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Curcumin is an early-acting stage-specific inducer of extended functional longevity in *Drosophila*

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ABSTRACT

Larval feeding with curcumin induces an extended health span with significantly increased median and maximum longevities in the adult fly. This phenotype is diet insensitive and shows no additive effect on longevity when combined with an adult dietary restriction (DR) diet, suggesting that curcumin and DR operate via the same or overlapping pathways for this trait. This treatment significantly slows the aging rate so that it is comparable with that of genetically selected long lived animals. The larval treatment also enhances the adult animal's geotactic activity in an additive manner with DR, suggesting that curcumin and DR may use different pathways for different traits. Feeding the drug to adults during only the health span also results in a significantly extended health span with increased median and maximum life span. This extended longevity phenotype is induced only during these stage-specific periods. Feeding adults with the drug over their whole life results in a weakly negative effect on median longevity with no increase in maximum life span. There are no negative effects on reproduction, although larval curcumin feeding increases development time, and also apparently accelerates the normal late-life neuromuscular degeneration seen in the legs. Gene expression data from curcumin-fed larvae shows that the TOR pathway is inhibited in the larvae and the young to midlife adults, although several other genes involved in longevity extension are also affected. These data support the hypothesis that curcumin acts as if it is a DR mimetic nutraceutical. These data also suggest that the search for DR mimetics may be enhanced by the use of stage-specific screening of candidate molecules.

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1. Introduction

The adult life span in *Drosophila* consists of a health span, a transition phase, and a senescent span (Arking et al., 2002a). Analysis of these life stages in model organisms showed that they are characterized by different patterns of gene expression such that the health span is defined by tightly regulated gene expression patterns which maximize tissue function while minimizing inflammatory and other damage responses; the transition phase is characterized by a qualitative decrease in the regulatory ability of the cell, and the senescent span is characterized by a stochastic pattern of degradation of the gene expression network (Arking, 2009; Park et al., 2009; Pletcher et al., 2002). Research on model organisms over the past decade has identified certain gene products, such as the insulin-receptor (InR) or the target of rapamycin (TOR) or histone deacetylase inhibitors (HDACI), as playing an important role in regulating the patterns of gene expression, and so altering the longevity of the experimental organisms (Kenyon, 2010). Other lines of research have focused on identifying small molecules which, when given orally to an organism, can affect these same regulatory processes by activating or inhibiting the gene product in situ. Examples of such effects are the longevity enhancing effects of 4-phenyl butyrate (4PB) on flies (Kang et al., 2002), resveratrol on flies (Antosh et al., 2011), rapamycin on flies (Bjedov et al., 2010; Harrison et al., 2010), and mice (Harrison et al., 2009; Miller et al., 2011), tetrahydrocurcumin on mice (Kitani et al., 2007) and/or curcumin on flies (Lee et al., 2010; Suckow and Suckow, 2006).

It is common to administer experimental prolongevity drugs over the entire adult life span of the test organism. In this paper, we present data which leads to a different paradigm of prolongevity drug

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intervention. Given that different stages of the life span have different patterns of gene expression, and given that longevity regulating gene products may have a gene-specific target pattern, then there is reason to believe that whole-life interventions will not always be the most effective intervention. In fact, the first genotropic compound demonstrated to have a beneficial effect on *Drosophila* longevity was 4PB (Kang et al., 2002), which was known to function as an HDAcI (Chen et al., 2002). This initial report suggested the existence of a stage-specific effect since 4PB feeding during the first 12 days of the adult life span (e.g., including a portion of the health span) was not as effective as feeding during the time period from 12 days to the end of life (e.g., including the transition and senescent periods).

Given that there is significant change (~23%) in genome wide transcript profiles with age in *Drosophila* (Pletcher et al., 2002), then the targets of genotropic compounds may well not be present in all life stages. Such compounds may thus have stage-specific positive effects in one part of the life span but neutral or negative effects in another part. If so, then whole-life treatments alone might not detect if a particular compound showed no effect because it really was not an effective pro-longevity drug, or because there was a masking effect such that deleterious effects in the health span, for example, preceded beneficial effects in the senescent span (or vice-versa). Given the potential of whole life experiments to yield false negative conclusions, then temporally targeted investigations may yield better insights into drug efficacy.

In this report, we tested the effects of curcumin on the longevity of our normal-lived Ra strain. Curcumin was chosen because it was known to bind to the TOR complex of flies in vivo (Bjedov et al., 2010) and human cells in vitro (Beevers et al., 2006; Yu et al., 2008; Zhou et al., 2010), although it is known to down-regulate other targets as well (Das et al., 2010; Sun et al., 2011). In addition, Kitani et al. (2007) showed that tetrahydrocurcumin had a stage-specific effect on mouse longevity. TOR is known to affect the longevity of *Drosophila* (Bjedov et al., 2010; Kapahi et al., 2004) and to play a central role in the cell signaling pathways of the fly (Nuzhdin et al., 2009). The stage-specific longevity alterations in our selected fly strains have been previously described (Arking et al., 2002b) and primarily consist of a significant extension of the health span and a consequent delayed onset of senescence.

We now report that curcumin has beneficial effects in the normal-lived Ra strain in that it increases longevity when administered in the developmental or health span stages, which may be due to systemic effects on the TOR complex. Curcumin has a negative effect when administered over the entire adult life span or over the senescent stage only. This may be related to an apparent curcumin-dependent accelerated neuromuscular degeneration observed in the legs of mid- and late-life treated animals. Curcumin is an early acting prolongevity drug which acts so as to yield the same longevity effects as does DR in our strains. Experiments designed to identify prolongevity drugs should take this stage specificity into account.

2. Experimental procedures

2.1. Experimental design

Our experimental design is based on stage-specific feeding of the chemical. We tested four life stages: the larval stage, and the three adult stages of the health span, transition span, and senescent span. The larval span is defined as the period of time from hatching from the egg (~22 h after the egg-laying midpoint) until pupation (~6 days). Neither the embryo nor the late larvae or pupae feed; thus the feeding time is limited to days −9 to −~5.5. We did not test the possibility of curcumin adsorption by the embryo. The adult health span is defined as the period of time when there are no intrinsic deaths and which is approximated by adult survival >90%. The adult transition period is recognized by the (usually) sharp decline in adult survival and defined by the time period between 90% and ~80% survival. The adult senescent period is recognized by the steady decline portion of the survival curve and defined by the period between the end of the transition period and the maximum longevity span (e.g., LT_{10}). There is some intrinsic variation in the ages when different cohorts reach these survival points, and so the actual transition times are based on the progress of contemporaneous control and experimental vials.

For the larval stage treatments, parental flies of the appropriate strain were allowed to mate for ~1 day before being transferred to a new vial and allowed to lay eggs on the curcumin-containing food for one day. Parental flies were transferred to new vials each day, and discarded after ~3–4 days of egg laying so as to avoid any curcumin effects on developing eggs. Thus, eggs were laid on day −10 before adult eclosion on day 1. The larvae fed on the chemical from ~days −9 to −~5.5 before they stopped feeding and pupated. Table S-1 shows that lower (μM) doses of curcumin have no effect on the median time of adult eclosion (AE_{50}) but Ra larvae fed on higher (mM) doses show a ~1–1.5 day delay in the AE_{50} value due to a slowdown of development. We observed no effect on adult eclosion rates relative to controls (Fig. S-2). For the adult stages, animals were exposed to the curcumin-containing food for a defined number of days as judged by contemporaneous control cultures. For the Ra strain in adult feeding experiments, these actual treatment ages were as follows: health span = 5–27 days, transition span = 27–40 days, senescent span = 38–89 days.

For molecular biology analyses, flies of the desired strain and age were raised under the indicated conditions, frozen and analyses done as indicated below.

2.2. Animals and doses

We used the normal-lived Ra strain which has been well characterized (Arking, 1987; Arking et al., 2002a; Soh et al., 2007). Curcumin was obtained from Sigma as, Fluka brand at >95% pure (#28260, lot #1355475) or from Fisher as Acros brands at 98% pure (#218580500, lot #A0284314) total curcumin plus curcuminoids (desmethoxycurcumin and bisdesmethoxycurcumin, ≥95.0% (TLC)). In control tests, we noted no consistent difference between the two brands (data not shown). Standard protocol for putting curcumin into solution involves dissolving it in ethanol or DMSO and then mixing it with the media. This limits the possible concentration of curcumin in the food to μM amounts. Test flies raised on AL food containing up to 1000 μM curcumin in DMSO showed only a slight increase in longevity (data not shown). Both Kitani et al. (2007) and Suckow and Suckow (2006) reported significant extensions of longevity in mice and flies by directly mixing their tetrahydrocurcumin or curcumin into the food in mg amounts. We tested this mode of incorporating curcumin into the food by direct vigorous mixing of the chemical with warm AL food at concentrations ranging from 10 to 200 mM. This runs the risk of curcumin hydrolysis (Wang et al., 1997) but it seems to have worked. The resulting media was a combination of dissolved and small but variably sized solute curcumin. Larvae raised on the food were observed to eat both forms of the chemical. Adults raised on the food showed a yellow coloration of their intestines indicating that they were eating significant amounts. We did not control for the possible effect of curcumin affecting adult food intake. The results presented herein show that this method of administering the drug is effective. Future work will involve a more precise titration of the effective dose. The fly media used is our standard yeast–sugar–cornmeal recipe which was previously described (Luckinbill et al., 1984). The AL version of this food contains 64 g of yeast and 108 g of sucrose per liter; the DR recipe contains 32 g of yeast and 54 g of sucrose per liter.

2.3. Stock maintenance

All stocks were maintained in incubators at 25 °C and a 12 h/12 h light/dark cycle. Humidity was maintained via water pans. Longevity experiments were initiated by collecting newly eclosed adults on day 1 from the appropriate strain and treatment, allowing them to mate

for several days, sexing the adults on days 3–5 after eclosion with CO_2 anesthesia treatment, and transferring them in groups of 25 same-sex flies to the appropriate food vials. Adults were transferred to new vials three times weekly and survival data was recorded for each vial. The data for each treatment group was compiled, and when all flies had expired, survival curves were plotted using both Excel and GraphPad Prism software. Statistical significance for longevity was calculated by using the Log-Rank test in Prism. Age-specific mortality rate (Gompertz) data was calculated from the relevant survival data, and the curve best fitting the data was determined using the maximum likelihood protocol in the Winmodest program (Pletcher et al., 2000), and graphed using Prism GraphPad v5 software.

2.4. Paraquat protocol

In order to find a working concentration of paraquat that could resolve changes in oxidative stress response, Ra flies were collected and exposed to concentrations ranging from 0 to 40 mM in 5% sucrose. Male and female flies exposed to 15 mM paraquat exhibited a standard S-shaped dose response curve with a median life span of 2–3 days (data not shown). In order to introduce positive and negative controls we sought genotypes that had been reported as either paraquat resistant or paraquat sensitive. The La strain is known to be very paraquat-resistant (Arking et al., 1991; Force et al., 1995), while the *bsk2* stock is much more susceptible to paraquat than are controls (Miller and Arking, unpublished data). These control genotypes were run in each experiment to verify that there were no technical problems.

2.4.1. Paraquat assays

For each test and control line, several replicates of 100 or more flies were analyzed for each cohort. Flies at the appropriate age were transferred to vials containing 5 Whatman 2.3 cm grade 3 filter discs saturated with 15 mM paraquat (Sigma) in a 5% sucrose solution. Flies were monitored every few hours and deaths recorded all animals had died or until the sugar-water only controls started to die (~5–7 days). Mortality data was plotted and analyzed for significance using the log-rank survival tests of Graphpad Prism 5. We determined the hours of exposure needed to kill 50% of the animals (LT_{50}) and used this median value as an index of their resistance to acute oxidative stress.

2.5. Behavioral analysis

We modified Groteweil's RING apparatus (Gargano et al., 2005) so as to yield an inexpensive device suitable for high throughput analysis. Basically, a clear Plexiglas box firmly holding six vials in place was built and placed in front of a neon white backlight equipped with a white diffuser. Thin wires across the front of the box optically divided the vials into four equal-sized quadrants. Each vial held 25 male or female flies, or 150 animals in total. A digital camera was mounted in front of the box. The box was tapped several times on a foam pad to knock all the flies to the bottom of each vial. Prior experiments (data not shown) indicated that, for the young Ra strain animals, a four second interval between tapping and taking the photo sufficed to give a good distribution of flies over the vertical axis of the vial. Each set of six vials was tested twice consecutively, and the mean climbing index obtained. This was done by counting the flies in each quadrant of each vial, multiplying that by the number of the quadrant (1 was lowest), summing this weighted number across all quadrants, and dividing that by the total number of flies to obtain the mean geotactic index for that cohort. Other statistical tests were done as described in Fig. 6.

2.6. Neuromuscular analysis

Ra larvae were fed on curcumin as described and raised as adults on standard AL food without curcumin. At ages ranging from 20 to 68 days, aliquots of adults were removed, anesthetized with CO_2, their legs cut off with fine micro-dissecting scissors, and immersed in *Drosophila* Ringer's solution. Using standard protocols, they were stained for double stranded DNA and mitochondria, and then analyzed by light microscopy. The reported observations were obtained from left or right hind legs. During this study, light microscopic observations were made of the fore-, mid-, and hind legs from the left and right sides of at least three and as many as five flies (e.g., 12 to 30 legs) from a single time point per experiment. Each experiment was repeated at least twice. Fields in which dsDNA-containing nuclei were observed in leg muscle showed at least 20 H33342-labeled nuclei, usually in a row along the edge of myofibers, as is typical for skeletal muscles. This value for number of nuclei went to zero at days for which nuclei were said to be absent.

2.6.1. Imaging of nuclear double-stranded DNA (dsDNA)

Imaging of nuclear double-stranded DNA (dsDNA) within fly legs and thorax was performed using the DNA-specific (AT-selective) bisbenzimide dye Hoechst H33342 (H33342; Invitrogen, peak $\lambda_{\text{Excitation peak w dsDNA}} = 350$ nm, peak $\lambda_{\text{Emission peak w dsDNA}} = 460$ nm). Dissected portions of flies were bathed in *Drosophila* Ringer's solution for up to 60 min, then bathed in 10 ng/ml H33342 in Ringer's solution, and labeled for up to 22 h at 4 °C. This concentration of H33342 is more than 3 orders of magnitude below the LD50 for H33342. Labeled and un-labeled control fly parts were washed for 2 min in Ringer's solution, then placed into a drop of FC-70 fluorochemical oil on a biocleaned microscope slide, covered with a biocleaned 0.17 mm thick coverslip. A double thickness of Parafilm® was used as a spacer to assure that the fly parts were not crushed by the coverslip. The resulting chamber was sealed with clear nail polish (Sally Hanson, Hard As Nails™) and the polish was allowed to dry at room temperature prior to mounting onto the microscope stage for observation. Fluorescence emission intensities of these samples were well within the linear range of our cameras operated at 12-bit bit-depth with $\gamma = 1.0$.

2.6.2. Localization and analysis of mitochondrial location, morphology, and number

Localization and analysis of mitochondrial location, morphology, and number were determined in hind legs of larval curcumin-fed and control flies using the mitochondria-selective fluorescent dye MitoTracker Deep Red 633 FM (Invitrogen; $\lambda_{\text{Excitation peak}} = 644$ nm, $_{\text{peak}}\ \lambda_{\text{Emission peak}} = 665$ nm). Cultured legs were labeled at minimal dye concentrations and illuminated only during image capture as described below. Taken together, these observations permit assessment of the location and amounts of mitochondria (MitoTracker) relative to the amount of nuclear dsDNA (H33342). Thus, these assays provide a functional catalog and quantitation of changes in location and level of nuclear and mitochondrial presence in a time-dependent manner.

2.6.3. Light microscopy

Light microscopy was performed by RBS with a Zeiss AxioObserver D1.m microscope equipped with a 1.4 NA condenser, optics for differential interference contrast (DIC) using linear polarized light with an LED illuminator and Senarmont compensator, Zeiss 40X/1.1 NA c-apochromat objective lens, episcopic illumination with an X-Cite® Series 120 illuminator and liquid light guide, appropriate filter cubes for fluorescence, a Zeiss AxioCam HSm high speed monochrome digital camera. Observation of the dsDNA-H33342 complexes was made with a Zeiss 48 filter cube. Observations in the green (fluorescein) channel were performed with a Zeiss 38 HW GFP filter cube. Observations of labeled mitochondria used a Zeiss Hq Cy5 filter cube. Zeiss AxioVision software (v 4.8) was used for instrument control, image acquisition, processing and analysis; image data were recorded onto a RAID 1 hard disk array.

2.7. Gene expression profiling

Ra parents laid eggs on vials with either AL control food or AL food containing 100 mM curcumin. Curcumin-fed or control-fed non-feeding late 3rd instar larvae were collected from some of the vials, and frozen as described below. Animals in the other vials were allowed to eclose and were aged on an AL diet until males were collected at the following days of adult life: 1 (eclosion), 5, 15, 30, and 45 days. At these time-points, the animals were sexed, counted, weighted in a tared tube, and then flash-frozen in liquid N_2, and subsequently stored at −80 °C until assayed. The mRNA expression levels of ten different genes in each of the six age cohorts were quantified using real-time PCR technique. Briefly, total RNA was extracted from ~30 to 40 larvae or adult control and experimental flies, and was considered as one sample. Total RNA was extracted from 3 to 4 samples of each condition using the TRIzol reagent for RNA (Gibco BRL, Rockville, MD, USA). First strand cDNA was synthesized from 1 μg RNA using random primers and purified using QIAquick PCR purification Kit (Qiagen, Valencia, CA). Expressions of ten genes involved in TOR signaling and/or longevity extension were quantified for each cohort using real time PCR with specific primers for the genes studied (Table 2). The difference in expression was calculated as the mean (±SEM) of fold differences of experimental relative to control flies. Briefly, the average of the control group for each age was used as a reference for that age. Each sample was individually calculated as a fold change relative to the reference value for that age. The fold changes for the control and the experimental samples at each age were compared for the relative difference in the mean and the variance.

3. Results

3.1. Effects of curcumin feeding during development stage

3.1.1. Dose response and genome specificity

We began our analysis with the larval stages. A dose–response test was done on the Ra strain by testing the effect of 0, 10, 100 and 200 mM curcumin food fed to larvae only, and assaying the subsequent longevity of the adult flies raised on the AL standard food. Fig. 1A shows that the optimal extended longevity response was displayed by the Ra adults fed 100 mM curcumin as larvae (days −9 to −5), which displayed an 80% increase in the length of their health span relative to the control. The 10 mM dose caused no discernable effect on any stage of the adult life span. The 200 mM dose showed no extension of the health span but did show an apparent extension of the senescent span only. We standardized on the 100 mM dose as this provided the optimal longevity effect, particularly the delayed onset of senescence, for this stage-specific feeding.

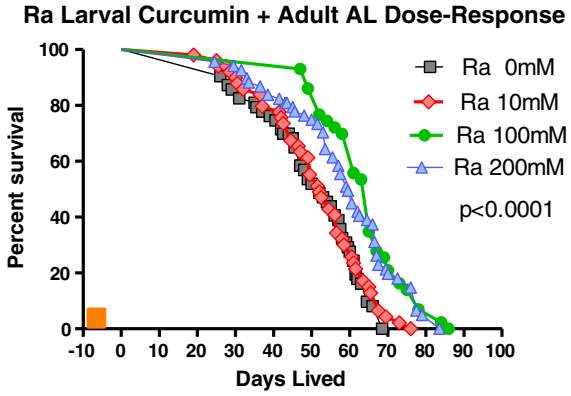

Fig. 1. Larval curcumin dose–response studies. Eggs were laid on curcumin-containing media and exposed to the chemical from −10 to −5 days as indicated by the solid box on the X-axis. Resulting female adults were raised on control AL food. The N values are: 0 mM-63; 10 mM-50; 100 mM-43; 200 mM-68. The normal-lived Ra animals show a maximum response at 100 mM (e.g., a ~85% delay in the age of onset of senescence relative to controls (48 vs 26 days)), and significant increases in median (65 vs 52 days) and maximum (LT_{90}; 77.5 vs 64.5 days) life spans relative to controls (log rank test, $X^2=26.79$, 1 df, $p<0.0001$). The 10 mM dose is not significantly different from the control ($p=0.6031$), while the 200 mM dose is less significant ($X^2=15.61$, $p<0.0001$) than the 100 mM dose relative to control (see above).

3.1.2. Larval feeding of curcumin results in an extended longevity adult phenotype which is diet-insensitive

In this experiment, we combined larval feeding of 100 mM curcumin with either the AL or DR type of adult food. The Ra control adults not treated with curcumin and raised on AL food show a normal-lived AL-type phenotype, while those raised on DR food show a long-lived DR-type phenotype (Fig. 2), as expected, based on our prior studies of dietary restriction in these animals (Soh et al., 2007). Importantly, Ra animals treated with 100 mM curcumin as larvae and raised on either AL or DR food show a long-lived phenotype which is diet-insensitive. Note that both sexes showed the same type of response. There is clearly a curcumin-sensitive developmental component to adult longevity.

3.1.3. Larval curcumin and adult dietary restriction are not additive for longevity

The data of Supplementary Fig. 1 makes clear that animals fed 100 mM curcumin as larvae and then raised on DR-type food as adults express an extended-longevity phenotype statistically indistinguishable

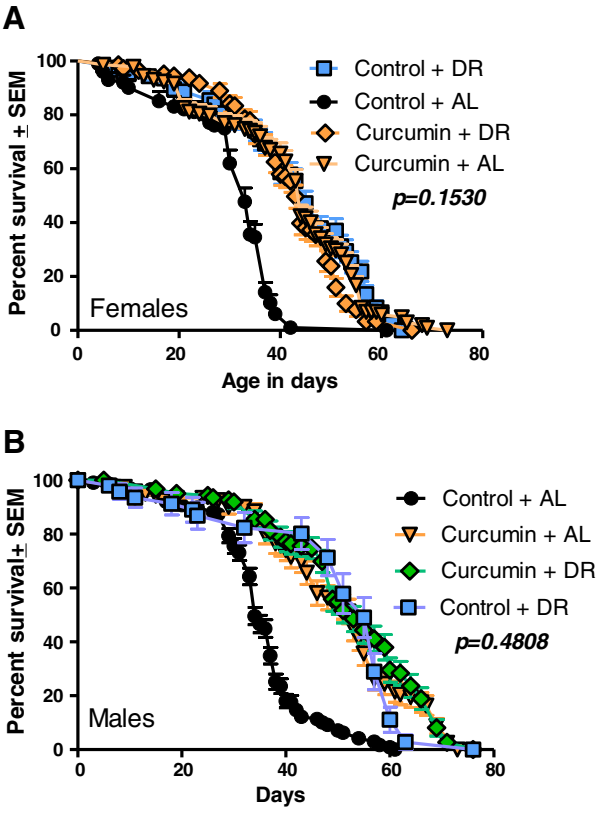

Fig. 2. The larval curcumin effect is adult diet insensitive. Animals were treated with 100 mM curcumin or not, as in Fig. 1 and the longevity of each sex assayed. In both females (A) and males (B), the control + DR, curcumin + AL and curcumin + DR had statistically identical (log-rank test, see p values in A and B) and longer mean and maximum life spans, while the control + AL cohort had a significantly shorter life span relative to the other cohorts in both A and B (log-rank test, $p<0.0001$). For females, the N, median, and LT_{90} values are respectively, control + DR = 124, 45 and 58 days; control + AL = 120, 33 and 38 days; curcumin + DR = 127, 43 and 53 days; and curcumin + AL = 87, 43 and 55 days. For males, the comparable N, median, and LT_{90} values are respectively, control + DR = 312, 34 and 61 days; control + AL = 201, 34 and 61 days; curcumin + DR = 145, 53 and 69 days; and curcumin + AL = 58, 53 and 67 days. See text for discussion.

Table 1
Curcumin-fed larvae yield adults with stage-specific resistance to acute antioxidant stress.

Age	N	Paraquat, mM	Sex	LT_{50} in hours of exposure time to paraquat needed to kill test animals[a]		p value	Relative % change in LT_{50} antioxidant resistance
				Control	Curcumin		
15	271	15.0	F	25.4	29.3	0.5055	−3.5%
	289	15.0	M	45.9	43.7	0.8781	−4.7%
30±1	465	1.0	F	121.4	140.5	0.0025	+15.7%
	393	1.0	M	135.8	143.4	0.0200	+5.6%
45	472	0.25	F	135.0	124.9	0.0003	+7.7%
	289	0.25	M	130.7	139.5	0.0003	−7.7%

[a] The PQ dose was adjusted so that each age group would have their LT_{50} value fall within the time bounds of the desiccation controls (not shown).

from a DR phenotype expressed by controls fed a DR-type diet as adults. Thus the two dietary interventions employed in Fig. 2 are not additive but yield statistically similar survival curves in both sexes, even though they were induced in different life cycle stages. This suggests that the curcumin effect is epistatic to the DR effect and that both interventions are using the same or shared pathways.

3.1.4. Effect of larval curcumin feeding on adult reproduction and body weight

It was necessary to determine if apparent side-effects of the treatment might account for the observed curcumin effect. Our preliminary data shows that larval curcumin feeding has no significant harmful effect on adult fertility or fecundity. In fact, it appears as if curcumin feeding increases fertility and has no detrimental effect on the egg-hatching rate (Supplementary Table 1). We did observe a small but consistent delay in development time such that the 100 mM curcumin fed Ra strain larvae took ~1.01 days longer to eclose as adults relative to the AL controls (Supplementary Fig. 2). We observed no detrimental or trade-off effects arising from this increased developmental time.

The data of Supplementary Table 2 shows that the adult body weight over the course of the life span is consistently significantly decreased in males, but not in females. We observed no detrimental effects arising from this sex-specific effect on body weight.

3.1.5. Effect of larval curcumin feeding on adult acute oxidative stress resistance

Oxidative stress is often correlated with extended longevity. We tested the ability of adults derived from curcumin-fed larvae to resist lethal doses of paraquat. There is no significant effect noted in young (15 day) animals, and a significant but sex-related effect noted in old (45 day) animals (Table 1). A modest but highly significant (log-rank test, $p = 0.0025$) increase in the median survival of both sexes is observed only in middle-aged (30 day) animals. This may reflect the early to mid-life stage-specific effects of curcumin.

3.1.6. Effects of larval curcumin feeding on adult age-specific mortality kinetics

Changes in longevity must flow from changes in mortality. Curcumin-fed Ra larvae yield adults of both sexes with significantly altered mortality kinetics (Fig. 3). The age specific mortality values of the curcumin treated females and males are significantly different ($p<0.0001$, F-test) than the controls, and are best described by different curves. Curcumin treatment changed the Gompertz–Makeham type curve of the control animals into the Gompertz curve of the experimental cohort. The Gompertz curves of the (untreated) long-lived La animals are shown in Fig. 3A for comparison. Note that the decreased slope and reduced early mortality of the curcumin treated Ra females approximate that of the Gompertz curve for the long-lived La female control. Curcumin significantly alters the male mortality response ($p<0.0001$, F-test) such that they are also best described by a Gompertz curve without additional mortality terms.

3.2. Effects of curcumin feeding during adult life stages

3.2.1. Feeding during the entire adult life span has no effect on longevity

We next investigated the effects of curcumin on the adult life cycle stages. When treated with 100 mM curcumin during their entire adult life span (e.g., from days 5 to 65), both males and females responded with obvious decreases in median longevity. This decrease is borderline

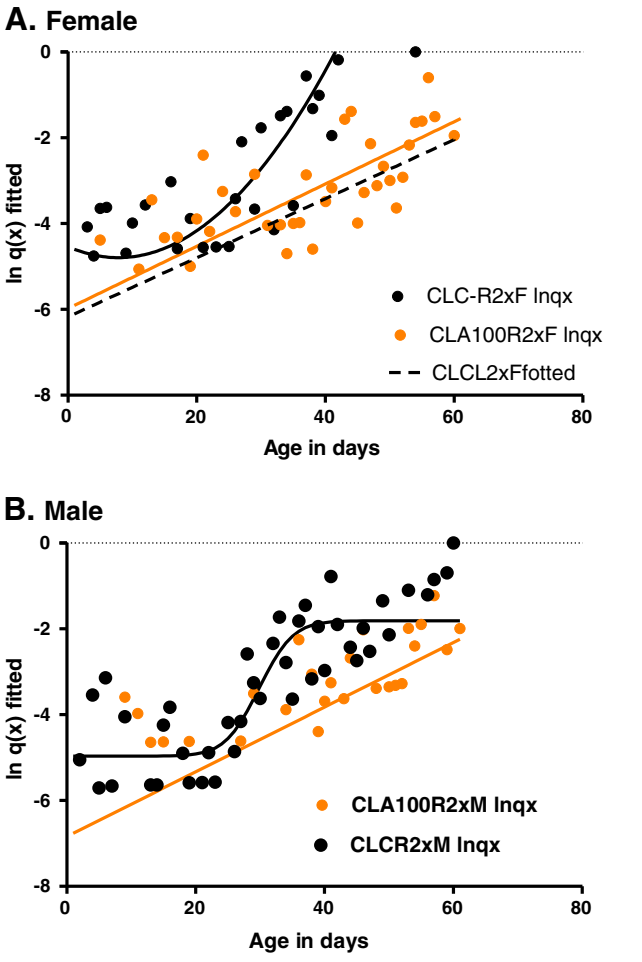

Fig. 3. Curcumin affects both Gompertz parameters and increases mortality rate doubling time in both sexes. The age-specific mortality rate (lnq_x) was calculated from the data of Fig. 2, plotted, and the best fit mortality curve fitted to the data using maximum likelihood analysis (Winmodest), calculated and graphed. The data symbols represent the actual lnq_x values. The solid lines represent the best fitted curves; a Gompertz–Makeham plot for the control (CLC-R2xF) and a Gompertz plot for the experimental (CLA100R2xF) cohort. The dotted line in (A) represent the Gompertz curves of the long-lived La females ($N = 188$) on control food (CLCL2xplotted) (Arking, unpublished data) for comparison. Note that the fitted Gompertz curve of the curcumin-treated Ra females approximates the comparable curve of the long lived La animals. See text for discussion. Control males = CLCR2xM; experimental males = CLA100R2xM).

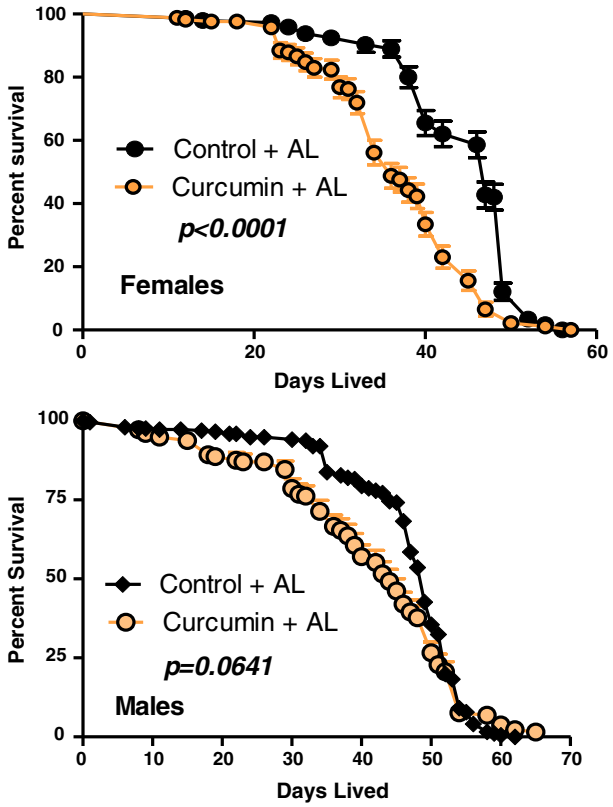

Fig. 4. Curcumin has an inhibitory effect if fed throughout the adult life span in either sex. Ra animals of each sex were fed 100 mM curcumin only during the adult life span. The curcumin-fed females (A, N = 156, median life span = 36 days) respond with a significant 30% decrease in median, but not maximum, life span relative to their controls (N = 230, median = 47 days). Curcumin-fed males (N = 178, median = 44 days) respond with a non-significant 10.2% decrease in median, but not maximum, life span (relative to their controls (N = 290, median = 49 days)) (see p values in Panels A and B). In both cases, the negative effects of lifetime feeding of curcumin manifest themselves in an earlier age of the end of the health span and an earlier age of onset of senescence relative to either controls or to animals fed curcumin only during the health span (Fig. 5A and D). See text for details.

significant in the Ra males but is highly significant in the females (Fig. 4). This may be attributed to either a sex-specific lack of sensitivity to curcumin in adult animals (Lee et al., 2010), or alternatively to the presence of multiple stage-specific sensitive periods in which the drug can exert either a positive or negative effect depending on the target gene expression pattern at that time. Note that there is no effect on maximum longevity in either sex.

3.2.2. Stage-specific feeding shows that curcumin is an early-acting drug

We tested the hypothesis of stage-specific sensitive periods towards curcumin by feeding the adults on curcumin only during one of the three functionally defined periods of adult life.

3.2.2.1. Health span feeding. When Ra males were fed an AL diet with curcumin only during their health span (days 5–27; defined as the time in which survival is >90%), and then transferred to an AL diet with no curcumin for the rest of their lives, they displayed a delayed onset of senescence, which resulted in a significant increase in their median (49%) and maximum longevities (49%) (Fig. 5A).

3.2.2.2. Transitional span feeding. When Ra males were fed an AL diet with curcumin only during their transition span (days 27–40; defined as the time about the inflection point from health (e.g., 90% survival) to senescence (defined as the time when survival <80%)), and fed with an AL diet with no curcumin before and after that period, they displayed a lesser increase in their median and maximum longevities of 25% (Fig. 5B).

3.2.2.3. Senescent span feeding. When Ra males were fed an AL diet with curcumin only during their senescent span (days 38–89; defined as the period of continuous decline in survivorship from <80% until death), and fed on an AL diet with no curcumin prior to day 38, they displayed a 4% decrease in median longevity and an ~11% increase in maximum longevity (Fig. 5C).

3.2.2.4. Female response to stage specific feeding. When Ra females were subjected to stage-specific curcumin feeding as described above, they also expressed stage specific responses in a manner comparable to the males. These data are shown in a composite form in Fig. 5D. Curcumin treatment during the health span resulted in a ~30% increase in median and ~38% increase in maximum longevities. Transition span treatment resulted in only a ~20% increase in median longevity and no change in maximum longevity; while curcumin exposure during the senescent span resulted in a ~20% decrease in median longevity and only a ~3% increase in maximum longevity.

These data make clear that whole life feeding of curcumin to either sex is ineffective at best, while feeding curcumin only during the health span yields a robust delayed onset of senescence in both sexes which yields an adult longevity comparable to that induced by DR treatments. Thus the effective periods of curcumin treatment are the larval stage and the health span portion of the adult stage.

3.3. Effects of curcumin larval feeding on adult trophic behaviors

We noticed that the adults derived from curcumin-fed larvae appeared to be more active than their controls. We investigated this phenomenon with a climbing behavioral assay procedure that allowed us to quantitate their distribution along the vertical axis of the vial under standard conditions. Age is the major factor in the loss of geotactic climbing ability, but its effects are significantly modulated by larval curcumin feeding in both sexes (Fig. 6). Curcumin significantly improves the animals' climbing ability regardless of their adult diet. Both sexes respond strongly for the first four weeks of life, with a progressively weaker response thereafter. Males (Supplementary Fig. 3) seem to respond better to the DR + Curcumin diet regime, while females seem to respond better to the AL + Curcumin regime. The curcumin effect seems to enhance and maintain the early and midlife climbing ability of both sexes, which begins to be lost at five weeks and thereafter.

3.4. Effects of curcumin larval feeding on neuromuscular structure in the leg

Curcumin feeding had a noticeable effect on neuromuscular organization in hind legs of flies from control and curcumin-fed larvae. The general appearance of the leg muscles differs across the time points studied, and between the control and curcumin-fed animals. Representative observations of hind leg femurs are shown in Supplementary Fig. 4, and overall observations are summarized in Table 2.

A blue channel autofluorescence emission was observed in the axons of hind leg nerves similar to that of H33342 in hind legs whether or not the leg was exposed to H33342. A diffuse green channel autofluorescence emission similar to that of green fluorescent protein (GFP) was observed throughout the hind legs of 45 day old flies of both conditions, i.e., control and curcumin-fed. The diffuse green autofluorescence, which was observed in specimens that received no exogenous green channel label, intensified in amplitude through 53 days of age.

Myofibers and their sarcomeres, as well as nuclei are readily distinguishable throughout the hind leg muscles in 38 day controls (Supplementary Fig 4, top left panel). By comparison, the hind legs of curcumin-fed animals exhibit poor delineation of muscle fibers and sarcomeres at each age observed (e.g., 38, 45 and 53 day old

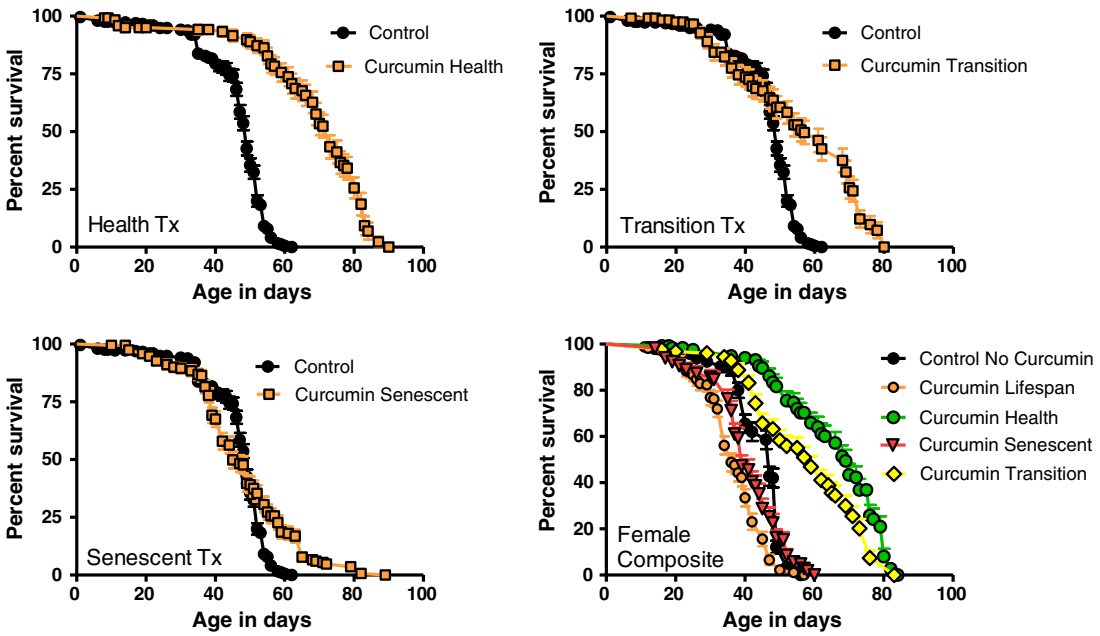

Fig. 5. Adult curcumin intervention has an early-life stage-specific effect in Ra males and females. Panel A (top left) compares effect of feeding curcumin during the Health span (orange, N = 125, median life span = 73 days) (days 5–27) relative to AL only male controls (black, N = 290, median = 49) (log rank test, $X^2 = 211.0$, 1df, $p < 0.001$). Panel B (top right) compares the effect of feeding curcumin (N = 123, median = 57) during the Transition span (days 28–40) to male controls (N = 290, median = 49) (log-rank test, $X^2 = 63.38$, 1df, $p < 0.0001$). Panel C (bottom left) shows the effect of feeding curcumin (N = 181, median 45) during the Senescent span (days 38–89) relative to male controls (N = 290, median = 49) (log-rank test, $X^2 = 12.91$, 1df, $p = 0.0003$). Panel D (bottom right) is a composite graph of female longevities following curcumin feeding during the entire life span (N = 147, median life span = 36 days); Health (N = 126, median = 69, $X^2 = 127.7$, $p < 0.0001$); Transition (N = 126, median = 59, $X^2 = 59.4$, $p < 0.0001$); or Senescent (N = 125, median = 39, $X^2 = 7.567$, $p = 0.0059$); spans relative to controls (N = 230, median = 47). Note that both sexes show the same stage specific response as well as the same decreased longevity after whole life feeding (Fig. 4). See text for discussion.

flies). Extensive microscopic searches revealed no discernible nuclei in the hind legs by or after 45 days in either control or curcumin-fed flies. In contrast, labeled nuclei were not observed in legs of curcumin-fed animals by or after 38 days age. Curcumin treatment apparently accelerates the age of muscular degeneration.

Mitochondrial labeling was observed in legs from control flies through 45 days of age. In contrast, mitochondrial labeling, especially within myofibers, was significantly reduced in legs from flies of 38 days of age and older that were fed curcumin while larvae. Some mitochondrial labeling remained in the curcumin fed flies at all ages observed. Punctate emissive mitochondria were observed along linear myofibrillar tracks with spacing consistent with their approximate 2 μm separation spacing in healthy leg muscle. Some punctate labeling was observed at the periphery of hind legs in the curcumin fed flies at all ages observed. Curcumin has been reported to open the mitochondrial permeability pore and alter the organelle's function (Morin et al., 2001). It is possible that this mitochondrial effect may underlie the observed acceleration in neuromuscular degeneration.

3.5. Effects of curcumin larval feeding on gene expression patterns

We noted that larvae fed on curcumin expressed an extended longevity beginning at about ~25 to 30 days of adult life (Figs. 1, 2). If there is a causal mechanism between the feeding of curcumin to larvae and the extended longevity of the adult, then it should be detectable at some intermediate age. We used qRT-PCR techniques to assay the ability of larval curcumin feeding to affect the expression level of ten candidate longevity genes during the larval and male adult life span The candidate genes were chosen so as to include three members of the TOR signaling pathway (*Tor*, *4e-BP* (*Thor*), and *S6k*); three chaperones known to be associated with extended longevity (*hsp 22*, *27* and *70*), two regulatory genes known to be associated with extended longevity (*Sir2*, *foxo*); and two downstream ROS scavenger genes (*Cat* and *Sod*).

Fig. 7 shows the profiles of gene expression for the *Tor* gene over the six ages assayed. Table 3 contains summary relative expression (e.g., curcumin/control) data for each of the ten genes, including *Tor*. The results are expressed as the mean ± SEM of fold change in curcumin relative to control expression at each age. *4e-BP* and *S6k* are downstream target branches from *Tor*, while *Foxo* is indirectly downstream on another branch from *Tor*. *Sir2* is a major longevity gene indirectly affecting *Tor*. The *hsps* are downstream genes involved in protein folding/protection and only indirectly involved in longevity. *Cat* and *Sod* are also downstream genes involved in reactive oxygen species scavenging and indirectly involved in longevity.

The striking curcumin-dependent increase in the expression of many genes on day 1 was unexpected and not yet understood. It is not likely to be a general phenomenon affecting all genes and related to the stresses of eclosion occurring at the beginning of day 1 since *hsp70* does not show such overexpression. In addition, neither the *Foxo* nor *Sod* genes show a larval repression following curcumin treatment. Thus only seven of the ten genes show a robust larval repression and an adult day 1 overexpression, and all of these are genes which the literature suggests have a major effect on longevity. The *Tor*/*4eBP*/*S6k* branch shows a general pattern of reduced expression, observed at both the larval stage as well as in adults 5 to 15 days of age. *Sir 2* shows a similar pattern of larval and young adult inhibition. This NAD-dependent HDAc inhibitor has multiple effects, one of which may be connecting TOR and/or the insulin-like signaling pathway with oxidative stress levels (Kenyon, 2010; Parella and Longo, 2010). The *hsp22* gene alone shows a robust overexpression on day 15. *Foxo* levels only transiently decrease in young adults before returning to parity with control at day 30. *Cat* and *Sod* show very different larval patterns even though these genes are thought to work together in scavenging ROS. Taken together, these data suggest that this experiment is revealing important clues about the categories of genes which respond to larval curcumin feeding. The involvement of multiple genes is consistent with the wide array of molecular effects described for curcumin (Das et al., 2010). Despite the preliminary nature of this experiment, it nevertheless seems as if the observed general pattern of larval inhibition followed by a long term inhibition in young adults is consistent with the known behavior of the TOR

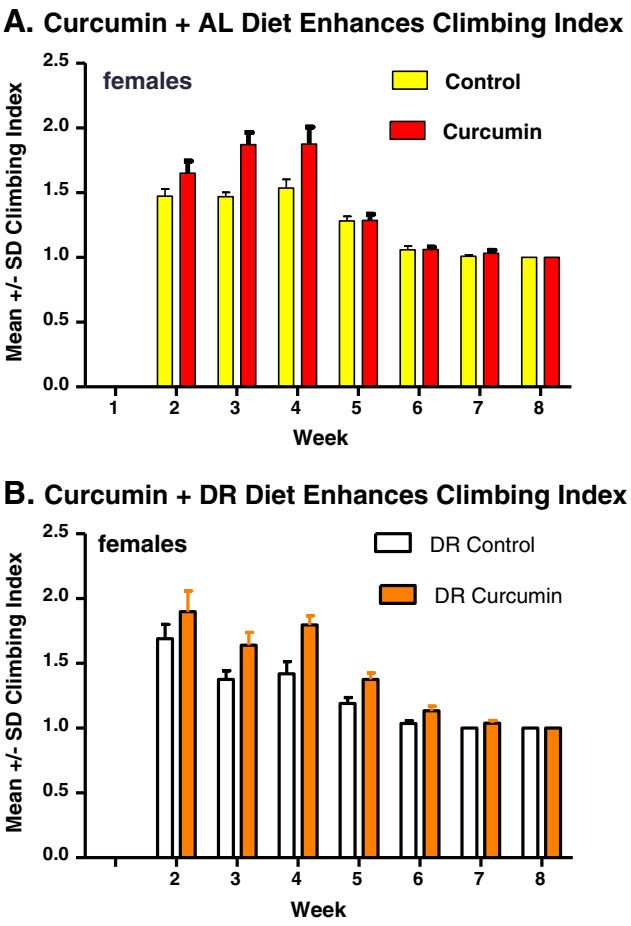

Fig. 6. Negative geotactic climbing behavior is enhanced by curcumin regardless of adult diet. Each column represents the mean ± sd of the climbing index compiled from 6 cohorts of 25 flies each which were tested twice consecutively (i.e., replicated tests of 150 animals/cohort/time point) and used to calculate the mean climbing index value for each cohort. Females raised on AL food with curcumin had a significantly higher climbing index than controls fed AL food only over weeks 2–4 (t-test, $t = 4.574$, $df = 2$, $p = 0.0441$). Females raised on DR food with curcumin had a significantly higher climbing index than controls fed DR food only over weeks 2–8 (t-test, $t = 3.342$, $df = 6$, $p = 0.0156$). There is no significant difference between the AL control and the DR control (t-test = 0.4053, $df = 6$, $p = 0.6993$). Thus in this functional test, curcumin enhanced the climbing behavior on both diets. Similar results were found with males (see Fig. S-3).

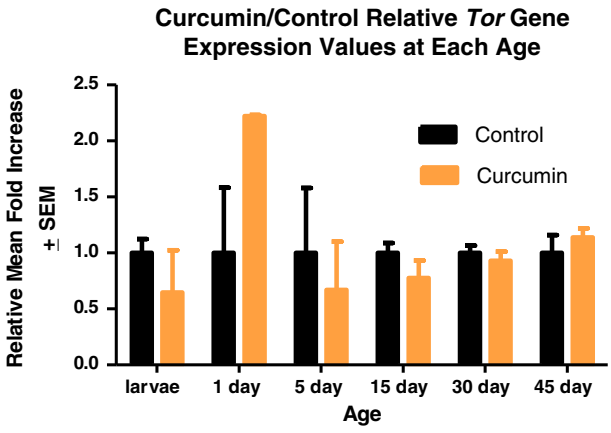

Fig. 7. Curcumin/control relative *Tor* gene expression values at each age. These preliminary qRT-PCR data for *Tor* gene expression were obtained from one experiment consisting of three dependent replicates of ~50 males/time point. Wildtype parents laid eggs on control or curcumin-containing food, and the F1 were flash-frozen in liquid N_2 at the indicated stages (larval = late non-feeding 3rd instar) or male adult ages. qRT-PCR was done using standard techniques. Results are expressed as the mean ± SEM of fold change in curcumin relative to control expression at each age. Table 3 shows the relative expression values of ten different longevity-related genes assayed during the same experiment. See text for discussion.

pathway and strongly supports our hypothesis that curcumin is working at least in part through an inhibition in TOR signaling (Lamming et al., 2012). A detailed mechanistic interpretation as to how these early alterations in gene expression patterns bring about the various traits comprising the extended longevity phenotype will be of interest.

4. Discussion

Our data shows that larval feeding with curcumin induces an extended longevity phenotype in the adult which is diet insensitive and has no detectable additive effect on longevity when combined with an adult DR diet (Figs. 1 and 2). This implies that curcumin may be epistatic to DR, and that both interventions may exert their effects via the same or overlapping signaling pathways. Larval curcumin treatment results in a slowing of the aging rate of the treated animals relative to controls (Fig. 3), and is correlated with systemic effects on TOR and its downstream genes (Table 3). The larval treatment also enhances the adult animal's geotactic activity (Fig. 6), although it does so in interaction with age and diet; indicating that DR and curcumin treatment might affect different traits via different pathways. Whole life feeding of curcumin results in a weakly negative effect on median longevity and no effect on maximum longevity (Fig. 4). Curcumin feeding has its strongest effect if given during the developmental span (Fig. 1) or during the adult health span (Fig. 5). Feeding during the transition span yields a weaker effect, while feeding in the senescent span is actually harmful to the organism's longevity. This latter effect may be related to the apparent acceleration of neuromuscular degeneration noted in the legs of treated animals (Table 2 and Fig. S-4). Taken together, these data suggest that 1), larval or adult feeding of curcumin to normal-lived animals acts, possibly via TOR, to significantly extend their health span and delay the onset of senescence; and 2), there exists a curcumin-sensitive component of development that has long-term delayed effects on the adult animal. It remains to be seen if this

Table 2
Latest age of normal hind leg morphology following curcumin treatment.

Morphology	Control age	Curcumin age	Age difference	Observed curcumin effect on neuromuscular morphology
Nuclei appear normal	38	32	−6	*Accelerate*
Sarcomeres with normal appearance	38	32	−6	*Accelerate*
Sarcomeres disrupted	45	38	−7	*Accelerate*
Mitochondria are discrete and discernable	38	32	−6	*Accelerate*
Macrophages on sarcomeres	45	32	−13	*Accelerate*
Axons with blue autofluorescence	38	32	−6	*Accelerate*
Ganglia with blue autofluorescence	45	45	0	*No effect*
Muscles with green autofluorescence	45	38	−7	*Accelerate*

Table 3

Gene	Stage	Curcumin/control relative gene expression				
	Larval	Day 1	Day 5	Day 15	Day 30	Day 45
Tor	0.65	2.22	0.67	0.78	0.93	1.14
4eBP	0.41	3.45	0.34	1.21	0.64	0.99
S6k	0.75	2.61	0.54	1.11	0.95	1.13
Sir 2	0.44	2.58	0.3	0.85	1.06	1.27
hsp22	0.54	1.6	0.16	2.37	0.94	1.38
hsp27	0.37	1.98	0.18	1.42	0.99	1.04
hsp70	0.05	0.86	0.1	1.03	0.67	1.11
Cat	0.77	2.5	0.53	0.75	0.95	1.49
Foxo	1.03	1.84	0.6	0.776	0.99	1.11
Sod	2.09	1.46	0.75	1.02	0.91	1.02

curcumin-sensitive developmental component of the Ra animal is the same or different from the larval density-sensitive developmental component previously described in the long-lived La strain (Buck et al., 1993). Both of these larval effects on adult longevity depend on stage-specific environmental stimuli. It has recently been suggested that DR may be mediated via epigenetic regulation (Li et al., 2011; Rando and Chang, 2012). Curcumin is known to function as a histone acetyltransferase (HAT) inhibitor, in addition to its other known processes (Das et al., 2010). The data of Fig. S-2, showing that larval curcumin and adult DR treatments are not additive, is consistent with that suggestion. Understanding the mechanistic nature of the Ra strain's sensitivity to curcumin would likely provide new insights into the developmental origins of this longevity extension mechanism(s). There are reports of developmental effects on longevity in *C. elegans* (Huang et al., 2011; Pincus et al., 2011), and there is a high probability that such effects are conserved across species.

A DR feeding regime often imposes trade-offs, particularly with respect to reproduction and body size. We observed no negative effects on reproduction (Fig. S-1 and Table S-1); in fact the curcumin treated females have non-significantly higher fertilities and fecundities than do normal controls. We did measure a significant male-specific effect on adult body weight (Table S-2). Larval curcumin feeding increases development time in the Ra strain but without any observed harmful effects (Table S-1). It also accelerates by about one week the apparent neuromuscular degeneration observed in the hind legs of non-climbing animals (Table 2). None of these effects appear capable of providing an alternative explanation for the observed extension of the health span.

Resistance to acute oxidative stress is enhanced in a stage-specific manner by curcumin treatment, being most effective in the 30 day old animal (Table 1). This is admittedly an extreme test and may not accurately reflect the subtleties of the organism's response to chronic or intermittent oxidative stress. Still, resistance to any form of oxidative stress may reflect an underlying resistance to inflammation, which is the precursor to much age-related loss of function (Salmon et al., 2010). Curcumin is known to inhibit both mTOR and NFkB in human cells (Sun et al., 2011). It may not be coincidental that the midlife stage specificity of the curcumin effect on delaying the age-related loss of negative geotactic climbing ability (Fig. 6) is approximately coincident with the midlife stage specificity of the acute oxidative stress resistance (Table 1). We also observed a similar effect of larval curcumin treatment on phototactic behavior, which will be reported elsewhere.

Curcumin is known to inhibit several important conserved regulatory genes, including *Tor*, in both humans and flies (Lee et al., 2010; Sun et al., 2011). Our data shows that *Tor*, as well as several known regulatory genes with a major role in life span determination is apparently inhibited in larvae and 5 to 15 day old males (Table 3). The *Tor*/*4eBP*/*S6k* branch shows a general pattern of reduced expression, observed at both larval stages as well as adults 5 to 15 days of age. Thus the curcumin effect is strongly associated with, but is not limited to, alterations in the activity of the *tor* pathway. There are at least three potential mechanisms which might bring about such an effect. First, curcumin has been reported to alter mitochondrial function by opening its permeability transition pore and so inhibiting ATP synthesis (Morin et al., 2001). Second, mTOR is reported to act as an ATP sensor independent of its ability to sense amino acid levels (Dennis et al., 2001). An ATP decrease would likely affect NADH levels, which could in turn affect *Sir 2* activity. Taken together, these facts might suggest an indirect effect of curcumin which would have its primary effect on the mitochondrion and secondarily on the TOR pathway. Second, it is known that curcumin can directly bind to mTOR and so is capable of having a direct effect on TOR pathway activity (Beevers et al., 2006). Third, it is possible that curcumin's HAT inhibitory effect may epigenetically induce a constitutive DR effect in the Ra animals similar to what we observed in our selected long-lived La strain animals (Soh et al., 2007). Deciding which of these effects is actually operative in our animals will be a topic for future studies.

In either event, it is noteworthy that this inhibition in larvae and 5–15 day old adults occurs after the feeding and prior to the expression of the extended longevity phenotype (Fig. 2A). The available data suggest that the curcumin effect may not be identical to the DR effect (e.g. *Sir2* inhibition is not seen in DR (Frankel et al., 2011)), and perhaps this might account for the different curcumin-DR effects seen in different traits (e.g., longevity vs. geotactic ability). Analysis and characterization of the gene-based signaling pathways involved in the etiology of both the curcumin- and DR-induced extended longevity phenotypes will provide information on the core genes involved in longevity extension (Antosh et al., 2011; Bauer et al., 2010; Miller, 2012), as well as on the genes differentiating one longevity intervention from another. The existence of observable effects of curcumin on diverse traits such as longevity (Figs. 2–5, S-2), adult neuromuscular-based behaviors (Fig. 6), and reproduction (Fig. S-1), suggests that stage-specific feeding of curcumin exerts systemic genotypic effects on the animal and so affects various physiological systems which together give rise to an extended longevity phenotype. We conclude that larval or adult feeding of curcumin during specific sensitive stages induces an extended health span and is associated with apparently systemic effects on the dTOR pathway, presumably via mTORC1 (Katewa and Kapahi, 2011; Lamming et al., 2012), and other genes known to be involved in longevity extension.

Our stage-specific feeding regime plus the use of higher doses yielded the robust data described above. Other labs have used a variety of life-time feeding protocols and reported small or no increases in median longevity coupled with strain-specific gender differences and no effect on maximum longevity (Lee et al., 2010). We replicated that life-time feeding experiment and also obtained small or no increases in longevity but in both sexes (Fig. 5A, D). We conclude that life-time feeding, and not the use of other techniques, is responsible for their observed failure of curcumin to significantly extend the health span and delay the onset of senescence.

Curcumin is similar to resveratrol and other plant compounds which are rapidly degraded, have a low bioavailability and so require a high dose to be effective (Anand et al., 2007). The direct mix approach we used accomplished this (see Experimental procedures). Now that we have defined the stage specificity of the drug, we can do more precise titration of the minimum concentration needed to induce the several changes at each sensitive change.

Our data supports a paradigm whereby genotropic drugs would be effective only during those life cycle stages during which their target molecules are actually available. A result of ineffectiveness derived from a whole life feeding regime may be a false negative result. Our data also supports the addition of curcumin to the roster of those interventions which are known to robustly delay the onset of senescence and so significantly extend a healthy longevity. Characterizing the curcumin-based mechanisms involved in stage-specific longevity extension should be of great value in aiding our deeper understanding of

inducible longevity pathways and may shed light on the genetic architecture of the health span in comparison to that of the senescent span (Pilling et al., 2012).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2012.09.007.

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Author contributions

Scientific responsibilities were as follows: RA conceived and provided overall direction of the project, and wrote the manuscript. JWS developed the larval assay and oversaw the adult feeding experiments. NM and TJN also took part in the adult feeding experiment, executed the behavioral assays and oversaw all other experiments. TM developed the behavioral assays and assisted in their execution. PS performed the acute oxidative stress experiments. AMR performed the fertility and fecundity experiments. NM provided the body weight data. RK, AU, and ARH performed the RNA isolations and the qRT-PCR experiments. RBS performed the light microscopy analysis of leg neuromuscular changes. Data analysis was done by RA, RBS, and ARH.

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