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Developmental Expression of *Drop-Dead* is Required for Early Adult Survival and Normal Body Mass in *Drosophila melanogaster*

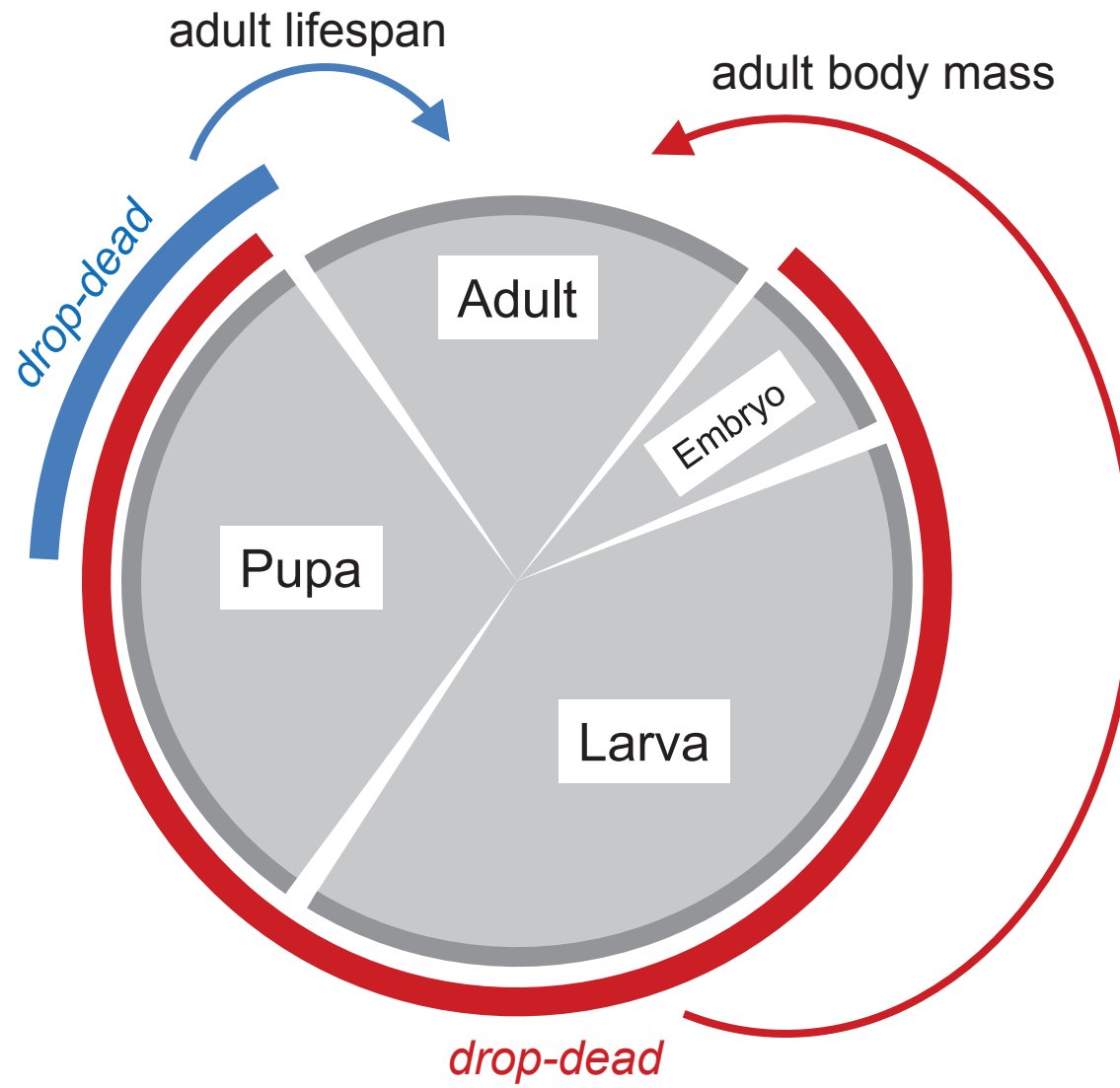
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*Graphical Abstract



Highlights

- Knockdown and rescue of *drd* expression was temporally controlled by heat shock.
- Adult lifespan was affected only when *drd* expression was manipulated during mid to late metamorphosis.
- Adult body mass was affected only when *drd* expression was manipulated through all of pre-adult development.

Developmental expression of *drop-dead* is required for early adult survival and normal
body mass in *Drosophila melanogaster*

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Abstract

In *Drosophila melanogaster*, mutations in the gene *drop-dead* (*drd*) result in early adult lethality, with flies dying within 2 weeks of eclosion. Additional phenotypes include neurodegeneration, tracheal defects, starvation, reduced body mass, and female sterility. The cause of early lethality and the function of the *drd* protein remain unknown. In the current study, the temporal profiles of *drd* expression required for adult survival and body mass regulation were investigated. Knockdown of *drd* expression by UAS-RNAi transgenes and rescue of *drd* expression on a *drd* mutant background by a *UAS-drd* transgene were controlled with the *Heat Shock Protein 70* (*Hsp70*)-*Gal4* driver. Flies were heat-shocked at different stages of their lifecycle, and the survival and body mass of the resulting adult flies were assayed. Surprisingly, the adult lethal phenotype did not depend upon *drd* expression in the adult. Rather, expression of *drd* during the second half of metamorphosis was both necessary and sufficient to prevent rapid adult mortality. In contrast, the attainment of normal adult body mass required a different temporal pattern of *drd* expression. In this case, manipulation of *drd* expression solely during larval development or metamorphosis had no effect on body mass, while knockdown or rescue of *drd* expression during all of pre-adult (embryonic, larval, and pupal) development did significantly alter body mass. Together, these results indicate that the adult-lethal gene *drd* is required only during development. Furthermore, the mutant phenotypes of body mass and lifespan are separable phenotypes arising from an absence of *drd* expression at different developmental stages.

Key words

Drosophila, lifespan, body mass, metamorphosis, *drop-dead*

1. Introduction

Over the past several decades, *Drosophila* has been a popular model organism for the study of neurodegeneration (Lessing and Bonini, 2009). While some such studies involve genes that were identified through their association with human disease (Lu and Vogel, 2009), many neurodegeneration-linked genes were originally discovered in the fly through forward genetic screens for adult lethality, retinal defects, or neurodegeneration itself (Cook et al., 2011; Knust, 2007; Kretzschmar, 2009; Min and Benzer, 1997). The very first adult lethal mutation connected to neurodegeneration, reported by Benzer and coworkers, was in the gene *drd* (Benzer, 1971). Flies that carry the strong alleles *drd*¹ or *drd*^{lwf} have a median survival of four days and display widespread degeneration of the brain at the time of death (Benzer, 1971; Blumenthal, 2008; Buchanan and Benzer, 1993). Despite the early discovery of *drd*, the function of this gene remains unknown.

Flies with mutations in *drd* manifest diverse and seemingly unrelated phenotypes, many of which may contribute to their short lifespan. Newly eclosed *drd* mutants display abnormal glial morphology that has been postulated to lead to the neurodegeneration that accompanies death (Buchanan and Benzer, 1993). Moreover, the tracheae in the brain appear fragile, and it is hypothesized that tracheal breakdown leads to hypoxia and subsequent neurodegeneration (Kim et al., 2012; Lehmann and Cierotzki, 2010; Tschäpe et al., 2003). Also, these flies exhibit a gut phenotype; the movement of ingested food from the crop into the midgut is impaired (Peller et al., 2009). The decreased ability to digest food results in depletion of triglyceride and glycogen stores, suggesting that the flies may also be starving to death. In addition to these phenotypes, *drd* mutants have a reduced

body mass on the day of eclosion and are female sterile (Blumenthal, 2008; Buchanan and Benzer, 1993).

The *drd* gene encodes a member of the NRF (nose resistant to fluoxetine)-domain protein family, a group of putative integral membrane proteins with limited homology to prokaryotic acyltransferases (Blumenthal, 2008; Choy and Thomas, 1999). The *drd* protein is localized to an intracellular membrane-bound compartment (Kim et al., 2012). To date, no biochemical function has been reported for DRD, the other 16 NRF-domain proteins encoded by the *Drosophila* genome, or any other eukaryotic NRF-domain protein.

The two phenotypes under investigation in this work are adult lifespan and body mass. These phenotypes share a dependence on signaling by insulin-like peptides (ilps) and by ecdysone and related steroid hormones (ecdysteroids). In holometabolous insects such as *Drosophila*, adult body mass is determined by body mass at the time of pupariation; this in turn is regulated by the timing of pupariation and the growth rate of the larva up until that point (Mirth and Riddiford, 2007; Riddiford, 2011; Shingleton, 2005). Larvae commit to pupariation upon reaching a “critical mass”; the growth rate up until this point determines the overall length of larval development, while the growth rate after this point affects the mass upon pupariation and the resulting adult mass. Pupariation itself is triggered by a pulse of ecdysteroid production by the prothoracic gland, and mutants that fail to produce this pulse do not pupariate (Garen et al., 1977). The overall growth rate of larval tissues is positively regulated by ilps in response to nutrient availability (Britton et al., 2002), and larvae with defects in ilp signaling become very small adults due to their lack of growth post-critical mass (Clancy et al., 2001; Tatar et al., 2001). In addition, both positive and negative interactions exist between the ilp and ecdysteroid signaling

pathways, resulting in a complex regulatory network controlling larval growth and development (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). In the adult, ilp signaling is a major determinant of lifespan; mutants in the ilp signaling pathway are long-lived (Clancy et al., 2001; Giannakou and Partridge, 2007; Tatar et al., 2001). Modest reductions in ecdysteroid receptor expression also increase lifespan in male flies while conflicting results have been reported in females (Schwedes and Carney, 2012; Simon et al., 2003; Tricoire et al., 2009). As in larvae, ilp and ecdysteroid signaling in the adult are highly interconnected (Francis et al., 2010; Tu et al., 2002).

The dual effects of ilp signaling mutations on body mass and lifespan result from a lack of ilp signaling at different stages of the insect lifecycle. Nutritional deprivation of late third-instar larvae, which inhibits ilp signaling and results in low body mass, has no effect on adult lifespan (Tu and Tatar, 2003). Conversely, inhibition of ilp signaling specifically in the adult fat body results in an extension of lifespan with no change in body mass (Giannakou et al., 2004; Hwangbo et al., 2004).

In this study, we ascertained the temporal requirement for *drd* expression in determining rapid adult lethality and body mass. We find that *drd* expression during mid to late metamorphosis is both necessary and sufficient for survival beyond the first month of adult life. Adult body mass requires a different period of *drd* expression that spans the larval-pupal transition and potentially encompasses all of pre-adult development. Together, these results demonstrate that *drd* is an essential developmental gene that separately modulates adult lifespan and body mass.

2. Materials and Methods

Drosophila Stocks

All fly stocks were maintained on standard cornmeal-yeast-agar food (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/molassesfood.htm) at 25°C on a 12h:12h light:dark cycle unless otherwise noted. UAS-RNAi stocks (*w*¹¹¹⁸; *P{GD3367}v37404* (FBst0461992) and *w*¹¹¹⁸; *P{GD15915}v51184* (FBst0469325)) were obtained from the Vienna Drosophila RNAi Center. Other stocks (*y*¹ *w*^{*}; *P{Act5C-GAL4}17bFO1/TM6B*, *Tb*¹ (FBst0003954), *w*^{*}; *P{GAL4-Hsp70.PB}2* (FBst0002077), and *w*¹¹¹⁸; *P{UAS-Dcr-2.D}2* (FBst0024650)) were obtained from the Bloomington Drosophila Stock Center. The genes and alleles referenced in this work include *drd* (FBgn0260006), *drd*^{lwf} (FBal0193421), and *rp49* (FBgn0002626). Stocks were not outcrossed prior to this study.

For RNAi experiments, the *UAS-Dcr-2* transgene was recombined onto the two UAS-RNAi chromosomes by a standard crossing scheme. Recombinants were identified by eye color.

Generation of UAS-drd transgenic flies

The entire coding sequence of *drd* was amplified from the cDNA clone RE74651 (Drosophila Genomics Resource Center, Bloomington, IN) by PCR with PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA) and the primers *drd* CDS 5' BglII 5' GTC AGA TCT ATG TCG CGT ATG TCG CAT ATG 3' and *drd* CDS 3' XbaI 5' GTC TCT AGA CCA GAC TAA TCC GAG TGC GG 3', which included BglII and XbaI sites, respectively. The PCR product was purified (QIAquick, Qiagen, Valencia, CA), and digested with XbaI and

BglII (New England Biolabs, Ipswich, MA). The digested insert was ligated into XbaI/BglII-digested pUAST vector (Quick Ligation kit, NEB) and transformed into competent DH5alpha bacteria (NEB). A single clone was isolated and minipreped (Wizard Plus SV, Promega, Madison, WI) and its integrity confirmed by sequencing the entire *drd* coding sequence. Maxi-prep DNA was prepared (QIAfilter Plasmid Maxi kit, Qiagen) and injected commercially to create transgenic lines (Genetic Services Inc., Cambridge, MA).

The resulting transgenic lines were crossed onto a *drd^{lwf}* background to screen for “leaky” expression of the *UAS-drd* transgene. Lines that did not display a reduced lifespan were discarded (four out of 11 autosomal insertions). The remaining lines were crossed with *y w; Act5C-Gal4/ TM6B* to drive ubiquitous overexpression of the *UAS-drd* transgene. Real-time PCR was performed on 3-day old flies and the line that displayed the greatest fold increase in *drd* expression relative to sibling controls was used for all experiments.

Synthesis of cDNA

RNA was isolated from whole flies or pupae using Trizol reagent (Life Technologies, Grand Island, NY). One µg total RNA was treated with either RQ1 RNase-free DNase (Promega) for 30 minutes at 37°C or DNase I, amplification grade (Life Technologies) for 20 minutes at room temperature and then cDNA synthesis was performed with qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD). To determine the genotype of individual pupae, PCR was performed (GoTaq Hot Start Polymerase, Promega) for the presence of Gal4. Primers used: Gal4 F: 5' GGC TAG AAA GAC TGG AAC AGC T 3' and Gal4 R: 5' AGG GCA AGC CAT CCG ACA TG 3'.

Quantitative Real Time PCR

Real-time PCR was performed on cDNA using the MyiQ thermocycler (Bio-Rad, Hercules, CA) and iQ SYBR Green supermix (Quanta Biosciences). Each sample was run in triplicate. A melt curve was performed directly after amplification to verify the authenticity of the PCR products. *drd* transcript levels, relative to the housekeeping gene *rp49*, were calculated in MyiQ software (Bio-Rad) using a dilution series of whole fly cDNA that was included in each PCR run. For the pupal heat shock experiments, these relative expression levels were then further normalized to the average expression at either the beginning of the heat pulse or 24 hours after the end of the heat pulse, depending on the experiment. Primers used: *rp49* F: 5' AAG ATC GTG AAG AAG CGC ACC AA 3', *rp49* R: 5' CTG TTG TCG ATA CCC TTG GGC TT 3', *drd* L: 5' GCT CTG AAG ATG CAC GAC TC 3', and *drd* R: 5' CCT CGA ATT GTG TGG CAA AA 3'.

Lifespan Assays

Flies were collected on the day of eclosion, transferred to fresh vials every 2-7 days, and scored daily for survival for 40 days, except for the experiment shown in figure 1b, which was continued until all flies died. A minimum of 50 flies per genotype were used for each survival curve.

Body Mass Assays

The mass of the flies was performed as previously described (Blumenthal, 2008). Flies were collected in the early afternoon of the day of eclosion, killed by placing on dry ice for 3 minutes, and weighed in groups containing 2-13 flies. The average fly mass for each

group was determined by dividing the total mass by the number of flies. The legend of figure 5 indicates the number of groups of flies weighed for each experimental condition and the total number of flies contained in those groups. Large, newly eclosed flies were excluded from analysis. We do not expect the measured differences in body mass to be biased by changes in mass that occur during the day of eclosion, as we have not observed any effect of *drd* on the timing of eclosion (E.M.B., unpublished observations).

Statistics and Data Analysis

Data were graphed and analyzed using GraphPad Prism v5 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). For survival curves (fig 1-3), pair-wise comparisons of each experimental group with its sibling control were carried out using a Mantel-Haenszel test. For body mass data (fig 5), each dataset was first tested for normality using a D'Agostino-Pearson normality test, and all sets were found to be Gaussian. The data for each experiment (based on the timing of the heat shock) was then analyzed by 1-way ANOVA followed by a Bonferroni Multiple Comparison Test to compare each experimental group with its sibling control. For real-time data (fig 4), a 1-way ANOVA followed by a Bonferroni Multiple Comparison Test was used to compare each time point either to the 0 hr value (start of heat shock) or the 24 hr value (recovery from heat shock).

3. Results

We first established a system for targeted knockdown and expression of *drd*. The UAS-RNAi lines $w^{1118}; \{GD15915\}v51184$ and $w^{1118}; \{GD3367\}v37404$ (referred to as 51184 and 37404, respectively) were obtained from the Vienna Drosophila RNAi center (Dietzl et al., 2007). The hairpin from 51184 is targeted to the first exon of *drd*, while the hairpin from 37404 is targeted against the sixth exon; neither is predicted to have any off-target effects (<http://stockcenter.vdrc.at>). Both UAS-RNAi lines were recombined with a UAS-*Dicer-2* line to ensure efficient cleavage of the dsRNA. All experiments using the UAS-RNAi lines also included the UAS-*Dicer-2* transgene. To test our ability to knock down *drd*, the UAS-RNAi lines were crossed with the ubiquitous *Actin5C-Gal4* driver. Male progeny from both crosses recapitulated the early lethality phenotype, with flies from the 51184 cross surviving 4 days and flies from the 37404 cross surviving 3 days (Fig 1a). Sibling controls that lacked the Gal4 driver survived the duration of the experiment (40 days, $p < 0.0001$ for pairwise comparisons of each knockdown population with its sibling control). Quantitative real-time PCR of 3 day-old males displayed 8.8 ± 0.8 fold and 7.9 ± 2.0 fold knockdown of transcript for progeny of the 51184 and 37404 crosses, respectively, relative to sibling controls ($n=3$, mean $\pm$ SD). For targeted expression of *drd*, we created a line carrying an inducible UAS-*drd* transgene (see methods). Flies carrying both the UAS-*drd* transgene and the *Act5C-Gal4* driver displayed a 32.7 ± 1.3 fold upregulation of *drd* transcript relative to sibling controls ($n=3$). This level of overexpression did not affect lifespan (Fig 1b, $p=0.59$). To test the ability of UAS-*drd* to rescue lifespan, the UAS-*drd* transgene was placed on a *drd^{lwf}* background and crossed with the *Act5C-Gal4* line. The progeny ubiquitously expressing *drd* survived for the entire experiment and sibling controls had a median

survival of 5 days (Fig 1c, $p < 0.0001$). This result indicated that we were able to rescue the early lethality phenotype and that the *UAS-drd* transgene is only expressed under Gal4 control.

To temporally control the expression and knockdown of *drd*, we crossed each UAS-RNAi line and the *UAS-drd* line with flies carrying the *Heat Shock Promoter 70 (Hsp70)-Gal4*; all experiments below were conducted on the progeny of these crosses. We first examined progeny at 24°C to ensure that the driver did not cause expression at a non-inducing temperature. Survival was wild-type for both of the UAS-RNAi lines (Fig 2a, $p = 0.06$ and 0.08 for 51184 and 37404, respectively) and the early lethality phenotype was observed in *drd^{lwf}* rescue flies (Fig 2b, $p = 0.60$). For the remainder of the experiments, we heat-shocked flies at 30°C for different stages of life to determine the temporal requirement of *drd* expression. This temperature is optimal for inducing and maintaining expression of a gene, while minimizing the adverse effects of increased temperature on lifespan (Chavous et al., 2001).

To test the necessity of *drd* expression in adults, flies were maintained at 24°C until eclosion and then moved to 30°C for the duration of the experiment. As shown in Figure 2c, there was no significant difference between *drd* knockdown flies and sibling controls ($p = 0.06$ and 0.70). Flies of all genotypes had a median survival between 24 and 27 days. We attribute the relatively short lifespan observed in this experiment to the increased temperature (Hollingsworth, 1968). Similarly, the complementary rescue experiment did not extend lifespan of the *drd* mutants, with both the experimental and control lines surviving a median of 5 days (Fig 2d, $p = 0.23$). These results indicate that *drd* expression in the adult fly does not affect the early lethality phenotype.

We next tested the requirement for *drd* expression during the embryonic and larval stages by crossing flies at 30°C and maintaining the progeny at this temperature until the wandering third larval instar. Under these conditions, knockdown of *drd* did not have an effect on lifespan; flies, experimental and control, survived the duration of the experiment for both UAS-RNAi lines (Fig 2e, $p=0.11$ and 0.67). Similarly, induction of *drd* expression during these developmental stages did not significantly rescue early adult lethality in *drd^{lwf}* mutants (Fig 2f, $p=0.77$).

In contrast to the previous experiments, expression of *drd* during metamorphosis did have an effect on lifespan. Knockdown of *drd* from the white prepupal stage until eclosion resulted in a median survival of 5 and 13 days for 51184 and 37404, respectively (Fig 2g). Sibling controls survived the duration of the experiment ($p<0.0001$ for each RNAi line). Similarly, induction of *drd* during this developmental stage resulted in complete rescue of the early lethality phenotype (Fig 2h, $p<0.0001$).

To narrow down the requirement for *drd* expression within the four days of metamorphosis, white prepupae were collected, immediately moved to 30°C for 48 hours, and then returned to 24°C. We observed partial effects when manipulating *drd* expression during the first half of metamorphosis. Knockdown of *drd* during this period resulted in a significant reduction in median lifespan to 12 and 9 days for 51184 and 37404, respectively (Fig 3a, $p<0.0001$ for each RNAi line). However, 30-40% of the experimental flies survived for the duration of the 40-day experiment. In addition, we observed a significant effect on survival ($p=0.03$) of rescuing *drd* expression during the first two days of metamorphosis, but an inspection of the survival curves indicates that this rescue was marginal (Fig 3b). To examine the second half of metamorphosis, white prepupae were collected and left at 24°C

for 48 hours and moved to 30°C until eclosion, after which they were returned to 24°C. Knockdown of *drd* during this stage resulted in a median survival of 7 and 9 days for 51184 and 37404, respectively (Fig 3c, $p < 0.0001$ for each RNAi line). Moreover, driving *drd* expression during the last two days of metamorphosis resulted in a complete rescue of early adult lethality (Fig 3d, $p < 0.0001$).

Quantitative real-time PCR was performed to determine the time course of *drd* knockdown and induction and subsequent recovery following the beginning and end of a 30°C heat shock starting on day 2 of metamorphosis. For both UAS-RNAi lines, there was a significant reduction in transcript abundance after 6 hours of heat shock. Moreover, 37404 showed significant knockdown of transcript by 3 hours (Fig 4a). The *UAS-drd* line required 6 to 24 hours to induce an increase in the transcript level (Fig 4b). For the UAS-RNAi line 51184, *drd* transcript levels recovered within 6 hours after removal from a 48-hour heat shock and in fact showed a slight overshoot. Transcript levels in the 37404 UAS-RNAi line returned to normal between 6 and 24 hours after the end of heat shock (Fig 4c). Recovery in the *UAS-drd* rescue line also appeared to take 6-24 hours, although there was no statistically significant change in transcript abundance from 3 hours after the end of the heat shock (Fig 4d).

On the day of eclosion, *drd* mutants have a significantly lower body mass than sibling controls (Blumenthal, 2008). We set out to determine when *drd* must be expressed during development to cause the reduced body mass phenotype. The same experimental strategy utilized for lifespan determination was employed for body mass assays. Heat shock was performed at different stages of development and then flies were weighed on the day of eclosion.

As expected, flies that did not undergo heat shock and were maintained at 24°C did not differ in body mass from their control siblings (Fig 5a). Since body mass is primarily determined during the larval stages, we next manipulated *drd* expression from embryogenesis until third instar wandering larvae. Knocking down and rescuing *drd* expression during this developmental time did not have a significant effect on body mass (Fig 5b). Moreover, there was no significant difference in body mass when *drd* was knocked down or rescued during metamorphosis (Fig 5c). However, when flies were heat-shocked throughout all of pre-adult development, we observed a significant difference in body mass. Knocking down *drd* throughout all of development resulted in a 10% decrease in body mass relative to sibling controls. Additionally, expressing *drd* throughout all of pre-adult development in a *drd^{lwf}* background resulted in flies that were 19% heavier than sibling controls (Fig 5d).

4. Discussion

The defining phenotype of *drd* mutants is an extremely short adult lifespan, and we have demonstrated that this phenotype results from an absence of *drd* expression during pupal metamorphosis. We can both rescue early adult lethality in *drd* mutants by temporarily expressing *drd* and recapitulate the adult-lethal phenotype in wild-type flies by temporarily knocking down *drd* expression. Therefore, we conclude that pupal expression of *drd* is both necessary and sufficient to ensure adult survival during the first weeks after eclosion. Consistent with this interpretation, manipulation of *drd* expression during embryogenesis, larval development, or adulthood had no effect on rapid adult lethality. In addition, ubiquitous overexpression of *drd* on a wild-type background had no apparent detrimental consequences.

Within the four days of metamorphosis, expression of *drd* is required during the later stages. Complete rescue/recapitulation of the adult lethal phenotype was achieved by heat-shocking pupae beginning 48 hours after prepupa formation and ending at eclosion. We can therefore rule out a requirement for *drd* expression during the first half of metamorphosis. It remains possible, however, that *drd* is required early in the first day of adult life because in this experiment *drd* transcript levels remained suppressed/elevated for several hours after eclosion and the end of the heat pulse. In the complementary experiment, heat-shocking pupae for the first 48 hours of metamorphosis had little effect on rescue of adult lethality but resulted in approximately half of the RNAi flies dying within two weeks of eclosion. There is some overlap in the developmental stages probed by these two experiments, as metamorphosis is accelerated at higher temperatures (Powsner, 1935). Taking our data as a whole, we conclude that for survival during early adulthood,

drd expression is required beginning in the middle of the third day of metamorphosis, and this critical period may extend into the first several hours following eclosion. Defining this critical period further will require not only shorter heat pulses but also knowledge of *drd* protein dynamics following the induction of expression or knockdown.

Our model for the timing of *drd* expression is consistent with recent immunostaining data, in which high levels of *drd* protein are reported in several pupal tissues (Kim et al., 2012). Our results also agree to some extent with the modENCODE temporal expression data, which show a modest 3-4 fold increase in *drd* expression on the third day of metamorphosis relative to the previous two days (Graveley et al., 2011; McQuilton et al., 2012). However, the modENCODE data also indicate that the highest level of *drd* expression occurs during the final third of embryogenesis, when expression levels are up to 7-fold higher than during metamorphosis. While our results are not consistent with a role for embryonic expression of *drd* in the determination of adult lifespan, it is possible that this early expression peak is required for other aspects of *Drosophila* development or physiology. No larval phenotypes have been reported for *drd* mutants, but to our knowledge this topic has not been thoroughly investigated.

One *drd* phenotype that could arise during metamorphosis is a defect in glial morphology. On the day of eclosion, glial cells in *drd* mutants appear morphologically immature and fail to wrap around the neurons (Buchanan and Benzer, 1993). The process of glial cell maturation and neuronal wrapping occurs in late pupae; eighty hours after pupal formation, glia in *drd* mutants are morphologically wild-type. It has been hypothesized that *drd* expression is required for glial maturation during late pupal

development and that the failure of glial maturation is related to the neurodegeneration that occurs several days after eclosion (Buchanan and Benzer, 1993).

Some of the phenotypes seen in adult *drd* mutant flies are hypothesized to be associated with defects in epithelial integrity or function. The tracheae and air sacs in mutant flies are reported to be fragile, and *drd* mutants express genes associated with hypoxia (Kim et al., 2012; Lehmann and Cierotzki, 2010; Tschäpe et al., 2003). Mutant flies also exhibit a specific defect in the movement of food from the crop through the cardia and into the midgut (Peller et al., 2009). It is noteworthy that both the foregut and the tracheae are lined with cuticle (Nation, 2002), and the cardia synthesizes another chitinous structure, the peritrophic matrix (PM) (Hegedus et al., 2009). The expression of multiple genes encoding cuticular or PM components, such as *Peritrophin A*, *Crystallin*, *resilin*, *Acp65Aa*, and 12 *Cpr* genes, peak on day 3 of metamorphosis (Graveley et al., 2011)—the same day on which *drd* expression is required for adult survival. The causal relationships among neurodegeneration, hypoxia associated with fragile tracheae, and starvation associated with defective food movement remain to be elucidated, as do the relative roles of these phenotypes in causing rapid adult death. However, all three of these phenotypes are potentially connected with developmental events that occur during the second half of metamorphosis.

The other phenotype examined in this study was adult body mass, which is significantly reduced in *drd* mutants (Blumenthal, 2008). In contrast to the effect on adult survival, knockdown/rescue of *drd* expression during metamorphosis had no effect on body mass. This result indicates that the reduced body mass observed in *drd* mutants is an independent phenotype, separate from adult lethality. In this respect, mutation of *drd* is

similar to mutations in *ilp* signaling, which, as reviewed above, have separable effects on body mass and lifespan. However, the control of lifespan by *ilp* signaling is an adult-specific effect, in contrast to the actions of *drd* during metamorphosis. Furthermore, knockdown/rescue of *drd* expression from embryogenesis through the wandering third larval instar, which encompasses the entirety of larval feeding, failed to affect body mass. Thus, the *drd* body mass phenotype appears to be distinct from the nutritionally regulated *ilp*-dependent control of larval growth rate discussed above. We were able to rescue/recapitulate the body mass phenotype by heat-shocking flies continuously from embryogenesis until eclosion. This effect was significant but did not fully eliminate the difference in body mass between the different genotypes; for example, even after heat shock, the experimental rescue flies still had a lower mass than the control RNAi flies. We attribute this residual mass difference to differences in the genetic backgrounds of the stocks used in this experiment. We conclude from these results that the critical period for the body mass effects of *drd* either extends throughout pre-adult development or encompasses the transition between the end of larval development and the beginning of metamorphosis. It is interesting to note that both insulin receptor activity and the *ilp* dILP6 are reported to have effects on adult body mass after the cessation of feeding, during the wandering third instar (Okamoto et al., 2009; Shingleton et al., 2005; Slaidina et al., 2009). Thus, our data are consistent with an interaction between *drd* and *ilp* signaling late in larval development. We also cannot rule out a role for *drd* in ecdysteroid production or signaling that would affect the timing of pupariation.

The primary finding of this work is that the early adult lethality observed in *drd* mutants arises from an absence of *drd* expression at an earlier stage of the life cycle. To

our knowledge, this is without precedent among neurodegeneration mutants. While many neurodegeneration-associated genes are expressed both during development and in adults, this expression has either been demonstrated or is believed to be required for the maintenance of larval, pupal, or adult neuronal integrity (Khodosh et al., 2006; Lee et al., 2011; Lim and Kraut, 2009; Mühlig-Versen et al., 2005; Sone et al., 2009). In contrast, *drd* expression is clearly not required for the maintenance of the adult nervous system. Rather, *drd* expression appears to be required for the development of one or more adult tissues during metamorphosis, and the improper development of such tissues in *drd* mutants leads, within 1-2 weeks, to the death of the adult. It has also been reported that appearance of several age-dependent markers or phenotypes is accelerated in *drd* mutant adults, suggesting that *drd* mutants age more quickly than wild-type flies (Reenan and Rogina, 2008; Rogina et al., 1997). In light of this finding, our data raise the intriguing possibility that the rate of aging in adults could be controlled to some degree by events that occur during metamorphosis.

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Figure Legends

Figure 1. Establishment of a system for knockdown and expression of *drd*. a) Ubiquitous knockdown of *drd* by crossing *y w; Act5C-Gal4/TM6B* males with either *w; {GD15915}v51184 UAS-Dcr-2* or *w; UAS-Dcr-2 {GD3367}v37404* females recapitulates the early lethality phenotype. b) Overexpression of *drd* on a wild-type background by crossing *y w; Act5C-Gal4/TM6B* males with *w; UAS-drd* females has no effect on lifespan. c) Ubiquitous expression of *drd* on a *drd^{lwf}* background by crossing *y w; Act5C-Gal4/TM6B* males with *drd^{lwf}/ FM7a; UAS-drd* females rescues the early lethality phenotype. n=50-57 flies/genotype.

Figure 2. Temporal requirement of *drd* expression for survival. Survival curves of adult male progeny from crossing *w; Hsp70-GAL4* males with either *w; {GD15915}v51184 UAS-Dcr-2* or *w; UAS-Dcr-2 {GD3367}v37404* females for *drd* knockdown (a,c,e,g) and *w; Hsp70-GAL4* males with *drd^{lwf}/ FM7a; UAS-drd* females for *drd* rescue (b,d,f,h). a,b) flies were maintained at 24°C for the entire life cycle. c,d) flies were maintained at 30°C after eclosion. e,f) flies were maintained at 30°C from embryogenesis until wandering third larval instar. g,h) flies were maintained at 30°C during metamorphosis. n=50-70 flies/genotype.

Figure 3. Requirement for *drd* expression within metamorphosis. Survival curves of adult male progeny of crosses as detailed in fig. 2 following knockdown (a,c) or rescue (b,d) of *drd* during the first 48 hours of metamorphosis (a,b) or the final 48 hours of metamorphosis (c,d). n=50-60 flies/genotype.

Figure 4. Time course of *drd* knockdown and rescue during and following heat shock in 2-day pupae. Pupae were derived from crosses as detailed in fig. 2. a) knockdown of *drd* and b) rescue of *drd* after different durations at 30°C. Transcript levels were normalized to the level at 0 hours. c,d) Recovery of *drd* transcript from knockdown (c) and rescue (d) at different times after the end of a 48-hour heat shock. Transcript levels were normalized to the level at 24 hours of recovery. Asterices indicate significant difference from 0 hours (a,b) and 24 hours (c,d) by 1-way ANOVA and Bonferroni post-test. *:p<0.05; **:p<0.01; ***:p<0.001. n=2. Error bars represent S.E.M.

Figure 5. Temporal requirement of *drd* expression for normal body mass on the day of eclosion. Crosses and heat shock were performed as in fig. 2. The legend shown in (a) applies to all four graphs. a) No heat shock. b) Heat shock from embryogenesis until wandering third larval instar. c) Heat shock throughout metamorphosis. d) Heat shock for all of pre-adult development from embryogenesis until eclosion. Asterices indicate significant effect of rescue or knockdown by 1-way ANOVA and Bonferroni post-test. *:p<0.05; **:p<0.01. n=50-55 flies weighed in 10-23 groups per condition. Error bars represent S.E.M.

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Figure 1

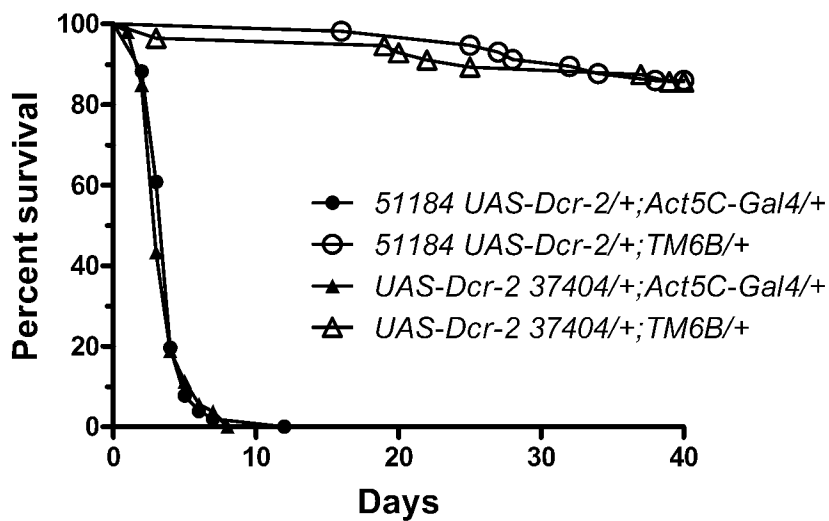
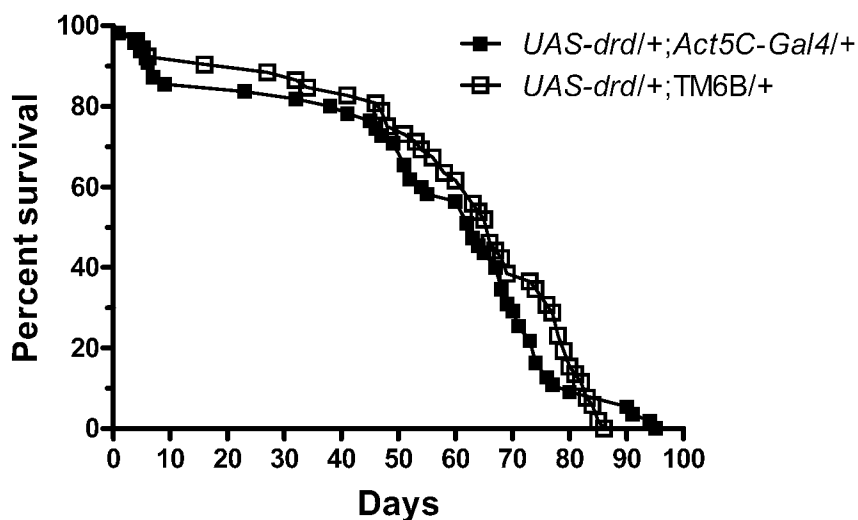
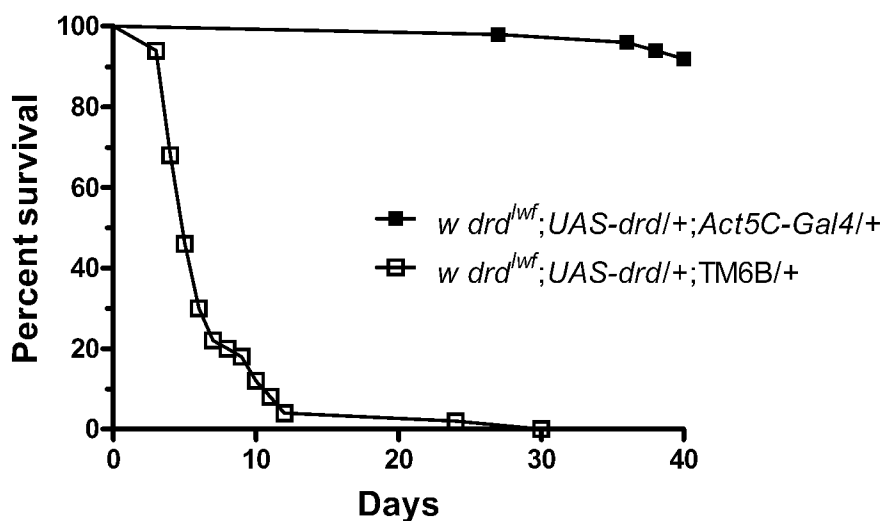
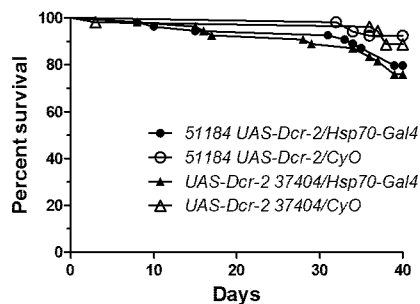
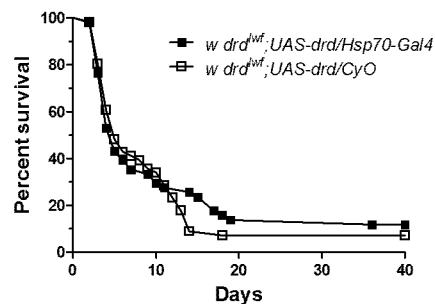
A**B****C**

Figure 2**A**

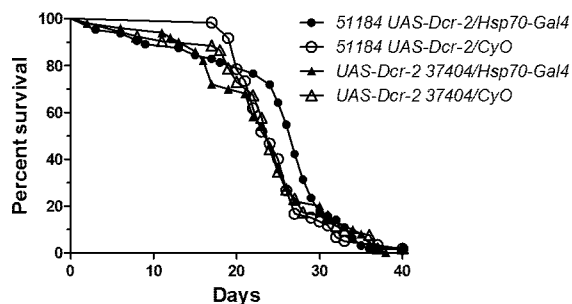
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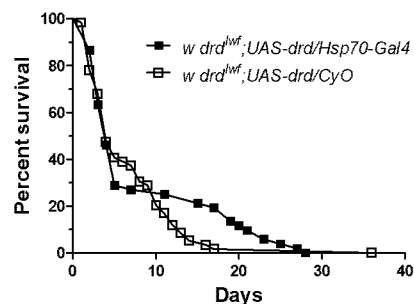
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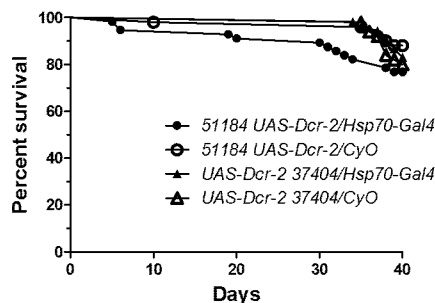
Heat shock: adults

**D**

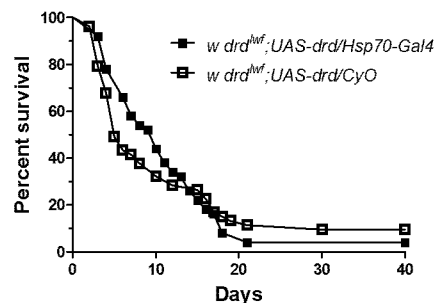
Heat shock: adults

**E**

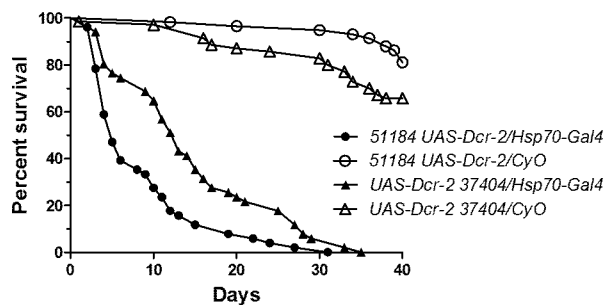
Heat shock: embryos and larvae

**F**

Heat shock: embryos and larvae

**G**

Heat shock: pupae

**H**

Heat shock: pupae

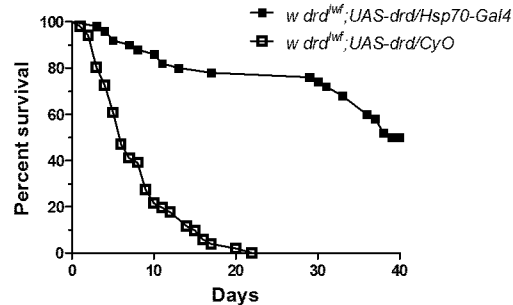
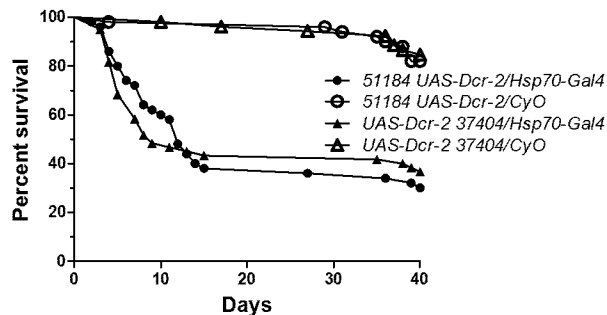
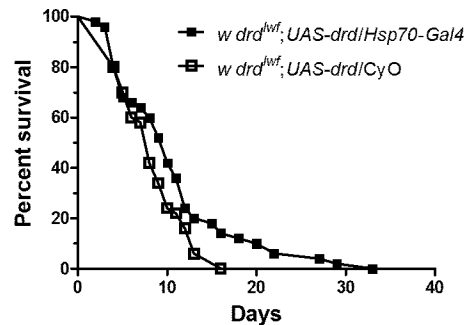


Figure 3

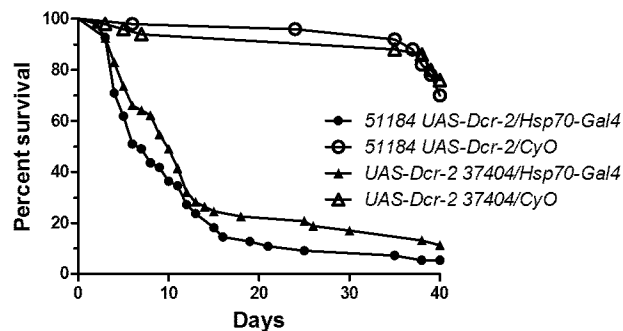
A Heat shock: first 48 hr. pupae



B Heat shock: first 48 hr. pupae



C Heat shock: second 48 hr. pupae



D Heat shock: second 48 hr. pupae

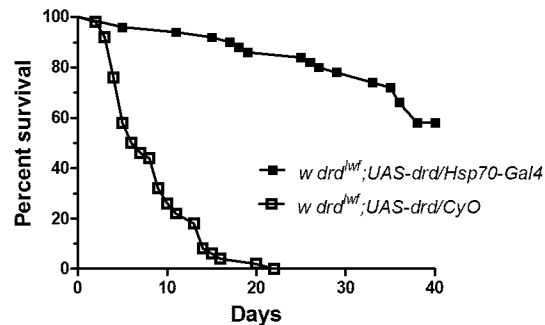
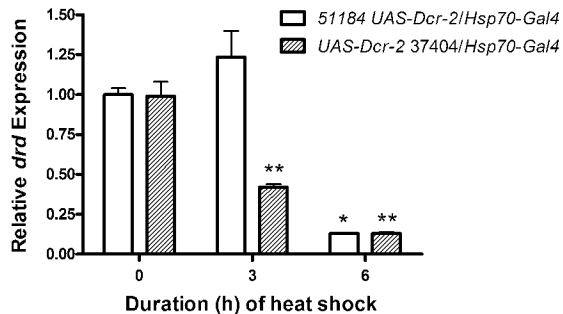
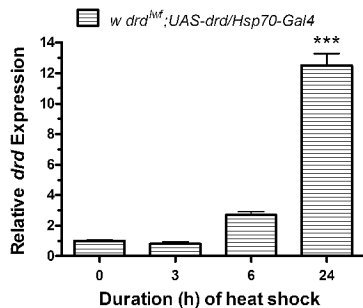


Figure 4

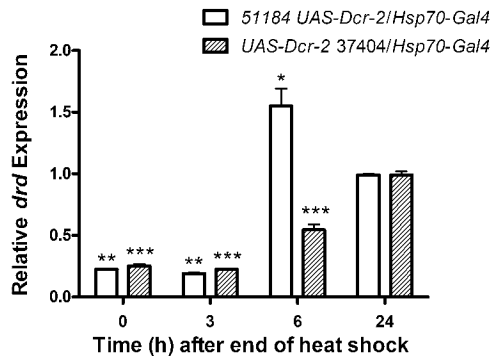
A



B



C



D

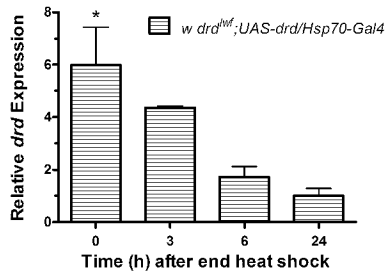
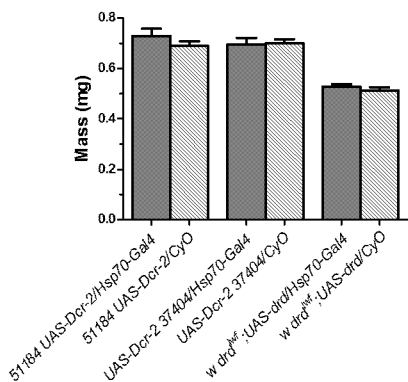
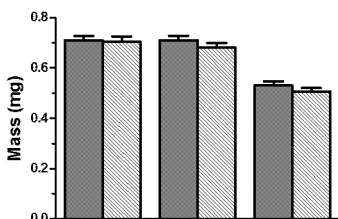


Figure 5**A**

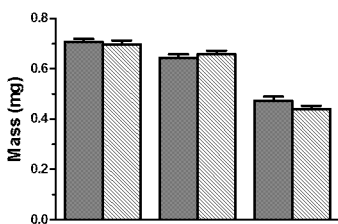
No heat shock

**B**

Heat shock: embryos and larvae

**C**

Heat shock: pupae

**D**

Heat shock: embryos, larvae, and pupae

