCR and samples for EIA experiments were collected at time points T0–T3.

doi:10.1371/journal.pone.0049105.g001
Ablating animals showed any evidence of tissue regeneration; all emerged as wingless adults (Figure S1).

Ecdysteroid Titers Following Tissue Damage

The developmental retardation observed following imaginal disc cell ablation suggested the presence of an underlying ecdysteroid deficiency in injured animals. To measure the ecdysteroid titers in Ablating and Non-Ablating larvae, we performed an enzyme immunoassay (EIA) utilizing an ecdysteroid antiserum (Cayman Chemical). We examined ecdysteroid levels at four time points (Figure 1B): (T0) 170 hrs AEL - immediately before cell ablation was induced, (T1) 190 hrs AEL - half-way through the cell ablation period, (T2) 210 hrs AEL - immediately after the completion of cell ablation, and (T3) 234 hrs AEL –24 hours after cell ablation treatment was complete. As shown in Figure 6, just prior to the induction of cell ablation (T0) there was no significant difference in ecdysteroid titers between Ablating (1.65 ± 0.98 pg 20E equivalents/mg tissue) and Non-Ablating (2.04 ± 1.99 pg 20E equiv/mg tissue) larvae. At T1 we detected a small (not statistically significant) difference between ecdysteroid concentrations in Ablating and Non-Ablating larvae; ecdysteroid concentrations were 1.65 ± 0.34 and 2.66 ± 1.03 pg 20E equivalents/mg tissue for larvae of the Ablating and Non-Ablating genotypes, respectively. Following the cell ablation period, ecdysteroid levels were significantly reduced (p<0.05) in Ablating larvae compared to their sibling Non-Ablating controls. At T2, ecdysteroid concentrations were 1.12 ± 0.28 and 3.42 ± 0.57 pg 20E equivalents/mg tissue for Ablating and Non-Ablating larvae, respectively while at T3 we detected 3.01 ± 0.44 and 6.11 ± 0.040 pg 20E equivalents/mg tissue for Ablating and Non-Ablating larvae, respectively.

Effects of Tissue damage on Ecdysteroid Biosynthesis

To examine the effects of tissue damage on ecdysteroid signaling, we used qRT-PCR to examine injury-induced changes in expression of genes involved in ecdysone synthesis and signaling. Total RNA was isolated from Ablating and Non-Ablating larvae at time points T0–T3 (Figure 1B). For each genotype (Ablating and Non-Ablating), transcript levels in larvae at each time point (T1–T3) were compared to transcript levels in larvae at T0 to determine relative changes in gene expression.

To assess how tissue damage influences ecdysone synthesis, we examined expression of genes including (1) pth, which encodes the neuropeptide that stimulates ecdysone synthesis in the ring gland [30], (2) genes encoding enzymes required for ecdysone synthesis in the ring gland including neverland (nvld) [62], spookier (spok) [63], disembodied (db) [64], phantom (phtm) [63], and shadow (sad) [66], and (3) genes encoding additional components required for ecdysone synthesis including ecdysone-
To further examine the effects of injury on ecdysone signaling, we examined expression of ecdysone inducible genes including Broad (br) [41,42], Eip74EF [39], Eip75B [40], Eip71CD [38], and Eip78C [37]. Expression of Eip75B, Eip74EF, and Eip78C were significantly reduced in Ablating larvae compared to Non-Ablating controls following cell ablation at time points T2 and T3 (Figure 7E, T3 Shown; Figure S3). There was no significant effect on tissue damage observed for br or Eip71CD (Figure 7E, T3 Shown; Figure S3).

Ecdysone Catabolism Following Localized Tissue Damage

Ecdysone oxidase (Eo) is an enzyme that catalyzes the conversion of ecdysteroids into inactive 3-dehydroecdysteroids [72]. This ecdysteroid inactivation results in decreased ecdysteroid titers and helps to regulate the sharp ecdysteroid peaks that trigger developmental transitions. Following cell ablation in the wing disc (time points T2 and T3), Eo expression was elevated in Ablating samples compared to Non-ablating controls (Figure 7E, T3 Shown; Figure S3).

Early Response to Injury

To identify potential differences between the early and late response to injury we examined expression of genes involved in ecdysteroid synthesis and signaling at an earlier time point, halfway through the cell ablation treatment (T2; Figure 1B). There was no significant difference for most genes examined in Ablating samples compared to Non-Ablating controls at T1 (Figure 8). Only five genes displayed reduced levels of expression in Ablating larvae compared to Non-Ablating controls at this early time point. At T1, ablation samples displayed significantly lower levels of expression of eip74EF, eip75b, eip78C compared to Non-Ablating controls (Figure 8).

Discussion

Previous studies have indicated that injury to imaginal tissues is associated with prolonged larval and pupal stages but the effects of injury on developmental timing is minimized or even reversed in older larvae, after the animal has passed an Injury Response Checkpoint (IRC) [10,17,20,47]. We find that cell ablation in wing imaginal discs delays all subsequent developmental transitions including the MIT (Figure 4), pupariation (Figure 5A), and adult eclosion (Figure 5B). We demonstrate for the first time that once larvae have progressed through the MIT, a time point that marks the initial steps of metamorphosis, injury no longer results in developmental retardation (Figure 3, 5,). These data suggest that the IRC coincides with the MIT and that events initiated at the onset of metamorphosis inhibit components of the systemic injury response.

Following wing disc cell ablation, the damaged tissues produce signals that retard development, thus providing time for imaginal tissue regeneration to occur [20,21,73,74]. Tissue regeneration is inhibited if tissue damage is inflicted following the IRC (Figure S1) [20,21]. It is possible that one or more of the genes that are up-regulated at the MIT may act to inhibit the injury response signals that mediate tissue regeneration and developmental retardation; however, interactions between injury response components, genes required for tissue regeneration, and genes that are differentially regulated at the MIT have not been explored.
Injury induced developmental delays are characterized by decreased hemolymph ecdysteroid titers. Halme et al [20] demonstrated that injury induced via x-irradiation triggers decreased expression of ptth, which encodes the cerebral neuropeptide that stimulates ecdysone synthesis in the ring gland. Similarly, here we have shown that targeted cell ablation in the wing imaginal disc leads to decreased expression of ptth and many genes required for ecdysone synthesis and signaling. Ptth has been shown to regulate expression of a number of genes required for ecdysone synthesis and signaling. Ptth has been shown to regulate expression of a number of genes required for ecdysone synthesis and signaling. 

Together, these studies indicate that multiple factors likely participate in mediating the injury response.

Based on the observation that most ecdysteroidogenic genes are decreased following cell ablation in the wing disc, it is likely that the reduction in the ecdysteroid titer is due to decreased ecdysone synthesis in the ring gland. Our data indicate that decreased ecdysone titers may also result from enhanced ecdysone inactivation (Figure 7F). We observed an increase in expression of ecdysone oxidase – an enzyme that catalyzes the oxidation of ecdysteroids - following tissue damage [72]. This suggests the presence of multiple mechanisms that act in concert to reduce circulating ecdysteroid levels following injury.

Ecdysone response genes are largely down regulated in response to tissue damage. Two exceptions are Eip71CD and br (Figure 7E). Eip71CD and br each show tissue specific responses to ecdysone and are induced in response to ecdysone in some tissues and repressed by ecdysone in other tissues [51]. Our observations likely represent the combined effects of tissue-specific responses of these genes to ecdysone.

To assess the early effects of injury we analyzed changes in gene expression mid-way through the cell ablation procedure (T1, Figure 1A). At this early time point, we found that most ecdysteroidogenic genes were not yet affected (Figure 8). In contrast, five genes (ptth, spok, dib, br, Eip78C) displayed reduced expression in Ablating samples compared to Non-Ablating controls at T1. It is possible that these genes represent direct targets of the injury response and that systemic injury...
Figure 5. Damage to Wing Imaginal Discs Delays Pupariation and Adult Eclosion. (A–F) Timing of pupariation and adult eclosion following induction of cell ablation in the wing disc at the indicated time. Fraction of larvae that had (A, C, E) pupariated or (B, D, F) eclosed as adults are plotted relative to time in hours AEL for Ablating (Red - w*; rmGAL4, UAS-egr, tubGAL80Δ7/Sgs3GFP) and Non-Ablating (Blue - w*; TM6, Tb1, tubGAL80/Sgs3GFP).
lakes. n = 3 independent populations (30 larvae each) were assayed for each ablation time. (A–B) Timing of (A) pupariation and (B) adult eclosion for larvae heat-treated at 173 hours AEL. Mean pupariation times are 286 and 227 hours AEL for ablating and non-ablating larvae, respectively. Mean eclosion times are 304 and 440 hours AEL for ablating and non-ablating larvae, respectively. Similar results were obtained with larvae heat-treated at 150, 162, or 184 hours AEL (data not shown). (C–D) Timing of (C) pupariation and (D) adult eclosion for larvae heat-treated at 198 hours AEL. Mean pupariation times are 279 and 230 hours AEL for ablating and non-ablating larvae, respectively. Similar results were obtained with larvae heat-treated at 190 hours AEL (data not shown). (E–F) Timing of (E) pupariation and (F) adult eclosion for larvae heat-treated at 223 hours AEL. Mean pupariation times are 263 and 265 hours AEL for ablating and non-ablating larvae, respectively. Mean eclosion times are 419 and 411 hours AEL for ablating and non-ablating larvae, respectively. Similar results were obtained with larvae heat-treated at 213 and 220 hours AEL (data not shown). doi:10.1371/journal.pone.0049105.g005

**Materials and Methods**

**Drosophila Stocks**

*w*; *y*; GAL4, UAS-egr, tubGAL80/*; TM6 Tb*, tubGAL80 [21] was a generous gift from I. Hariharan. *w*; *P{Sgs3GFP}3* [58], which expresses an Sgs3GFP fusion under the control of Sgs3 in an otherwise wild-type (Canton-S) background, and *w*1118 were obtained from the Bloomington Stock Center. Unless otherwise indicated, flies were maintained at 22–25°C on a standard cornmeal-yeast-agar medium (Bloomington recipe).

**Developmental Timing Measurements**

Fertilized eggs were collected at room temperature on grape juice agar plates. Collections were done in four hour intervals after which plates were transferred to 18°C. First instar larvae were collected from these plates 48 hours after egg laying and transferred (in groups of 30) to vials containing standard cornmeal-yeast-agar medium. Larvae were maintained at 18°C. For developmental progression, larvae were scored in 24 hour intervals. Larval stages were determined by observing mouth hook morphology. Pupariation was determined by observing contraction of larval body, eversion of spiracles, and onset of pigmentation of the puparium. Mean developmental times were tested for significant differences via two-sample t-test.

**Minimum Weight to Pupariate**

Larvae were cultured at 18°C. First-instar larvae were collected 48 hours after egg laying and were transferred in batches of 30 to vials containing fresh cornmeal-yeast-agar medium. At the designated time, larvae were weighed in batches of 3–5 and transferred to a 35 x 10 mm plate filled with either grape-juice agar (fed) or 2% agar in water (starved). Pupariation was scored in 12 hour intervals for fed and starved animals.

**Cell Ablation Strategy**

Cell ablation [Figure 1] was induced essentially as described by Smith-Bolton et al (2007) with the following modifications: *w*1118/+ or *w*; *P{Sgs3GFP}3* females were crossed to *w*; *y*; *y*; GAL4, UAS-egr, tubGAL80/*; TM6 Tb*, tubGAL80 males [21]. Flies were conditioned for two days on fresh yeast paste and embryos were collected at room temperature in four hour intervals on grape juice agar supplemented with a small amount of fresh yeast paste. Plates were incubated at 18°C. First-instar larvae (48 hours after egg laying) were transferred in groups of 30 larvae to a vial containing standard cornmeal-yeast-agar medium. Larvae were allowed to develop at 18°C until the designated time for ablation induction. At the designated time vials were transferred to 30°C for 40 hours, returned to 18°C and monitored daily to document the time to Sgs3GFP expression, pupariation or eclosion. Ablating animals were *w*; *y*; *y*; GAL4, UAS-egr, tubGAL80/*; P{Sgs3GFP}3 or *w*; *y*; *y*; GAL4, UAS-egr, tubGAL80/*; + (collectively referred to as Ablating). Mock-ablated discs were the siblings of the ablating animals which were *w*;*y*;*TM6 Tb*, tubGAL80/*; P{Sgs3GFP}3 or *w*;*y*;*y*;GAL4, *TM6 Tb*, tubGAL80/*; + (collectively referred to as Non-Ablating).

**Larval Collection**

Cell ablation was induced at 170 hours AEL as described (Cell Ablation Strategy; Figure 1A). Larvae were collected at the following time points: T0 just before induction of cell ablation (170 hrs AEL), T1 half-way through cell ablation (190 hrs AEL), T2 immediately after cell ablation (210 hrs AEL), and T3 24 hours after cell ablation (234 Hrs AEL). For qRT-PCR, three sets of larvae (five larvae/set) were collected for each genotype (Ablating: *w*1118+;*y*;GAL4, UAS-egr, tubGAL80/*; and Non-ablating: *w*1118+;*TM6 Bb*, tubGAL80/*; +) at each time point. For ecdysteroid titer measurements, two sets of larvae (15 larvae/set) were collected for each genotype (Ablating/Non-Ablating) at each time point (T0–T3). Larvae were flash frozen in liquid nitrogen and stored at −80°C for future use.
Ecdysteroid Titer Measurements

Ecdysteroid levels were quantified via competitive Enzyme Immunoassay (EIA) (Cayman Chemicals, Inc., USA) [76,77] using 20E (Sigma) and 20E acetylcholinesterase (Cayman Chemicals, Inc., USA) as the standard and enzymatic tracer, respectively. The antiserum detects ecdysone, 20-hydroxyecdysone and other ecdysteroid metabolites including 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone [76,77,78]. The standard curve was obtained with 20E (Sigma-Aldrich, USA) and results are expressed as 20E equivalents. For sample preparation, 15 staged larvae were weighed and preserved in...
performed in triplicate. and absorbance was read at 415 nm. All assays were
Chemicals, Inc., USA) was used for the chromogenic reaction
BSA in 0.1 M phosphate buffer). Ellmann reagent (Cayman
immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1%
Real-Time PCR

Larvae were homogenized in Trizol reagent (Invitrogen). RNA concentration was determined by spectrophotometric analysis. RNA was flash frozen in liquid nitrogen and stored at −80°C. Reverse transcription was carried out using qScript cDNA Supermix (VWR/Quanta). First Strand synthesis reactions were incubated as follows: 5 minutes [25°C]; 30 minutes [42°C]; 5 minutes [85°C]; hold [4°C]. cDNA was diluted 1:5 and 5 μl was used for each qRT-PCR reaction. qRT-PCR was performed using PerfeCTa SYBR Green FastMix (VWR/Quanta). Reactions were incubated in a real time thermal detection system (Stratagene MX3000P) as follows: 95°C [2 minutes]; 40 cycles [95°C (1 second); 60°C (30 seconds)]. Fluorescence intensity was recorded at the end of each elongation phase. A dissociation curve was added to the end of the thermal cycle program. Results were analyzed by using the MxPro qPCR Software version 4.1 (Stratagene) and relative expression levels were normalized to mRNA for ribosomal protein L32 (RpL32/Rp49). Primers used for qRT-PCR are shown in Table 1. Statistical analyses were performed using the nonparametric Mann-Whitney U test.

| Target Gene | Fwd Primer | Rev Primer |
|-------------|------------|------------|
| Pth         | 5'-TGTTGCAACCAAAAGCAATGATG-3' | 5'-ATCAGAAAGCAGGAGAATGCGA-3' |
| EcR-RA      | 5'-ATACGATGCTGCTGCGTCCTTG-3' | 5'-AGAATGCCTGCTGCTGCTGCTG-3' |
| EcR-RE      | 5'-TAGACGATGCTGCTGCTGCTGCTG-3' | 5'-ACATGATGCTGCTGCTGCTGCTG-3' |
| Usp         | 5'-CCTGCCAACAGGCTGCGACATAA-3' | 5'-ATCCAAGCCGCTGCGACATAA-3' |
| eip74EF     | 5'-TTTTCATCAAGGACGAACCCGGGA-3' | 5'-CATTTTCATTGGATGTCATATTG-3' |
| eip75B      | 5'-ATGATCGACTGCTGCTGCTGCTG-3' | 5'-TAGAATGCTGCTGCTGCTGCTG-3' |
| eip71CD     | 5'-AGAAGACCCGCTGCGACATAA-3' | 5'-AACTAGCTTGGATGTCATATTG-3' |
| eip78C      | 5'-TTTGGGACTGCTGCTGCTGCTGCTG-3' | 5'-TTTGGGACTGCTGCTGCTGCTGCTG-3' |
| Br          | 5'-TGCTGCTGCTGCTGCTGCTGCTG-3' | 5'-TTTGGGACTGCTGCTGCTGCTGCTG-3' |
| Control     | 5'-AAAGAAGCCGCAACCAAGCATTATC-3' | 5'-CTTGGGGCTGATAAACAACCCC-3' |

doi:10.1371/journal.pone.0049105.t001

Supporting Information

Figure S1 Wing Phenotypes Following Cell Ablation.
All flies shown were heat treated at 198 hours AEL to induce cell ablation. (A) Control (Non-Ablating) fly. (B-E) Flies from the Ablating genotype representing the range of wing phenotypes obtained following cell ablation at 198 hours AEL. (TIF)

Figure S2 qRT-PCR Analysis of Ecdysteroidogenic Enzymes Following Cell Ablation for time points T0–T3. (TIF)

Figure S3 qRT-PCR Analysis of Ecdysteroidogenic Genes and Ecdysone Oxidase Following Cell Ablation for time points T0–T3. (TIF)

Figure S4 qRT-PCR Analysis of Ecdysteroidogenic Components Following Cell Ablation for time points T0–T3. Two primers sets (EcR-RA and EcR-RE) that each amplify a region common to all EcR isoforms were utilized to characterize the EcR response. (TIF)

Figure S5 qRT-PCR Analysis of Ecdysone Response Genes Following Cell Ablation for time points T0–T3. (TIF)

Acknowledgments

We thank Iswar Hariharan for fly strains.
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Author Contributions
Conceived and designed the experiments: JFH PC. Performed the experiments: JFH OZ. Analyzed the data: JFH OZ. Contributed reagents/materials/analysis tools: PC. Wrote the paper: JFH. Assisted in revision of manuscript: OZ PC.

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