© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

RESEARCH ARTICLE

Assessing the influence of curcumin in sex-specific oxidative stress, survival and behavior in *Drosophila melanogaster*

Abigail R. Esquivel[1], Jenna C. Douglas[1], Rachel M. Loughran[1], Thomas E. Rezendes[1], Kaela R. Reed[2], Tobias H. L. Cains[1], Sarah A. Emsley[1], William A. Paddock[3], Patrick Videau[1], Marc J. Koyack[2,*] and Brie E. Paddock[1,*]

ABSTRACT

Oxidative stress, which occurs from an imbalance of reactive oxygen and nitrogen species (RONS) and both endogenous and exogenous antioxidants, promotes aging and underlies sex-specific differences in longevity and susceptibility to age-related neurodegeneration. Recent evidence suggests that curcumin, a yellow pigment derived from turmeric and shown to exhibit antioxidant properties as a RONS scavenger, influences the regulation of genetic elements in endogenous antioxidant pathways. To investigate the role of curcumin in sex-specific *in vivo* responses to oxidative stress, *Drosophila* were reared on media supplemented with 0.25, 2.5 or 25 mmol l^{-1} curcuminoids (consisting of curcumin, demethoxycurcumin and bisdemethoxycurcumin) and resistance to oxidative stress and neural parameters were assessed. High levels of curcuminoids exhibited two sex-specific effects: protection from hydrogen peroxide as an oxidative stressor and alterations in turning rate in an open field. Taken together, these results suggest that the influence of curcuminoids as antioxidants probably relies on changes in gene expression and that sexual dimorphism exists in the *in vivo* response to curcuminoids.

KEY WORDS: Curcumin, *Drosophila*, Neural development, Oxidative stress

INTRODUCTION

Oxidative stress, which is the accumulation of reactive oxygen and nitrogen species (RONS), occurs as a result of a mismatch of endogenous and exogenous antioxidant pathway activities and oxidative load. Over-abundance of intracellular RONS creates a toxic environment, ultimately leading to denaturation and degradation of proteins and DNA (Liu et al., 2012), lipid peroxidation, tissue damage, and, consequently, cellular apoptosis and cytotoxicity (Nguyen et al., 2018). The oxidative stress hypothesis suggests that accumulation of RONS underlies functional losses associated with aging, such as sarcopenia and neuronal loss (reviewed in Sohal et al., 2002). The high lipid content of the brain makes this organ particularly vulnerable to lipid peroxidation, one of the primary damaging pathways of RONS.

Sexual dimorphism of oxidative stress and endogenous antioxidants may underlie sex differences in aging and age-related pathologies (Camus et al., 2012; Ruszkiewicz et al., 2019). Human females exhibit a more robust antioxidant response, consistent with greater longevity and decreased risk of age-related diseases (Bellanti et al., 2013; Signorelli et al., 2006; Viña and Borrás, 2010). In *Drosophila melanogaster* and mice, preservation of mitochondrial functionality and longevity as an adaptive response to oxidative stress is female specific (Pomatto et al., 2017; Valle et al., 2007). *Drosophila* provide a well-established model for investigating the sex-specific impacts of antioxidant treatment on longevity, oxidative stress and neural function. Sex differences in multiple physiological processes, including metabolic and neural functions, are well documented in *Drosophila* (Chen et al., 2017; Lnenicka et al., 2006; Pomatto et al., 2017).

Curcumin, a yellow pigment derived from turmeric, demonstrates promise as a neuroprotectant due to its ability to alleviate oxidative stress through RONS scavenging and interaction with central nervous system cells and processes in multiple model systems (Han et al., 2012; Jaroonwitchawan et al., 2017). Recent evidence suggests extensive involvement in regulation of endogenous antioxidant pathways by manipulation of genetic elements (Han et al., 2012; Huang et al., 2018). Pre-treatment with curcumin decreases many biomarkers of oxidative damage after exposure of *Drosophila* and mice to oxidative stressors (Chen et al., 2017; Seong et al., 2015; Strong et al., 2013). Administering sub-toxic doses of curcumin in neuronal and somatic human cell lines alleviates the effects of paraquat-induced oxidative stress by activation of autophagy-related pathways (Daverey and Agrawal, 2016; Han et al., 2012).

Here, we assessed the sex-specific role of curcuminoids (curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin) in oxidative stress, development and behavior in wild-type *Drosophila*. We confirmed that treatment with dietary curcuminoids reduced peroxidated lipids, extended longevity and demonstrated sex-specific differences in the impact of curcuminoids on motility and oxidative stress in *Drosophila*.

MATERIALS AND METHODS

Drosophila husbandry and curcuminoid treatment

All *Drosophila melanogaster* were maintained at 25°C in a 12 h:12 h light:dark cycle at 60% humidity. All experiments were conducted on the Oregon R strain, which was reared on Nutri-Fly German formulation (GF) medium, prepared according to the manufacturer's directions (Genessee Scientific, San Diego, CA, USA). Curcuminoid treatments were accomplished by inclusion of the appropriate amount of curcuminoids, derived from *Curcuma longa* (C1386, Sigma-Aldrich, St Louis, MO, USA; 98% pure standard consisting of curcumin, demethoxycurcumin and bisdemethoxycurcumin and referred to as curcuminoids hereafter) to generate media with 0.25, 2.5 and 25 mmol l^{-1} curcuminoids. No standard concentration of curcumin or curcuminoids has been developed in *Drosophila,* so this broad range of curcuminoid concentrations was chosen to encompass

[1]Department of Biology, Southern Oregon University, Ashland, OR 97520, USA. [2]Department of Chemistry, Southern Oregon University, Ashland, OR 97520, USA. [3]Department of Institutional Research, Arcadia University, Glenside, PA 19038 USA.

*Authors for correspondence (paddockb@sou.edu; koyackm@sou.edu)

W.A.P., 0000-0003-2649-9180; P.V., 0000-0003-2686-845X; M.J.K., 0000-0002-3454-4933; B.E.P., 0000-0002-2558-1222

Received 21 February 2020; Accepted 30 September 2020

Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

the association properties of many potential binding partners in diverse tissues.

Gut motility and larval preparation

The rate of gut clearance of third instar larvae was determined as previously described with minor modifications (Shell et al., 2018). Briefly, Oregon R embryos were reared to eclosion on standard Nutri-Fly GF medium and on medium supplemented with Blue 1 dye. Wandering third instar larvae from each medium were collected while minimizing the transfer of media components and soaked in phosphate-buffered saline (PBS) for 30 min intervals up to 1.5 h. After each interval, five groups of ten larvae were removed from the PBS, blotted dry on Kimwipes to remove liquid and any debris, and each group of ten larvae was saved in a 1.5 ml Eppendorf tube. Aliquots of 100 µl PBS were added to each tube and the larvae were homogenized with a Kontes motor and pellet pestle (Kimble, Rockwood, TN, USA). The homogenate was centrifuged at maximum speed for 10 min (∼14,000 ***g***), the supernatant was filtered through a 0.22 µm filter, and the absorbance at 625 nm was measured on a BioSpectrometer kinetic photometer (Eppendorf, Hamburg, Germany). Each set of five homogenates was measured in triplicate.

Liquid chromatography–mass spectrometry methods

Liquid chromatography grade water, acetonitrile, methanol and high-purity formic acid were purchased from Sigma-Aldrich and used without further purification. The ratio of curcumin, demethoxycurcumin and bisdemethoxycurcumin in the purchased standard was found to be 67:29:4, respectively, based on high-resolution liquid chromatography–mass spectrometry (HR LC-MS) peak areas determined as described below. For compound analysis, a 5 mg ml^{-1} curcuminoid standard stock was prepared in methanol, passed through a 0.22 µm filter, and stored at room temperature in an amber vial for no more than 2 weeks before use.

Larval preparation and extraction

Groups of 10 third instar larvae were collected, rinsed and visually inspected to ensure that no food or curcuminoid remained on the body surface. Larvae were soaked in PBS for 1 h and prepared as described above for gut motility assessment, transferred to pre-weighed 1.5 ml Eppendorf tubes, larval masses determined, and tubes frozen at −20°C for fewer than 4 days before extraction. For the tissue specificity studies, individuals were soaked in 1× PBS solution for 1 h and dissected and divided into brain, gut and compositional tissue as previously described (Brent et al., 2009). Gut tissue samples were also collected from larvae in which the 1 h PBS soak was omitted to further assess gut clearance. All tissue components were individually collected with forceps and dissecting scissors, which were rinsed in ethanol and then water between each use to avoid potential chemical carryover, and larvae were rinsed with PBS between the collection of each component. All samples were collected in pre-weighed 1.5 ml Eppendorf tubes with 40 of each larval component individually per tube (N=5 tubes each). Tissue masses were determined, and tubes were frozen at −80°C for less than a week before extraction.

Aliquots of 200 µl methanol were added to each tube and the larvae were ground for roughly 1 min using a motorized Kontes pellet pestle rod system. To limit carryover, fresh pestles were used for each tube of larvae. Ground larvae in methanol were then sonicated on ice using a Qsonica Q125 sonicator (Melville, NY, USA) with a 0.25-inch probe for three pulses of 10 s at 20% power with about 10 s between each pulse. Tubes were then centrifuged at maximum speed (∼14,000 ***g***) for 2 min, the methanol was transferred into new 1.5 ml Eppendorf tubes, and 200 µl of methanol was added to resuspend the larval pellets. The process of sonication, centrifugation and decanting methanol was repeated for a total of three rounds, and the total methanol extract from each tube of larvae was combined. The final combined methanol extracts were centrifuged at maximum speed for 4 min, the extract was transferred into a syringe while care was taken not to disturb the pellet, and the extract was filtered through a 0.22 µm filter directly into LC-MS vials for analysis. For brain and compositional tissue samples, the clarified extracts were dried *in vacuo*, water was removed via lyophilization, and the samples were resuspended in either 90 or 200 µl of methanol, respectively, before injection. Extraction efficiency was assessed by spiking a 2 µg aliquot of curcuminoid standard into each tube containing 10 larvae (N=10 tubes). The tubes were extracted as above and direct HR LC-MS determination and quantitation were conducted as described below. Extraction efficiency was determined to be 90.5±7.5%, and all data were scaled relative to this measurement.

High-resolution liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry analysis of curcuminoids

High-resolution (HR) liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-TOF-MS) analysis was performed using an Agilent 1260 high performance liquid chromatography (HPLC) system equipped with a quaternary pump, an autosampler and a 1260-diode array detector upstream of a 6230 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using a Dual ESI source in positive ionization mode. A 10 µl aliquot from each sample was injected and separation was achieved using an Acclaim RSLC 120 C18 column (3.0×110 mm, 2.2 µm, 120 Å; Thermo Scientific, Waltham, MA, USA) at 30°C with a flow rate of 0.75 ml min^{-1} where line A was water+0.1% (v/v) formic acid, and line B was acetonitrile+0.1% (v/v) formic acid with the following program. The column was pre-equilibrated in 60% A–40% B and this composition was held for 5 min post-injection (Chao et al., 2018). The composition of mobile phase was then changed to 42% A–58% B over 15 min, and then brought to 0% A–100% B, with both transitions utilizing a linear gradient. This composition was held for 5 min followed by changing back to 60% A–40% B over 4 min utilizing a linear gradient. The column was equilibrated in 60% A–40% B for 5 min prior to the next injection. Under these chromatographic conditions, bisdemethoxycurcumin, demethoxycurcumin and curcumin eluted at roughly 7.9, 8.8 and 9.7 min, respectively. A standard curve was created via dilution of the 5 mg ml^{-1} stock of curcuminoid standard such that 10–400 ng of the compounds was applied to the column and analysed in triplicate via the HR LC-MS program described above. The peak areas for the curcuminoids were summed to account for the presence of three compounds in the masses of the standard applied to column. The mass spectrometer had the following settings: mass range, 100–3000 m/z in profile mode utilizing the extended dynamic range (2 GHz); gas temperature, 325°C; drying gas, 8 liters min^{-1}; nebulizer, 25 psig; capillary voltage, 3500 V; fragmentor, 150 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; acquisition rate, 1 spectrum s^{-1}; time, 1000 ms per spectrum; transients per spectrum, 9982. Data were processed with MassHunter Workstation software, version B.06.00, build 6.0.633.0 (Agilent Technologies).

HPLC analysis of curcuminoids

HPLC analysis was performed using an Agilent 1100 HPLC system equipped with a binary pump, degasser, an autosampler and a diode array detector. A 50 µl aliquot from each sample was injected and separation was achieved using a Zorbax Eclipse XDB-C8 guard column (4.6×150 mm, 5 µm, 80 Å; Hewlett Packard, Palo Alto, CA, USA) upstream of an Ultra C18 column (4.6×250 mm, 5 µm, 10 Å; Restek, Bellefonte, PA, USA) at 30°C with a flow rate of 1.0 ml min^{-1} where line A was water+0.1% (v/v) formic acid, and line B was acetonitrile+0.1% (v/v) formic acid with the following program. The column was pre-equilibrated in 70% A–30% B and this composition was held for 3 min post-injection. The composition of mobile phase was then changed to 100% B over 15 min utilizing a linear gradient. This composition was held for 3 min followed by changing back to 70% A–30% B over 2 min utilizing a linear gradient. The column was equilibrated in 70% A–30% B for 3 min prior to the next injection. Under these chromatographic conditions, bisdemethoxycurcumin, demethoxycurcumin and curcumin eluted at roughly 13.7, 14.0 and 14.4 min, respectively, by measuring absorbance at 425 nm. A standard curve was created via dilution of the 5 mg ml^{-1} stock of curcuminoid standard such that 1–1000 ng of the compounds was applied to the column and analysed in triplicate via the HPLC program described above. The peak areas for the curcuminoids were summed to account for the presence of the three compounds in the masses of the standard applied to the column.

Thiobarbituric acid reactive substances assay

Oregon R embryos were reared to eclosion on four different concentrations of curcuminoids (0, 0.25, 2.5 and 25 mmol l^{-1}). Wandering third instar larvae were rinsed in PBS, visually inspected to ensure that no media or curcuminoid remained on the surface, and sexed. For the acute oxidative stress challenge, larvae were soaked for 1 h in either 3% H_2O_2 as an oxidative stressor, or PBS. Groups of 10 larvae of each sex were saved in pre-weighed 1.5 ml Eppendorf tubes, and larval weight was recorded. Tubes of larvae were stored for less than a week at −80°C before testing, and all manipulation was conducted on ice unless otherwise specified. The thiobarbituric acid reactive substances (TBARS) protocol was conducted essentially as previously described with minor modifications (Ohkawa et al., 1979). Briefly, 150 µl of 20% trichloroacetic acid (TCA) was added to each tube, larvae were ground for roughly 1 min using a motorized Kontes pellet pestle rod system, and the homogenate was incubated on ice for 10 min. The homogenate was centrifuged for 5 min at maximum speed (∼14,000 ***g***) and the clarified supernatant was transferred to a new 1.5 ml Eppendorf tube taking care not to disturb the pellet. A 150 µl aliquot of 0.5% thiobarbituric acid (TBA) in 20% TCA was added to each tube, vortexed briefly to mix, and incubated at 90°C for 15 min. After incubation, the tubes were centrifuged for 5 min at maximum speed, the absorbances at 532 nm (malondialdehyde; MDA) and 600 nm (non-specific turbidity) were measured in triplicate on an Eppendorf BioSpectrometer kinetic photometer, and the MDA–TBA product concentration was determined by comparison with a standard curve made with tetramethoxypropane as previously described (Ohkawa et al., 1979).

Survival and negative geotaxis assays

Oregon R embryos were reared to eclosion on four different concentrations of curcuminoids (0, 0.25, 2.5 and 25 mmol l^{-1}). Within 24 h of eclosion, adult flies were split by sex and grouped into vials containing medium with 0, 0.25, 2.5 or 25 mmol l^{-1} of curcuminoids, with no more than 10 individuals per vial to prevent overcrowding. Flies were transitioned to fresh medium every 4–8 days as needed to prevent F2 eclosion and maintain medium integrity. For the survival analysis, deaths were scored every 48 h and any escapees were excluded from the analysis. Negative geotaxis was performed as previously described (Le Bourg and Lints, 1992). Briefly, flies were tapped to the bottom of a vial and any that passed a 5 cm mark within 18 s were recorded as a passing event. Three replicates were collected for each vial for each time point.

Acute oxidative stress challenge

Oregon R embryos were reared to eclosion on four different concentrations of curcuminoids (0, 0.25, 2.5 and 25 mmol l^{-1}). Within 24 h of eclosion, 5–25 adult flies per vial were split by sex and grouped into vials containing media with 0, 0.25, 2.5 or 25 mmol l^{-1} of curcuminoids. At 3 days after emergence (DAE), flies were starved on water-soaked filter paper for 3–6 h, and then transferred to a 3% H_2O_2 and 5% sucrose solution. Death events were visually scored every 12 h until completion of the assay.

Larval locomotion

Oregon R larvae were reared from hatching on four different concentrations of curcuminoids (0, 0.25, 2.5 and 25 mmol l^{-1}). Wandering third instar larvae were sexed and groups of 5–7 larvae were placed on a 9×13-inch agar dish and allowed 30 s of acclimation. After the acclimation period, the agar dish was centred on a light box and motility was recorded for 3–4 min. The camera was placed approximately 16 inches above the agar plate, and an image of a ruler was used to scale the video in the analysis software. The videos taken were reduced to 180 s, cropped to frame and converted to MP4 file format using iMovie (https://www.apple.com/uk/imovie/). The MP4 files were subsequently imported into Adobe Photoshop and exported as an image sequence at one image per second. The image sequence was then uploaded to ImageJ (https://imagej.net/Welcome) and converted into an image stack (image>stack>images to stack). The stack was inverted in color, converted to an 8-bit type, and the threshold was set at maximum intensity to ensure the larvae were the only objects highlighted in color. The wrMTrck plug-in (http://www.phage.dk/plugins/wrmtrck.html) was utilized to measure distance, length, average speed, maximum speed and body length per second. Parameters and settings for wrMTrck were used in accordance with a previous study focused on optimization of wrMTrck for measuring *Drosophila* larval motility (Brooks et al., 2016). The number of contractions, contraction rate and distance per contraction were measured manually.

Analyses

Kaplan–Meier survival analyses were used to assess both the longevity and acute hydrogen peroxide adult data, and significance was determined with Mantel–Cox and Wilcoxon tests. For the negative geotaxis assay, pass rates from 2 to 16 DAE cohorts were analysed by three-way ANOVA to reveal the effects of age, curcumin concentration and sex on nervous system function. All analyses were completed in R (https://www.r-project.org/). Graphical preparation of data was conducted using GraphPad Prism software (GraphPad, San Diego, CA, USA).

RESULTS

Curcuminoids are present in *Drosophila* larvae

A primary focus of this work was to determine the influence of curcumin on various developmental and phenotypic parameters of fruit flies as a means of establishing a baseline upon which future research may build. To achieve such outcomes, media were

Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

supplemented with varying concentrations of a curcuminoid standard and adult flies were allowed to breed and lay eggs in these media. The eggs and larvae would spend the duration of development in the presence of the curcuminoids and thus the influence of these compounds could be compared with flies reared in media lacking curcuminoids. A 98% pure curcuminoid standard derived from the *C. longa* plant was utilized for supplementation, which contained curcumin, demethoxycurcumin and bisdemethoxycurcumin in a 67:29:4 ratio. The underlying assumption of this work is that the larvae ingest or are permeable to curcuminoids in the medium such that some level of these compounds is present in tissues to modulate developmental and functional events.

To determine whether *Drosophila* larvae accumulate curcuminoids in their tissues, third instar larvae reared on media supplemented with 25 mmol l^{-1} of curcuminoids were harvested in sets of 10 larvae per tube (N=8 tubes), extracted with methanol, and the amount of extracted curcuminoids was quantified using HR LC-MS. Larvae were incubated in PBS for 1 h prior to methanol extraction to both rinse their exteriors and allow the contents of their gut to pass. Supplementation of growth medium with Blue 1 dye, soaking for up to 1.5 h in 30 min increments, homogenization, and spectrophotometric analysis of soluble extracts indicated that the blue dye consumed in the medium passed out of the larval guts within an hour, consistent with previous findings on gut motility (Fig. S1; Shell et al., 2018).

Analysis of the pure curcuminoid standard with HR LC-MS found three peaks with retention times of roughly 7.9, 8.8 and 9.7 min, and masses corresponding to bisdemethoxycurcumin (calcd [M+H]+= 309.1121, 2.6 ppm error), demethoxycurcumin (calcd [M+H]+= 339.1227, 0.6 ppm error) and curcumin (calcd [M+H]+=369.1333, 0.6 ppm error), respectively (Fig. 1; Fig. S2). Extracts from third instar larvae reared on medium without curcuminoids lacked peaks with any of the curcuminoid masses at these retention times (Fig. 1). Spiking the curcuminoid standard into these control extracts revealed two new peaks, which correspond to curcumin and demethoxycurcumin in both masses and retention times. Three peaks were not observed because bisdemethoxycurcumin is a minor constituent of the standard mix and is only observed during analysis of high standard concentrations. Extracts from third instar larvae reared on media supplemented with 25 mmol l^{-1} curcuminoids displayed two peaks, which are identical to curcumin and demethoxycurcumin in both masses and retention times. Spiking the curcuminoid standard into these larval extracts did not create new peaks for curcumin and demethoxycurcumin, no new masses were observed for these compounds, and the integrated area of the existing curcuminoid peaks increased. Integration of curcuminoid peak areas and comparison with a standard curve determined that larvae harbored 103±42 ng curcuminoids mg^{-1} of larval tissue. Taken together, these data indicate that fruit fly larvae accumulate curcuminoids in their tissues, and that the HR LC-MS methodology used here is sufficient to unambiguously detect and quantify these compounds from larval extracts.

Although HR LC-MS is a sufficient method to detect curcuminoids in larval extracts, it is not cost effective or readily available to many research groups. Informed by the HR LC-MS results, an HPLC method was utilized that was also able to separate the three curcuminoids with retention times of roughly 13.7, 14.0 and 14.4 min corresponding to bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively (Fig. S3). Like the HR LC-MS results, two peaks corresponding to curcumin and demethoxycurcumin were observed in extracts from third instar

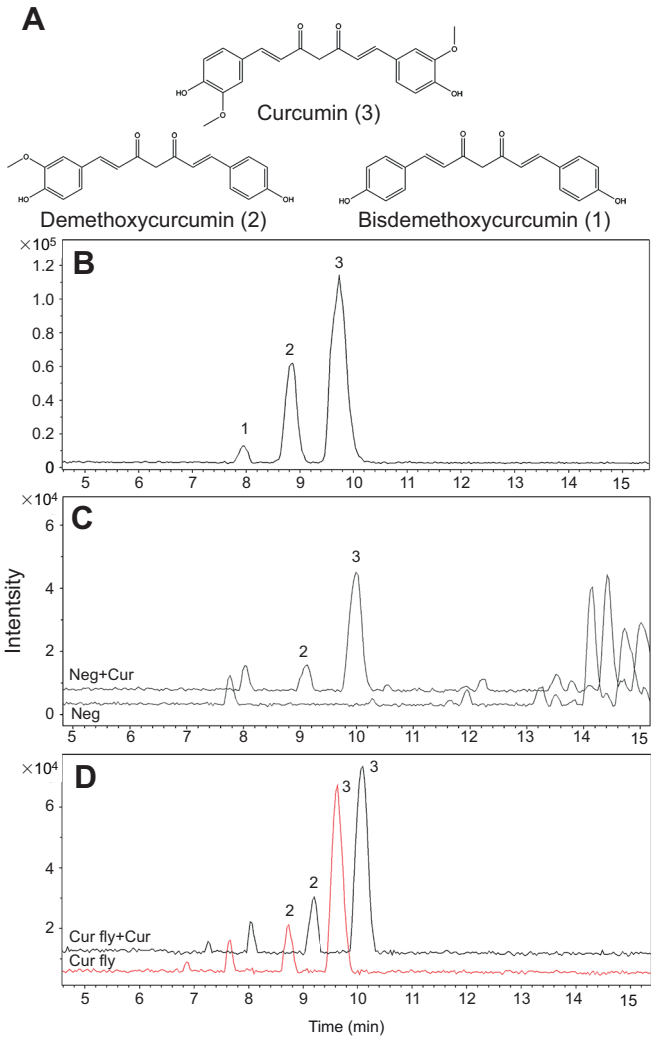

Fig. 1. Curcuminoids are present in the tissues of *Drosophila melanogaster* larvae reared on media supplemented with curcuminoids. The curcuminoid standard contains three compounds (A) and displays three peaks when run on HR LC-MS corresponding to these compounds (B). Extracts from larvae were grown on medium lacking curcuminoids (C; Neg) and with curcuminoids spiked into this extract (C; Neg+Cur). Extracts from larvae were grown on medium supplemented with 25 mmol l^{-1} curcuminoids (D; Cur Fly) and with additional curcuminoids spiked into this extract (D; Cur Fly+Cur). Representative chromatograms are presented, and the data sets are offset from one another by 0.3 min in panels C and D.

larvae reared on media supplemented with 25 mmol l^{-1} of curcuminoids, and no peaks were present in larvae reared on media without curcuminoids. Similarly, spiking the curcuminoid standard into negative controls resulted in new peaks with retention times like those of the standard and did not produce new peaks in larval extracts displaying curcuminoid peaks. Integration of curcuminoid peak areas and comparison with a standard curve determined that third instar larvae reared on media supplemented with 25 mmol l^{-1} curcuminoids harbored 139±70 ng curcuminoids mg^{-1} of larval tissue or 257±130 ng curcuminoids per larva (N=10 tubes), which is not significantly different from the value obtained from HR LC-MS analysis (t-test, P=0.21). Assessment of the curcuminoids harbored in the tissue of larvae raised on 2.5 or 0.25 mmol l^{-1} of curcuminoids found 44±5 and 2±1 ng curcuminoids mg^{-1} of larval tissue (N=10 tubes each), respectively. These data collectively demonstrate that HR LC-MS and HPLC can identify and quantify curcuminoids in larval

Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

extracts and that all of the larvae used in the following experiments contain curcuminoids in their tissues.

The results presented above indicate that the supplemented dietary curcuminoids are present in the larvae, but their tissue-specific accumulation remained a question. Due to the lipophilic nature of curcuminoids, it is possible that uptake is poor, they are not incorporated into cells, or never leave the gut, which is why studies in medicinal chemistry are working to derivatize curcumin for improved uptake kinetics. To determine the levels of curcuminoids present in the brain, gut and compositional tissues, 40 larvae reared on media supplemented with 25 mmol l^{-1} curcuminoids were dissected, their tissues saved individually, and extraction and HPLC analysis conducted as described above. Only one curcuminoid supplementation condition was used for analysis because quantification from smaller amounts of sample, rather than whole larvae, would only provide results within the detectable range if derived from the highest accumulating larvae. Integration of curcuminoid peak areas and comparison with a standard curve determined that third instar larvae reared on media supplemented with 25 mmol l^{-1} curcuminoids harbored 44±29 ng curcuminoids per larva in their compositional tissue, 85±53 ng curcuminoids per larva in their gut, and curcuminoids were not observed above the detectable limit from brain samples (N=5 tubes each). The combination of the composition tissue and gut curcuminoids is 129±81 ng curcuminoids per larva, which is not significantly different from the value obtained from the whole-larvae extractions (t-test, P=0.12). As a verification of the blue dye gut clearance assay described above, the guts of larvae not incubated in PBS for 1 h prior to extraction yielded 225±133 ng curcuminoids per larva. A visual inspection of the guts of larvae directly off media displayed far more contents than the largely emptied guts of larvae incubated in PBS for 1 h. These data indicate that curcuminoids remain in the gut after the majority of contents clear, accumulate in the compositional tissues, but were not observed in brain samples.

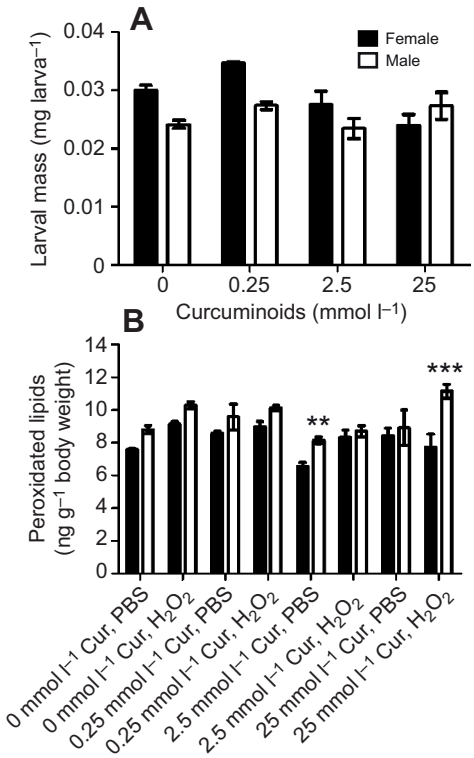

Fig. 2. Curcuminoids protect *Drosophila* larvae against acute oxidative stress in a sex-dependent manner. Oregon R third instar larvae were reared from hatching on indicated concentrations of curcumin (Cur), then soaked in either hydrogen peroxide (H_2O_2) as an oxidative challenge, or 1×PBS as a control. (A) Masses of 6–9 sets of 10 larvae were determined, the average mass per larva is presented, and the error bars represent the s.e.m. (B) Lipid peroxidation was quantified with a TBARS assay and the mean peroxidated lipids per gram of wet body tissue are reported. The average of three replicates of 10 larvae are presented, and errors bars represent the s.e.m.; **P<0.01, ***P<0.001.

Curcuminoids protect against acute and chronic oxidative stress challenges

Previous research has indicated that treatment with curcuminoids modulates organismal responses to oxidative stress via radical scavenging abilities (Daverey and Agrawal, 2016; Jaroonwitchawan et al., 2017). To validate the presumed protective role of dietary curcuminoids, larval and adult fruit flies were assessed for their responses to both acute and chronic oxidative stress. Oregon R *Drosophila* were reared on a range of curcuminoid concentrations, third instar larvae were sexed, and then exposed to a 1 h acute oxidative challenge by soaking in 3% hydrogen peroxide. Larvae were homogenized, their peroxidated lipids were quantified using a TBARS assay, and the results were analysed using a three-way ANOVA to determine the interactions of sex, curcuminoid pre-treatment and hydrogen peroxide challenge on the amount of peroxidated lipids. Curcuminoid pre-treatment had an interaction with the hydrogen peroxide challenge, confirming the antioxidant properties of curcuminoids to an acute oxidative stressor in third instar larvae (F=1.27, P=0.029; Fig. 2). Sex has a significant impact on the amount of peroxidated lipids (F=37.98, P=1.38×10^{-8}; Fig. 2), which is consistent with previous literature (Ruszkiewicz et al., 2019). Based on these data, the longevity of *Drosophila* adults reared on curcuminoids from hatching to adulthood was determined as a proxy for assessing lifetime oxidative stress levels.

To test whether curcuminoid pre-treatment resulted in an improvement in resistance of adult *Drosophila* to an acute oxidative challenge, we assessed the survival of adults reared on various concentrations of curcuminoid when challenged with 3% H_2O_2. Rearing of females on all curcuminoid concentrations tested resulted in a higher survival rate than females reared without curcuminoids (Fig. 3A). Interestingly, males reared on 0.25 mmol l^{-1} curcuminoids did not exhibit a higher survival rate than males reared on 0 mmol l^{-1} curcuminoids (Mantel–Cox, P>0.05; Fig. 3B), although higher curcuminoid concentrations resulted in higher survival rates than 0 mmol l^{-1} curcuminoid-reared males (Mantel–Cox, P<0.0001, for both 2.5 and 25 mmol l^{-1} curcuminoids; Fig. 3). Consistent with previous studies, we found that females had a higher median survival at every curcuminoid pre-treatment concentration (Fig. 3C; Pomatto et al., 2017). Taken together, these results indicate that dietary curcuminoids have a protective effect against an acute oxidative stress challenge, and extend the life of flies exposed to the chronic effects of oxidative stress.

Adult *Drosophila* reared from hatching on all concentrations of curcuminoids exhibit a longer lifespan and median survival compared with those reared on untreated media (Kaplan–Meier test, P<0.0001; Fig. 4). Interestingly, we observed a sexual dimorphism in the median survival of flies reared at the lower curcuminoid concentrations tested. Males flies reared on 0.25 mmol l^{-1} curcuminoids exhibited greater survival compared with males reared on 0 mmol l^{-1} curcuminoids (Mantel–Cox test, P<0.0001), but a lower median survival than females from the same 0.25 mmol l^{-1} curcuminoid treatment

Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

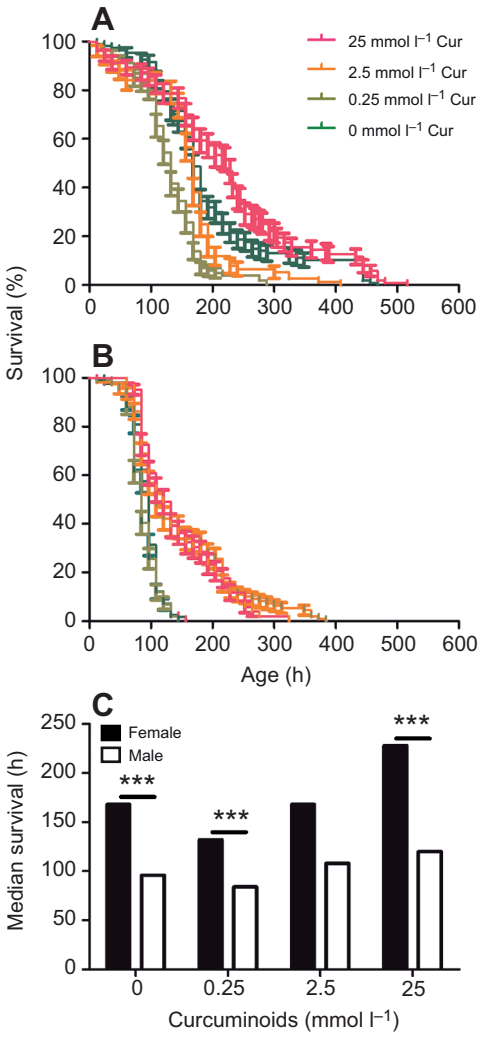

Fig. 3. Curcuminoids protect adult *Drosophila* against acute oxidative stress. (A) Female and (B) male Oregon R *Drosophila* [*N*=102–109 per sex for each curcuminoid (Cur) concentration] were reared on the indicated concentrations of curcuminoids. (C) At 3 days after emergence, larvae were challenged with 3% hydrogen peroxide in 5% sucrose solution, and deaths were scored to determine median survival in hours, as determined by a Kaplan–Meier survival analysis. Error bars represent the s.e.m.; ***P<0.001.

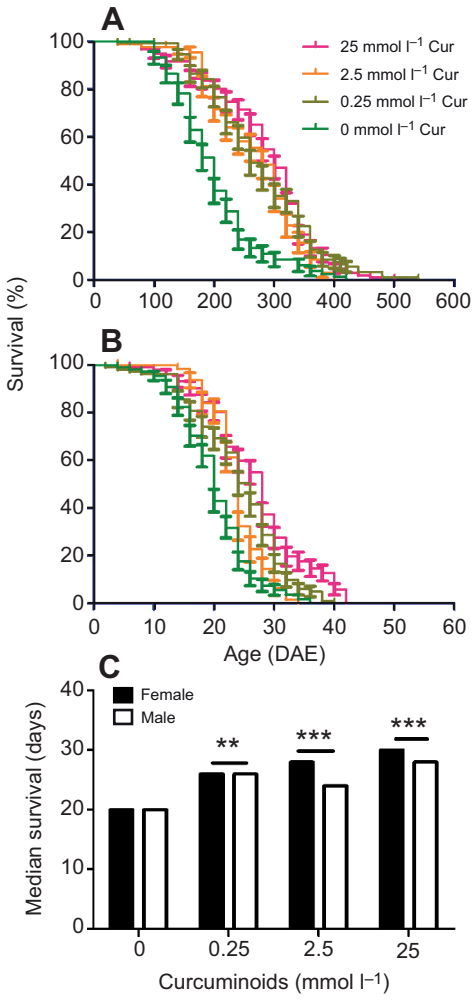

Fig. 4. Curcuminoids extend the lifespan of wild-type *Drosophila*. (A) Male and (B) female flies [*N*=85–115 adults per sex for each curcuminoid (Cur) concentration] were reared on media with the indicated concentrations of curcuminoids, and deaths were scored to determine median survival in days (C), as determined by a Kaplan–Meier survival analysis. Error bars represent the s.e.m.; **P<0.01, ***P<0.001.

(Mantel–Cox test, P=0.0010; Fig. 4). Similarly, males reared on 2.5 mmol l^{-1} curcuminoids exhibited a lower survival than females also reared on 2.5 mmol l^{-1} curcuminoids (Mantel–Cox test, P=0.0001), but a greater survival than males reared on 0 mmol l^{-1} curcuminoids (Mantel–Cox test, P<0.0001; Fig. 4). Females reared on the highest curcuminoid concentration examined (25 mmol l^{-1}) did not exhibit a greater median survival than males reared on the same concentration (Mantel–Cox test, P>0.05), although both survived longer than flies of the matched sex without curcuminoid treatment (Fig. 4).

Curcuminoids influence fruit fly motility in a sex-dependent manner

Brain tissue has the second highest abundance of lipids in the body after adipose tissue, and oxidation of brain lipids is associated with neural dysfunction and death (Butterfield et al., 2010). Analysis of larval locomotion provides a rapid, accessible assessment of several aspects of *in vivo* function of pharmacological treatments, including impacts on nervous system function (Kohsaka et al., 2017). Third instar crawling larvae exploring an open field have a bipartite locomotion pattern consisting of runs and reorientation phases, and genetic or pharmacological manipulations can independently alter each component of locomotion (Berni, 2015; Günther et al., 2016). To determine if curcuminoid treatment changed either component of larval locomotion, we analysed the exploratory behavior of freely crawling third instar larvae in an open field. Third instar larvae reared on the indicated curcuminoid concentrations were recorded exploring an agarose open field. Parameters of their locomotion were analysed with wrMTrk, and two-way ANOVAs were used to compare the effects of sex and curcuminoid concentration on velocity, distance moved per contraction, and reorientation of larvae in the field.

Curcuminoid treatment increases the turn rate in freely exploring larvae (ANOVA, F=8.7, P=8.9×10^{-5}). Although several curcuminoid treatment concentrations resulted in increased male turn rates compared with females from the same treatment concentration, a robust interaction between sex and curcuminoids on the turn rate of freely exploring larvae was not detected (F=1.16, P=0.334; Fig. 5). Treatment with curcuminoids did not alter distance traveled or velocity of freely moving larvae on an open field

Journal of Experimental Biology (2020) 223, jeb223867. doi:10.1242/jeb.223867

(Fig. 5). No sexual dimorphism was evident in either distance traveled or velocity in any treatment group (Fig. 5).

To examine whether curcuminoids have a sex-dependent effect on neural function in *Drosophila*, the reflexive locomotor response of negative geotaxis in curcuminoid-treated flies was tested as they aged. This assay has frequently been used to assess neural function in aging and neurodegenerative models (Le Bourg and Lints, 1992; Prasad and Muralidhara, 2014; Rhodenizer et al., 2008; Yang et al., 2012). We found age to be the strongest predictor of performance in the negative geotaxis assay (ANOVA, F=89.59, P=3.12×10^{-46}), consistent with previous studies (Le Bourg and Lints, 1992; Rhodenizer et al., 2008). Curcuminoids interacted with the sex component (F=9.36, P=1.22×10^{-5}), indicating a sexual dimorphism in the effect of curcuminoids on negative geotaxis in the aging flies (Fig. 6). It is important to note that sex alone did not affect the locomotor function (F=0.4192, P=0.519), nor was there an interaction between sex and age with regard to neural function (F=1.547, P=.0.157). The three-way interaction between all variables

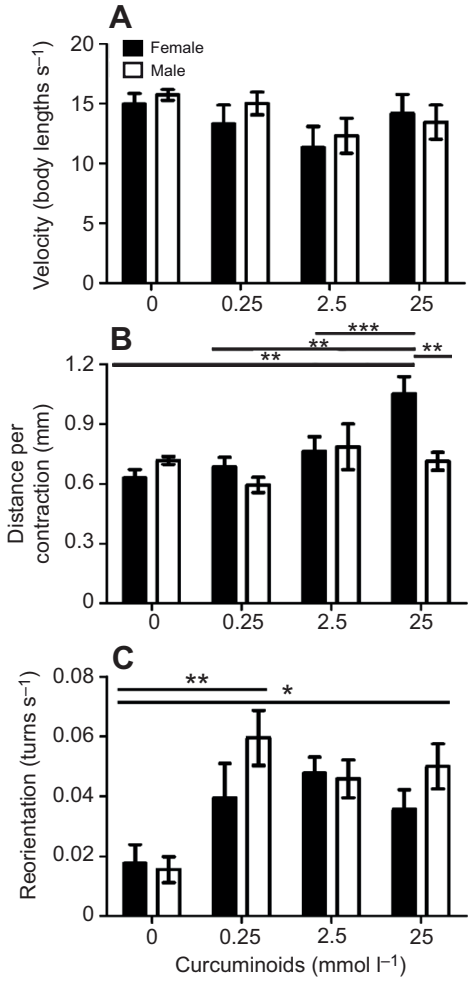

Fig. 5. Curcuminoids increase turning rate, but not distance and velocity, of wild-type *Drosophila* larvae crawling in an open field. Third instar wandering larvae reared on the indicated curcuminoid concentrations were recorded exploring an empty field. Parameters of larval crawling paths from 6–8 larvae per sex per treatment were analysed with the wrMTrck plug-in of ImageJ. The velocity (A; body lengths per second), total distance traveled (B) and rate of turns (C) were determined in ImageJ. *P<0.05, **P<0.01, ***P<0.001.

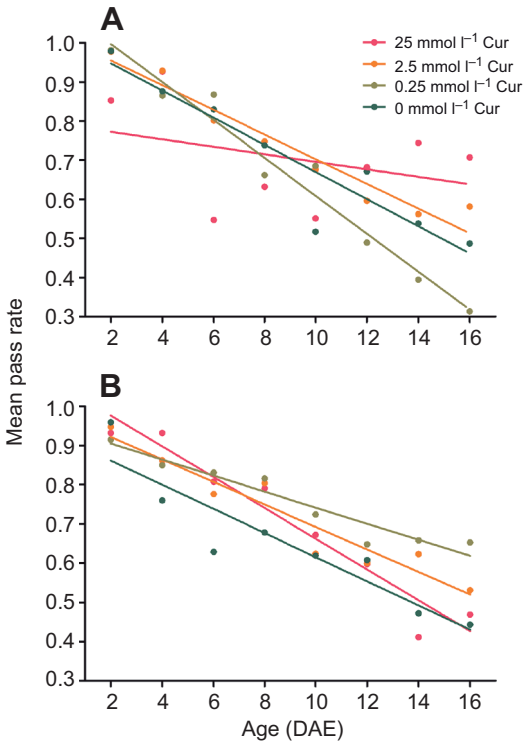

Fig. 6. Curcuminoids protect against age-related loss in climbing function in females and males, as measured by negative geotaxis. (A) Females; (B) males. The interaction plot displays mean pass rate of the negative geotaxis assay as a function of age (days after emergence, DAE) of *Drosophila* reared on 0 mmol l^{-1} (N=89–97), 0.25 mmol l^{-1} (N=74–90), 2.5 mmol l^{-1} (N=60–76) and 25 mmol l^{-1} (N=50–59) curcuminoids (Cur), with linear regressions displayed.

(curcuminoid concentration, sex and age) was significant (F=5.42, P=5.07×10^{-10}), indicating that the interaction between curcuminoids and sex altered the rate of loss of locomotor function. Treatment of females with 0.25 mmol l^{-1} curcuminoids accelerated the loss of neural function compared with 0 mmol l^{-1} in females (Fig. 6A; green versus brown line). In contrast, the 0.25 mmol l^{-1} curcuminoid concentration slowed the rate of neural function loss in males compared with males without curcuminoids (Fig. 6B; green versus brown line).

DISCUSSION

The purpose of this work was to determine the role of curcuminoids in phenotypic and developmental processes in an *in vivo* model system. Absorption of all curcuminoid components was confirmed with HR LC-MS (Fig. 1) prior to the assessment of phenotypic parameters. Curcuminoid treatment resulted in protection from an acute hydrogen peroxide challenge (Figs 2 and 3). Fewer peroxidated lipids were detected in two groups: females compared with males and curcuminoid-treated versus non-supplemented third instar larvae after an acute oxidative challenge (Fig. 2). Curcuminoid treatment also resulted in increased longevity in both male and female *Drosophila*, although some concentrations resulted in less protection for males than females (Fig. 4A–C). Freely crawling third instar larvae treated with curcuminoids demonstrated no changes in either velocity or distance moved per contraction, parameters frequently controlled by muscle or motor neuron function (Fig. 5A,B). In contrast, an increase in the rate of turning was observed in curcuminoid-treated larvae (Fig. 5C), a process controlled by the

subesophageal ganglia (SEG) of the brain (Kendroud et al., 2018; Tastekin et al., 2015). Taken together, these data suggest that chronic treatment with curcuminoids has sex-dependent impacts on oxidative stress and brain function.

Our data show curcuminoid presence in *Drosophila* tissues (Fig. 1), consistent with curcuminoid absorption via the gut, and additionally our data also support the hypothesis that curcuminoids have an impact on neural function. Curcuminoid treatment resulted in changes to negative geotaxis (Fig. 6) and larval reorientation rate (Fig. 5), behaviors centrally controlled by mushroom body dopaminergic afferent neurons and the SEG, respectively (Sun et al., 2018; Tastekin et al., 2015). Previous studies have demonstrated that treatment with dietary curcuminoids can ameliorate metal and chemical-induced neurotoxicity in rat brains, as measured by HPLC and TBARS (Candan and Tuzmen, 2008), and influence central nervous system (CNS) cell composition and molecular expression (Sandur et al., 2007). Furthermore, curcuminoids are detectable following oral administration in mice and rat brain tissues, despite limited bioavailability (Schiborr et al., 2010; Suresh and Srinivasan, 2007; Tsai et al., 2011). Although curcuminoids were not observed in brain tissue in this study, this might be a function of the much smaller brain size and need for a much larger sample, which is the subject of further investigations by our group. Taken together, we speculate that curcuminoid treatment may influence neural function by modulating changes to the aforementioned locomotive associated CNS structures. However, whether these changes are by way of direct or indirect alterations and, additionally, the potential involvement of the gut–brain axis, requires further investigation.

Curcuminoid treatment was not associated with changes in larval speed, larval distance per contraction, or adult negative geotaxis at 2 DAE (Fig. 5A–C). These metrics arise largely from the function of motor neurons and muscles (Camuglia et al., 2018; Rhodenizer et al., 2008), which indicates that either curcuminoids have no effect on these components or that the effect was below assay detection level. The complex behavior of freely crawling larvae allowed the analysis of other locomotory components, including turning frequency. Curcuminoid treatment resulted in an increased turning frequency (Fig. 5C), which is a behavior that frequently follows reorientation processing within the CNS (Günther et al., 2016), and data suggest that these reorientation phases are controlled by the SEG (Tastekin et al., 2015). The SEG is a brain region responsible for incorporation of sensory cues (Kendroud et al., 2018), and recent evidence has demonstrated sexual dimorphism in the *Drosophila* SEG (Ye et al., 2012). In light of the findings described above, the data presented here suggest that curcuminoid treatment could modulate or exacerbate sex-dependent differences in the SEG, leading to the observed phenotypes.

Acknowledgements
We are grateful to Nancy Shough (Southern Oregon University, SOU), David K. Marshall (SOU) for technical support, and Lauren Millman (SOU) for administrative support.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.R.E., J.C.D., P.V., M.J.K., B.E.P.; Methodology: A.R.E., J.C.D., R.M.L., T.E.R., T.H.L.C., S.A.E., P.V., M.J.K., B.E.P.; Formal analysis: W.A.P., B.E.P.; Investigation: A.R.E., J.C.D., R.M.L., T.E.R., K.R.R., T.H.L.C., S.A.E., P.V., M.J.K., B.E.P.; Resources: M.J.K., B.E.P.; Data curation: W.A.P.; Writing - original draft: A.R.E., P.V., M.J.K., B.E.P.; Writing - review & editing: J.C.D., P.V., B.E.P.; Visualization: W.A.P., P.V., B.E.P.; Supervision: P.V., M.J.K., B.E.P.; Project administration: B.E.P.; Funding acquisition: B.E.P.

Funding
This work was supported by start-up funds from Southern Oregon University (SOU) to B.E.P., M.J.K. and P.V. and a Carpenter I grant from SOU to B.E.P. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.223867.supplemental

References
Bellanti, F., Matteo, M., Rollo, T., De Rosario, F., Greco, P., Vendemiale, G. and Serviddio, G. (2013). Sex hormones modulate circulating antioxidant enzymes: impact of estrogen therapy. *Redox Biol.* **1**, 340-346. doi:10.1016/j.redox.2013.05.003

Berni, J. (2015). Genetic dissection of a regionally differentiated network for exploratory behavior in *Drosophila* larvae. *Curr. Biol.* **25**, 1319-1326. doi:10.1016/j.cub.2015.03.023

Brent, J. R., Werner, K. M. and McCabe, B. D. (2009). *Drosophila* larval NMJ dissection. *J. Vis. Exp.* **24**, 1107. doi:10.3791/1107

Brooks, D. S., Vishal, K., Kawakami, J., Bouyain, S. and Geisbrecht, E. R. (2016). Optimization of wrMTrck to monitor *Drosophila* larval locomotor activity. *J. Insect Physiol.* **93-94**, 11-17. doi:10.1016/j.jinsphys.2016.07.007

Butterfield, D. A., Bader Lange, M. L. and Sultana, R. (2010). Involvements of the lipid peroxidation product, HNE, in the pathogenesis and progression of Alzheimer's disease. *Biochim. Biophys. Acta* **1801**, 924-929. doi:10.1016/j.bbalip.2010.02.005

Camuglia, J. M., Mandigo, T. R., Moschella, R., Mark, J., Hudson, C. H., Sheen, D. and Folker, E. S. (2018). An RNAi based screen in *Drosophila* larvae identifies fascin as a regulator of myoblast fusion and myotendinous junction structure. *Skelet. Muscle* **8**, 12. doi:10.1186/s13395-018-0159-9

Camus, M. F., Clancy, D. J. and Dowling, D. K. (2012). Mitochondria, maternal inheritance, and male aging. *Curr. Biol.* **22**, 1717-1721. doi:10.1016/j.cub.2012.07.018

Candan, N. and Tuzmen, N. (2008). Very rapid quantification of malondialdehyde (MDA) in rat brain exposed to lead, aluminium and phenolic antioxidants by high-performance liquid chromatography-fluorescence detection. *Neurotoxicology* **29**, 708-713. doi:10.1016/j.neuro.2008.04.012

Chao, I.-C., Wang, C.-M., Li, S.-P., Lin, L.-G., Ye, W.-C. and Zhang, Q.-W. (2018). Simultaneous quantification of three curcuminoids and three volatile components of *Curcuma longa* using pressurized liquid extraction and high-performance liquid chromatography. *Molecules* **23**, 1568. doi:10.3390/molecules23071568

Chen, D., Sitaraman, D., Chen, N., Jin, X., Han, C., Chen, J., Sun, M., Baker, B. S., Nitabach, M. N. and Pan, Y. (2017). Genetic and neuronal mechanisms governing the sex-specific interaction between sleep and sexual behaviors in *Drosophila*. *Nat. Commun.* **8**, 154. doi:10.1038/s41467-017-00087-5

Daverey, A. and Agrawal, S. K. (2016). Curcumin alleviates oxidative stress and mitochondrial dysfunction in astrocytes. *Neuroscience* **333**, 92-103. doi:10.1016/j.neuroscience.2016.07.012

Günther, M. N., Nettesheim, G. and Shubeita, G. T. (2016). Quantifying and predicting *Drosophila* larvae crawling phenotypes. *Sci. Rep.* **6**, 27972. doi:10.1038/srep27972

Han, J., Pan, X.-Y., Xu, Y., Xiao, Y., An, Y., Tie, L., Pan, Y. and Li, X.-J. (2012). Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. *Autophagy* **8**, 812-825. doi:10.4161/auto.19471

Huang, L., Chen, C., Zhang, X., Li, X., Chen, Z., Yang, C., Liang, X., Zhu, G. and Xu, Z. (2018). Neuroprotective effect of curcumin against cerebral ischemia-reperfusion via mediating autophagy and inflammation. *J. Mol. Neurosci.* **64**, 129-139. doi:10.1007/s12031-017-1006-x

Jaroonwitchawan, T., Chaicharoenaudomrung, N., Namkaew, J. and Noisa, P. (2017). Curcumin attenuates paraquat-induced cell death in human neuroblastoma cells through modulating oxidative stress and autophagy. *Neurosci. Lett.* **636**, 40-47. doi:10.1016/j.neulet.2016.10.050

Kendroud, S., Bohra, A. A., Kuert, P. A., Nguyen, B., Guillermin, O., Sprecher, S. G., Reichert, H., VijayRaghavan, K. and Hartenstein, V. (2018). Structure and development of the subesophageal zone of the *Drosophila* brain. II. Sensory compartments. *J. Comp. Neurol.* **526**, 33-58. doi:10.1002/cne.24316

Kohsaka, H., Guertin, P. A. and Nose, A. (2017). Neural circuits underlying fly larval locomotion. *Curr. Pharm. Des.* **23**, 1722-1733. doi:10.2174/1381612822666161208120835

Le Bourg, E. and Lints, F. A. (1992). Hypergravity and aging in *Drosophila melanogaster*. 4. Climbing activity. *Gerontology* **38**, 59-64. doi:10.1159/000213307

Liu, Z., Celotto, A. M., Romero, G., Wipf, P. and Palladino, M. J. (2012). Genetically encoded redox sensor identifies the role of ROS in degenerative and mitochondrial disease pathogenesis. *Neurobiol. Dis.* **45**, 362-368. doi:10.1016/j.nbd.2011.08.022

Lnenicka, G. A., Theriault, K. and Monroe, R. (2006). Sexual differentiation of identified motor terminals in *Drosophila* larvae. *J. Neurobiol.* **66**, 488-498. doi:10.1002/neu.20234

Nguyen, T. T., Vuu, M. D., Huynh, M. A., Yamaguchi, M., Tran, L. T. and Dang, T. P. T. (2018). Curcumin effectively rescued Parkinson's disease-like phenotypes in a novel *Drosophila melanogaster* model with dUCH knockdown. *Oxid. Med. Cell Longev.* **2018**, 2038267. doi:10.1155/2018/2038267

Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351-358. doi:10.1016/0003-2697(79)90738-3

Pomatto, L. C. D., Carney, C., Shen, B., Wong, S., Halaszynski, K., Salomon, M. P., Davies, K. J. A. and Tower, J. (2017). The mitochondrial Lon protease Is required for age-specific and sex-specific adaptation to oxidative stress. *Curr. Biol.* **27**, 1-15. doi:10.1016/j.cub.2016.10.044

Prasad, S. N. and Muralidhara. (2014). Neuroprotective effect of geraniol and curcumin in an acrylamide model of neurotoxicity in *Drosophila melanogaster*: relevance to neuropathy. *J. Insect Physiol.* **60**, 7-16. doi:10.1016/j.jinsphys.2013.10.003

Rhodenizer, D., Martin, I., Bhandari, P., Pletcher, S. D. and Grotewiel, M. (2008). Genetic and environmental factors impact age-related impairment of negative geotaxis in *Drosophila* by altering age-dependent climbing speed. *Exp. Gerontol.* **43**, 739-748. doi:10.1016/j.exger.2008.04.011

Ruszkiewicz, J. A., Miranda-Vizuete, A., Tinkov, A. A., Skalnaya, M. G., Skalny, A. V., Tsatsakis, A. and Aschner, M. (2019). Sex-specific differences in redox homeostasis in brain norm and disease. *J. Mol. Neurosci.* **67**, 312-342. doi:10.1007/s12031-018-1241-9

Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Murakami, A., Sethi, G., Limtrakul, P., Badmaev, V. and Aggarwal, B. B. (2007). Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis* **28**, 1765-1773. doi:10.1093/carcin/bgm123

Schiborr, C., Eckert, G. P., Rimbach, G. and Frank, J. (2010). A validated method for the quantification of curcumin in plasma and brain tissue by fast narrow-bore high-performance liquid chromatography with fluorescence detection. *Anal. Bioanal. Chem.* **397**, 1917-1925. doi:10.1007/s00216-010-3719-3

Seong, K. M., Yu, M., Lee, K.-S., Park, S., Jin, Y. W. and Min, K.-J. (2015). Curcumin mitigates accelerated aging after irradiation in *Drosophila* by reducing oxidative stress. *Biomed. Res. Int.* **2015**, 425380. doi:10.1155/2015/425380

Shell, B. C., Schmitt, R. E., Lee, K. M., Johnson, J. C., Chung, B. Y., Pletcher, S. D. and Grotewiel, M. (2018). Measurement of solid food intake in *Drosophila* via consumption-excretion of a dye tracer. *Sci. Rep.* **8**, 11536. doi:10.1038/s41598-018-29813-9

Signorelli, S. S., Neri, S., Sciacchitano, S., Pino, L. D., Costa, M. P., Marchese, G., Celotta, G., Cassibba, N., Pennisi, G. and Caschetto, S. (2006). Behaviour of some indicators of oxidative stress in postmenopausal and fertile women. *Maturitas* **53**, 77-82. doi:10.1016/j.maturitas.2005.03.001

Sohal, R. S., Mockett, R. J. and Orr, W. C. (2002). Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free Radic. Biol. Med.* **33**, 575-586. doi:10.1016/S0891-5849(02)00886-9

Strong, R., Miller, R. A., Astle, C. M., Baur, J. A., de Cabo, R., Fernandez, E., Guo, W., Javors, M., Kirkland, J. L., Nelson, J. F. et al. (2013). Evaluation of resveratrol, green tea extract, curcumin, oxaloacetic acid, and medium-chain triglyceride oil on life span of genetically heterogeneous mice. *J. Gerontol. A Biol. Sci. Med. Sci.* **68**, 6-16. doi:10.1093/gerona/gls070

Sun, J., Xu, A. Q., Giraud, J., Poppinga, H., Riemensperger, T., Fiala, A. and Birman, S. (2018). Neural control of startle-induced locomotion by the mushroom bodies and associated neurons in *Drosophila*. *Front. Syst. Neurosci.* **12**, e07046. doi:10.3389/fnsys.2018.00006

Suresh, D. and Srinivasan, K. (2007). Studies on the in vitro absorption of spice principles – curcumin, capsaicin and piperine in rat intestines. *Food Chem. Toxicol.* **45**, 1437-1442. doi:10.1016/j.fct.2007.02.002

Tastekin, I., Riedl, J., Schilling-Kurz, V., Gomez-Marin, A., Truman, J. W. and Louis, M. (2015). Role of the subesophageal zone in sensorimotor control of orientation in *Drosophila* larva. *Curr. Biol.* **25**, 1448-1460. doi:10.1016/j.cub.2015.04.016

Tsai, Y.-M., Chien, C.-F., Lin, L.-C. and Tsai, T.-H. (2011). Curcumin and its nano-formulation: the kinetics of tissue distribution and blood–brain barrier penetration. *Int. J. Pharm.* **416**, 331-338. doi:10.1016/j.ijpharm.2011.06.030

Valle, A., Guevara, R., García-Palmer, F. J., Roca, P. and Oliver, J. (2007). Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions. *Am. J. Physiol. Cell. Physiol.* **293**, C1302-C1308. doi:10.1152/ajpcell.00203.2007

Viña, J. and Borrás, C. (2010). Women live longer than men: understanding molecular mechanisms offers opportunities to intervene by using estrogenic compounds. *Antioxid. Redox Signal.* **13**, 269-278. doi:10.1089/ars.2009.2952

Yang, D., Li, T., Liu, Z., Arbez, N., Yan, J., Moran, T. H., Ross, C. A. and Smith, W. W. (2012). LRRK2 kinase activity mediates toxic interactions between genetic mutation and oxidative stress in a *Drosophila* model: suppression by curcumin. *Neurobiol. Dis.* **47**, 385-392. doi:10.1016/j.nbd.2012.05.020

Ye, X., Deng, Y. and Lai, Z.-C. (2012). Akt is negatively regulated by Hippo signaling for growth inhibition in *Drosophila*. *Dev. Biol.* **369**, 115-123. doi:10.1016/j.ydbio.2012.06.014

Supplementary Information

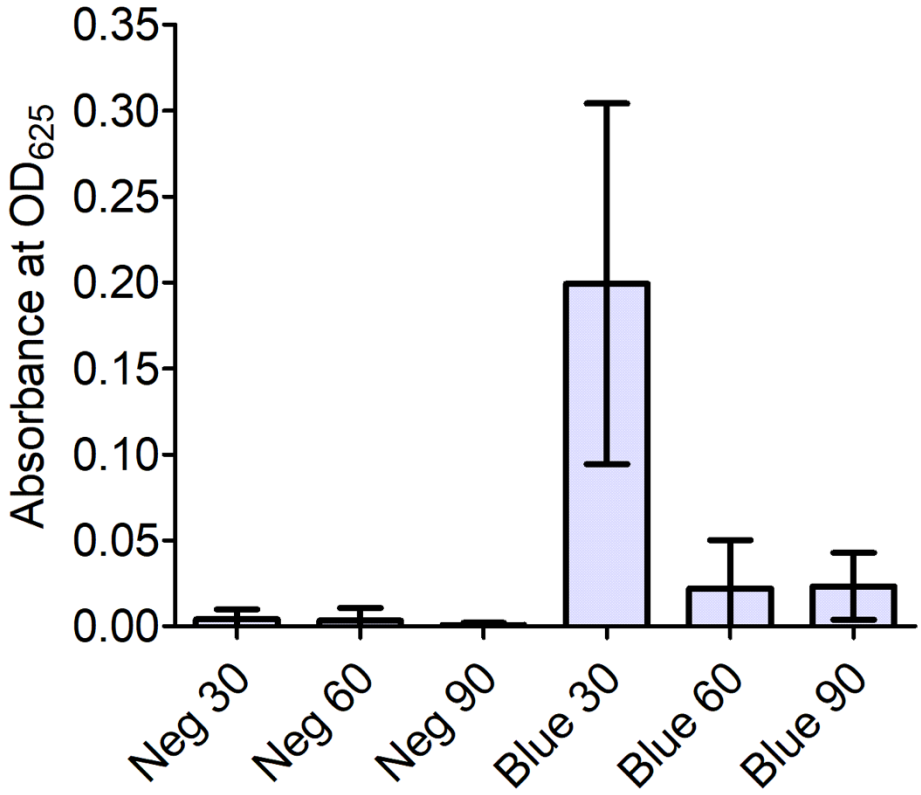

Figure S1. Clearance of blue food dye from larvae. Oregon R larvae were reared on media containing blue dye. Third instar larvae were removed from media and soaked in PBS for the indicated time (min), homogenized, and the OD_{625} was determined to measure clearance of media from gut. The average from five replicates of 10 larvae per tube is presented and the error bars represent the standard deviation.

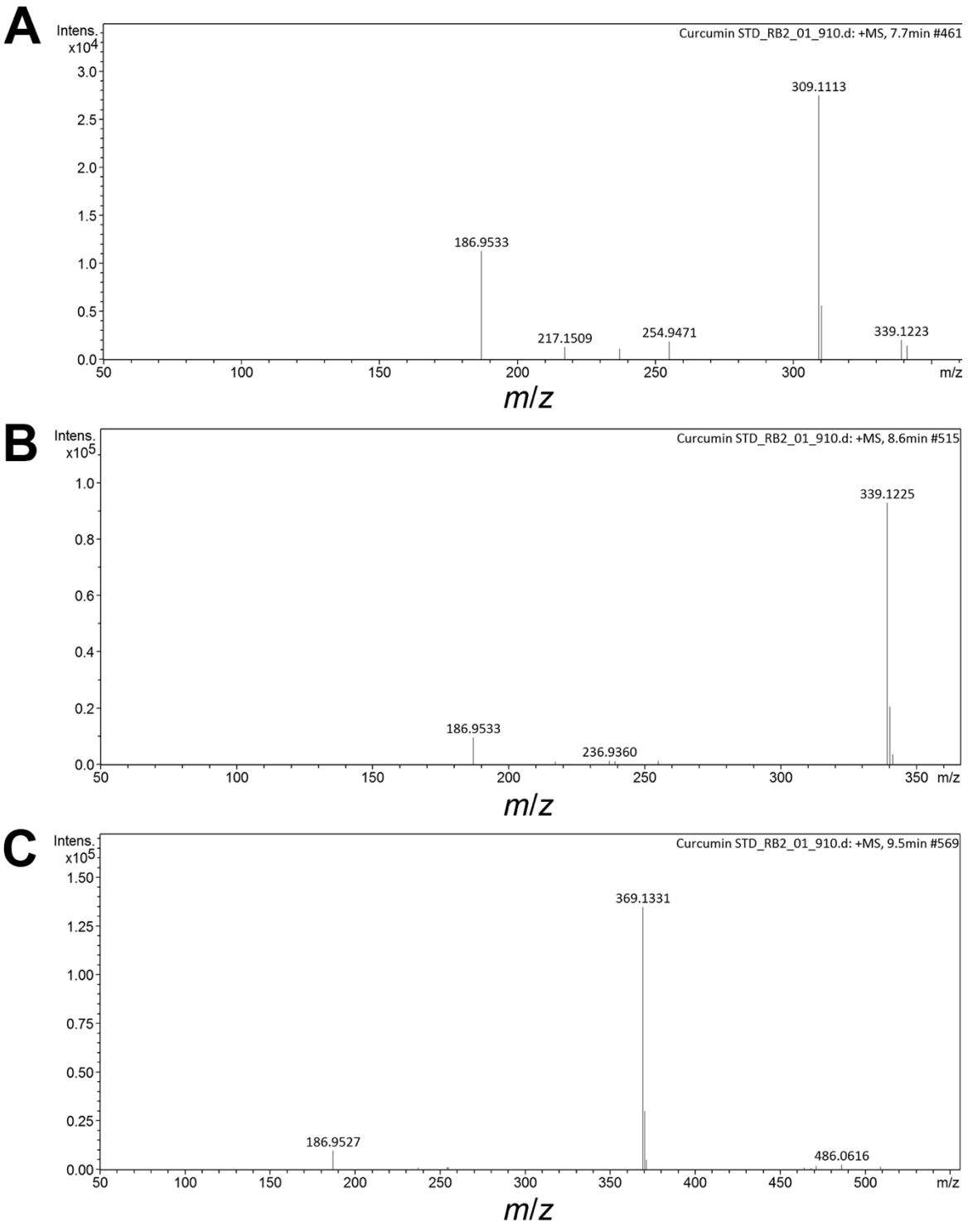

Figure S2. Accurate masses of the three curcuminoids tested, bisdemethoxycurcumin (A), demethoxycurcumin (B), and curcumin (C), as measured by HIRES-LCMS.

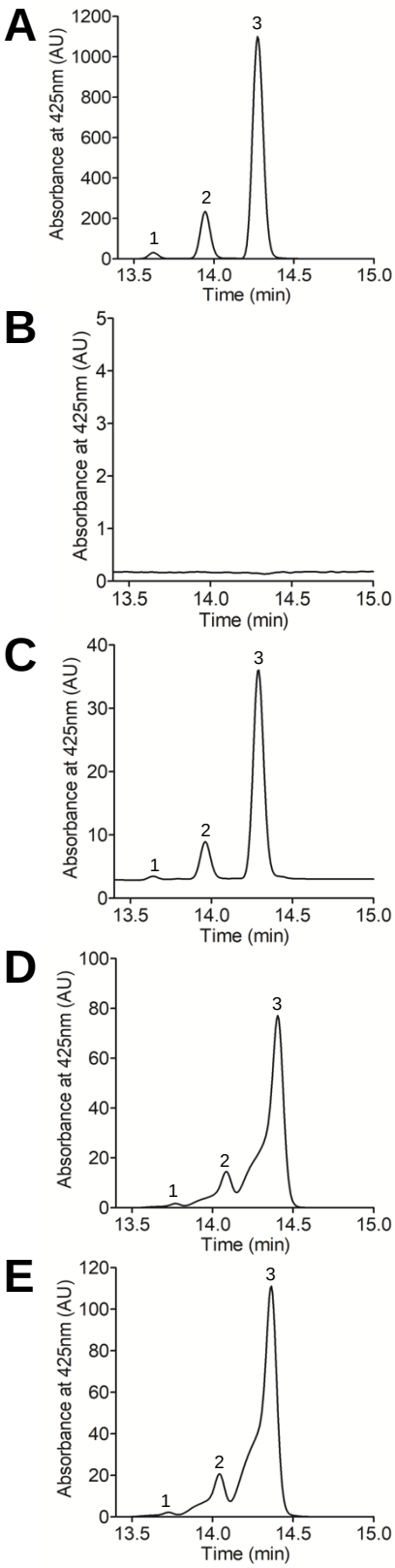

Figure S3. Curcuminoids in the tissues of larvae reared on media supplemented with curcuminoids are observed by HPLC at an absorbance of 425 nm. The curcuminoid

standard contains three compounds (A) and displays three peaks when run on HPLC, which correspond to bisdemethoxycurcumin (1), demethoxycurcumin (2), and curcumin (3). Extracts from larvae grown on medium lacking curcuminoids (B) and after curcuminoids were spiked into this extract (C). Extracts from larvae grown on medium supplemented with 25 mM curcuminoids (D) and after additional curcuminoids were spiked into this extract (E). Representative chromatograms are presented.