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Extension of *Drosophila* lifespan by cinnamon through a sex-specific dependence on the insulin receptor substrate *chico*

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Abstract

Cinnamon is a spice commonly used worldwide to flavor desserts, fruits, cereals, breads, and meats. Numerous health benefits have been attributed to its consumption, including the recent suggestion that it may decrease blood glucose levels in people with diabetes. Insulin signaling is an integral pathway regulating the lifespan of laboratory organisms, such as worms, flies, and mice. We posited that if cinnamon truly improved the clinical signs of diabetes in people that it would also act on insulin signaling in laboratory organisms and increase lifespan. We found that cinnamon did extend lifespan in the fruit fly, *Drosophila melanogaster*. However, it had no effect on the expression levels of the 3 aging-related *Drosophila* insulin-like peptides nor did it alter sugar, fat, or soluble protein levels, as would be predicted. In addition, cinnamon exhibited no protective effects in males against oxidative challenges. However, in females it did confer a protective effect against paraquat, but sensitized them to iron. Cinnamon provided no protective effect against desiccation and starvation in females, but sensitized males to both. Interestingly, cinnamon protected both sexes against cold, sensitized both to heat, and elevated HSP70 expression levels. We also found that cinnamon required the insulin receptor substrate to extend lifespan in males, but not females. We conclude that cinnamon does not extend lifespan by improving stress tolerance in general, though it does act, at least in part, through insulin signaling.

Keywords

cinnamon; botanical extract; insulin signaling; lifespan; aging; *Drosophila*

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Conflicts of interests

The authors have no conflicts of interests.

1. Introduction

Cinnamon is a spice commonly used in various cuisines around the world to flavor desserts, fruits, cereals, breads, and meats, and is derived from the bark of trees in the genus *Cinnamomum*, most commonly, *C. cassia*. The extract has been reported to exhibit numerous health benefits, including anti-inflammatory, antimicrobial, antioxidant, and anti-cancer action (Hong et al. 2002; Huss et al. 2002; Kim et al. 2007; Friedman et al. 2002; Lopez et al. 2005; Osawa et al. 1991; Lin et al. 2003; Kim et al. 1995; Jaganathan and Supriyanto 2012; Koppikar et al. 2010). There has been a recent interest in the ability of cinnamon to have a positive effect on diabetes. In particular, several clinical studies on the anti-diabetic properties of cinnamon may create an important role for this botanical in the management of diabetes and prediabetes (Lu et al. 2012; Ranasinghe et al. 2012; Davis and Yokoyama 2011; Kirkham et al. 2009).

The precise molecular action of cinnamon is not known. However, cinnamon and one of its putative active compounds, cinnamaldehyde, have been shown to elevate the expression levels of peroxisome proliferator-activated receptor γ (PPAR γ) and activate AMP kinase (AMPK) (Sheng et al. 2008; Huang et al. 2011). These activities mimic the action of the thiazolidinediones and metformin; commonly used antidiabetic drugs (Lee et al. 2011; Scarsi et al. 2007; Zhou et al. 2001; Zhang et al. 2007). Both PPAR γ and AMPK elevate metabolism in part by activating mitochondrial biogenesis (Alaynick 2008; Haemmerle et al. 2011; Hardie 2011). Cinnamon has also shown a complex relationship with insulin signaling where it activates insulin-like growth factor 1 (IGF1) signaling in fibroblasts, but down-regulates insulin signaling in adipocytes (Takasao et al. 2012; Cao et al. 2010). Another compound present in cinnamon, β -caryophyllene oxide, has been suggested to activate the mammalian target of rapamycin (mTOR) (Park et al. 2011), which is a complex of proteins that centrally regulates numerous cellular and metabolic processes (Schmelzle and Hall 2000; Wullschleger et al. 2006). In addition, cinnamon, cinnamaldehyde, and eugenol, another constituent compound, have been shown to directly modulate mitochondrial physiology (Usta et al. 2002). Thus, the potential anti-diabetic effects of cinnamon could possibly occur through the elevation of cellular metabolism.

In various model organisms, several pathways such as Insulin and insulin-like growth factor signaling (IIS), mTOR, cyclic-AMP signaling, autophagy, and mitochondrial function have been implicated (Bartke 2011; Giannakou and Partridge 2007; Fontana et al. 2010; Kapahi et al. 2004; Bjedov et al. 2010; Lu et al. 2011; Stenesen et al. 2013; Dai et al. 2009; Esposito et al. 1999; Lee et al. 2010; Morrow et al. 2004; Tong et al. 2007; Eisenberg et al. 2009; Schriner et al. 2005). Since cinnamon appears to modulate all of these pathways, it may be expected to extend lifespan in a short-lived model organism. This was previously shown to be the case in the worm, *Caenorhabditis elegans* where cinnamon appeared to require several molecular pathways, including IIS, to extend lifespan (Yu et al. 2010). Here, we found that cinnamon is able to extend lifespan the fruit fly, *Drosophila melanogaster* with an apparent requirement of IIS in males, but not in females. Cinnamon also appears to have little or no effect on stress tolerance, including oxidative stress in males, and is unlikely to act as an antioxidant.

2. Materials and Methods

2.1 Dietary supplements

Cinnamon was obtained from McCormick & Company, Sparks MD. Coumarin and cinnamaldehyde were obtained from Sigma-Aldrich, St. Louis, MO. Cinnamaldehyde, coumarin, cinamic alcohol, and cinnamic acid levels in the cinnamon extract were measured by high-performance liquid chromatography. Two hundred mg of cinnamon powder was suspended in 25 mL of 50% methanol and sonicated for 30 min and then filtered through a 0.2 μm Whatman filter. The samples were run on an ACE C18 column with a mobile phase initially composed of 10% acetonitrile, which was then ramped up to 70% over 30 min. The cinnamon powder used in this study was found to contain 3.8% cinnamaldehyde, 1.4% coumarin, and 0.9% cinnamic acid. No cinnamic alcohol was detected. Cinnamaldehyde and coumarin concentrations were selected based on the highest levels of what is detected in commercial preparations (Woehrlin et al. 2010).

2.2. Fly stocks

The *w¹¹¹⁸* control (Stock 3605) and *chico¹* (Stock 10738) flies were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. The JIV flies used in this study were derived from an Amherst, Massachusetts's population established by P.T. Ives in 1975 (Rose and Charlesworth 1981, Rose, 2002 #149). This population has been cultured at moderate to large population sizes and controlled densities (50–80 eggs per vial) for more than 700 generations with discrete generations cultured every 2 weeks.

Flies were reared by placing approximately 100 males and 100 females in a plastic cylinder with a screen top, housing at the bottom a 10 cm petri dish filled with banana-molasses food composed of 9% carbohydrate content and 10% yeast content. These flies were allowed to lay eggs for one day. The food around the edge of the dish was then cut into approximately 1-cm² squares and placed into 8-dram vials. Each of these vials then contained approximately 35 eggs. Two weeks later, approximately 30 flies/vial were recovered after eclosion, which were then used for our lifespan studies or other experiments.

2.3 Feeding and lifespan assays

Flies were fed Cinnamon, coumarin, and cinnamaldehyde on the methods described in Jafari *et al.* (Jafari et al. 2007). For all experiments, feeding was initiated immediately post-eclosion and, other than lifespan assays, were completed when the flies were 10 days of age. Cinnamon or chemicals were dissolved in a yeast solution (4% yeast in 1% acetic acid), and 75 μL of this mixture was overlaid on a banana-molasses food composed of 9% carbohydrate content and 3.6% yeast content. Flies were maintained at $22 \pm 1^\circ \text{C}$ under a 12 h light: 12 h dark cycle for all experiments. For lifespan studies, flies were housed 12 per vial (6 males and 6 females). This density was maintained as long as feasible. Flies were given fresh food every two days and deaths were recorded at these times. Survival analyses were calculated based on the number of deaths recorded and evaluated by the log-rank Mantel-Cox test. No deaths were censored. Flies were also housed 12 per vial (6 males and 6 females) for all other experiments, independent of the total number needed, and transferred to fresh food every other day.

2.4 Measurement of fecundity and locomotion

For the fecundity assays, 40 total pairs per treatment group, collected less than 12 h post eclosion, were placed in 8 dram vials (one male and one female per vial) containing 5 mL of a sugar-charcoal media covered with 75 μ L yeast solution (1 g yeast/mL 1% acetic acid). Pairs were permitted to lay eggs for 24 hours and then transferred to new vials every 24 h for 10 ten days. Eggs were counted every 24 hours.

Vertical locomotion (climbing) was assessed by the rapid iterative negative geotaxis (RING) assay (Gargano et al. 2005). For each treatment group, 15 flies were placed in an 8 dram vial. The vial was gently shaken until all flies were displaced to the bottom of the vial. Flies were then permitted to climb for 4 seconds, and then photographed. All four groups (males and females, cinnamon-fed and controls) were assayed simultaneously. The assay was repeated 3 times with independent groups of flies. The distance climbed was measured in Adobe Photoshop and the speed was calculated by distance/4 sec. Horizontal locomotion was measured by the *Drosophila* activity monitor (DAM) assay (Zordan et al. 2007) by the number of beam breaks over a 24-hour period.

2.5 Gene expression assays

Approximately 400 Flies were fed 25 mg/mL Cinnamon or an identical control diet for 10 days, and frozen in groups of 25. RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Samples were treated with DNase (New England Biolabs, Ipswich, MA) at 37° C for 10 min to remove contaminating DNA. DNase was heat inactivated by incubation at 75° C for 10 min in the presence of 5 mM EDTA. RNA was then purified by use of the RNeasy kit (Qiagen, Hilden, Germany). RNA quantity and quality was measured by spectrophotometry. One μ g of RNA from each sample was converted to DNA by the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Samples were diluted 100-fold. Quantitative PCR was performed on a MiniOpticon real-time PCR system with SYBR green dye (Bio-Rad, Hercules, CA). Relative amplification was calculated by the threshold cycle of each respective gene divided by the threshold cycle of the reference gene, RNA polymerase II. Primer sequences, listed in Table S1, were designed by NCBI/Primer-BLAST. All primers were designed to have a melting temperature of 60° C.

2.6 Stress assays

One hundred twenty flies per sex per treatment were fed for 10 days with 25 mg/mL cinnamon or control diet. For the H₂O₂, paraquat, and iron toxicity assays, flies were fed each compound at the concentrations indicated in the figure captions diluted in a 5% glucose solution supplied on filter paper. Iron was supplied in the form of iron-nitritotriacetate (Fe-NTA) (Awai et al. 1979). For the desiccation assay, flies were housed in empty vials and deaths were recorded every 2 h. For the starvation assay, flies were housed in vials containing 2% agarose to provide moisture, but no nutritional value. Deaths were recorded every 4 hours. For the heat assay, flies were housed in empty vials at 37° C, and deaths were recorded every hour. For the cold tolerance assay, flies were housed in empty vials at 4° C for 72 h. They were then returned to 22° C, and the numbers of alive and dead were

recorded after 12 hours. Survival for cold tolerance was calculated by Fisher's exact test. Survival for all other assays was determined by the Mantel-Cox log-rank test.

2.7 Measurement of protein, water and fat content and body weight

Flies were fed for 10 days with 25 mg/mL Cinnamon or a control diet. The flies were then collected with CO₂ and weighed. For sugar content, flies were homogenized in ice-cold 500 µL 50 mM Tris-HCl buffer, pH 7.8, and then centrifuged for 10 min at 10,000 × g at 4° C. Ten µL was removed for protein measurement, and the remainder was incubated for 10 min at 95° C. The samples were then incubated overnight at 37° C with 0.05 U/mL trehalase to convert trehalose to glucose. Glucose concentrations were then measured by an increase in absorbance at 340 nm in 50 mM Tris-HCl buffer, pH 7.8 containing 1 mM Mg-acetate, 0.66 mM NAD⁺, 0.4 mM ATP, and 2 mU/mL each of hexokinase and glucose-6-phosphate dehydrogenase (Carroll et al. 1971). For the soluble protein assay, 50 flies per sample were homogenized in 500 µL 100 mM Potassium phosphate buffer, pH 7.4. The samples were centrifuged for 10 min at 10,000 x g. Protein in the supernatant was measured by reaction with Coomassie Brilliant Blue and correlated to a standard curve generated with bovine serum albumin (Bradford 1976) and normalized to fly weight. For water content, 10 flies per sample were weighed, dried for 48 h at 70° C, and then weighed again. The difference in weights divided by the initial weight was taken to be the water content. To determine fat content, the samples used for water measurement were then incubated at RT for 24 h in diethyl ether. The ether was removed and the samples were allowed to dry, and then weighed. Fat content was taken to be the difference in the weights before and after diethyl ether treatment divided by the initial weight (prior to drying at 70° C).

2.8 Measurement of mitochondrial content and respiration

Flies were fed for 10 days. Fifty flies per sample were homogenized in ice-cold isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% fatty acid free BSA, PH 7.2) using a glass-teflon dounce homogenizer, and then filtered through two layers of cotton gauze. A mitochondrial-enriched pellet was obtained by centrifugation for 10 min at 6000 x g and re-suspended in 150 µL ice-cold isolation buffer. Mitochondrial respiration rates were measured using a Clark-type oxygen electrode and monitoring system (Hansatech Instruments, Norfolk, England). An NADH-linked substrate (pyruvate in combination with malate) was added to isolated mitochondria suspended in 1 mL of respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH₂PO₄, pH 7.2) at 30° C. To this, 125 nmol ADP was added to generate state 3 respiration. State 3 respiration represents the maximal ability to generate ATP, and is generally 5–6-fold higher than state 4 respiration which is determined after ADP is consumed. The respiratory control ratio (RCR) is defined as the ratio of state 3 to state 4. Uncoupled (maximal rate of O₂ consumption) respiration rates were measured the after addition of the uncoupler FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone).

Mitochondrial content was measured by fumarase activity and mtDNA copy number. For fumarase activity, 50 flies were homogenized in 50 mM potassium phosphate buffer, pH 7. 4 and centrifuged for 10 min at 10,000 x g at 4° C. Fumarase activity in the supernatant was measured by the conversion of 50 mM malate (in 50 mM potassium phosphate buffer, pH 7.

4) to fumarate at 240 nm (Kuff 1954). Activities were normalized to soluble protein content. Mitochondrial DNA copy was estimated by the relative amplification of a mitochondrial target (16s ribosomal RNA gene) to that of a nuclear target (ribosomal protein Rp49). Twenty-five flies were homogenized in DNA isolation buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% SDS) and supplemented with proteinase K (0.5 mg/mL final concentration) and digested overnight at 55° C. The samples were then extracted by standard phenol-chloroform procedures and precipitated by ethanol. Quantitative PCR procedures were identical to those listed above except in this case the reference gene used was RP49.

2.9 Statistical analyses

Data were presented as the mean \pm sem except for lifespan curves. Statistical analyses were conducted using Prism software (GraphPad, La Jolla, CA). The tests used and sample sizes for each experiment are indicated in the figure captions and in the methods and results sections.

3. Results

3.1 Cinnamon, but not coumarin or cinnamaldehyde, increases *Drosophila* lifespan

We examined whether cinnamon could extend lifespan in the fly at 3 different doses: 2.5, 25, and 75 mg/mL. The lowest dose had a marginal effect in males and no significant effect in females (Table 1). There was a dose dependent effect in female flies, with 75 mg/mL exhibiting a greater impact on mean lifespan (Table 1). We chose 25 mg/mL as the best dose as it increased lifespan in both sexes. We examined the effect of 25 mg/mL on survival in two types of *Drosophila*, *w¹¹¹⁸*, a commonly used inbred strain, and JIV, an outbred population derived from one originally collected in 1978 (Rose and Charlesworth 1981; Rose et al. 2002). Cinnamon increased lifespan up to 37% in both of strains indicating its effects are not specific to a single genotype (Fig. 1 and Table 1). The precise active component in cinnamon is not known. However, we tested two of the better-known, and potentially toxic, compounds, coumarin (Cohen 1979; Abraham et al. 2010), and cinnamaldehyde (Bickers et al. 2005). Neither compound was able to extend *Drosophila* lifespan (Fig. 2). Since cinnamaldehyde and coumarin concentrations were selected based on the highest levels of what is detected in commercial preparations (Woehrlin et al. 2010), the lack of a beneficial effect is unlikely to be explained by an insufficient amount, and may explain the slight toxicity of cinnamaldehyde in female flies (Fig. 2D).

3.2 The effect of cinnamon on fecundity and physical activity

A significant issue with measuring lifespan-extending effect of pharmaceuticals and botanicals is that they may deter the animal from eating due to a repulsive taste or smell. Then if the animal consumed less food, lifespan may be increased due to a calorie restriction effect, rather than a biochemical effect of the drug. This possibility can be indirectly addressed by measuring fecundity (egg laying) as a reduction in caloric or protein intake would result in decreased fecundity. We found that 25 mg/mL of cinnamon had no effect on fecundity, whereas 75 mg/mL did have a moderate negative effect (Fig. 3A). Because of the

positive effect on lifespan without a negative effect on fecundity, we used cinnamon at a dose of 25 mg/mL for the remainder the experiments.

We then examined whether cinnamon would have any effect on physical activity in the fly. We found that it dramatically improved vertical climbing ability (Fig. 3B). Since flies have an inherent desire to climb (negative geotaxis), this increased vertical climbing ability demonstrates a positive effect on physical performance. Horizontal locomotion is used to assess whether a drug acts as a stimulant or sedative. We found that cinnamon had no effect on horizontal locomotion (Fig. 3C).

3.3 The action of cinnamon through insulin signaling

Our original hypothesis was that cinnamon would extend lifespan by acting through insulin and insulin-like signaling (IIS) in the fly. We found that cinnamon required the insulin receptor substrate to extend lifespan in males (Fig. 4A), but the extract was able to extend lifespan in females in its absence (Fig. 4B). Of the 7 *Drosophila*-like insulin peptides (Dilps) present in flies, 3 are associated with increased lifespan. However, cinnamon had no effect on the expression levels of any of these 3 *Dilps* (Fig. 4C).

3.4 Cinnamon has sex-specific and variable effects on stress tolerance

Improved stress tolerance is strongly associated with enhanced lifespan (Kirkwood and Austad 2000; Vermeulen and Loeschcke 2007). We therefore examined whether cinnamon might act by enhanced stress tolerance. We found marked differences between males and females. In males, cinnamon had no protective effect against the superoxide generator paraquat, H₂O₂, or iron (Fig. 5A, C, E) and sensitized males to desiccation and starvation (Fig. 6A, C). Females fed cinnamon were marginally protected against paraquat (Fig. 5B). However, cinnamon afforded no protective effect in females against H₂O₂, desiccation, or starvation, and sensitized them to iron (Figs. 5D, F and 6B, D). Two commonalities between the sexes, was that cinnamon protected both against cold (Table 2), and sensitized both to heat (Fig. 6E, F).

3.5 The effect of cinnamon on sugar, fat, and protein contents and on body weight

Insulin signaling and dietary restriction are known to regulate total body sugar, fat, and soluble protein content (Hwangbo et al. 2004; Magwere et al. 2006; Bai et al. 2012; Schriner et al. 2013). We found that cinnamon had no effect on these parameters in either sex (Fig. 7A, B, C). However, Cinnamon did decrease body weight in females, but not males (Fig. 7D).

3.6 Cinnamon has no effect on mitochondrial function, but up-regulates HSP70

Since cinnamon increased physical performance and protected flies against cold and sensitized them to heat, we hypothesized that cinnamon might modulate mitochondrial function or content. The extract could also increase the expression levels of uncoupling proteins (UCPs). However, we found that cinnamon had no effect on total mitochondrial content (Fig. 8A, B) or respiration rates (Table 3). The extract had no effect on UCP expression (Fig 8C), nor did it alter the expression levels of the mitochondrial HSP22 (Fig. 8D). However, cinnamon did notably increase the expression levels of HSP70 (Fig. 8D).

4. Discussion

Cinnamon has been used to flavor food for thousands of years. Recently, it has been reported that cinnamon may have positive effects on the management of diabetes (Lu et al. 2012; Ranasinghe et al. 2012; Davis and Yokoyama 2011; Kirkham et al. 2009), suggesting that it might affect insulin signaling. Surprisingly, in laboratory animals, perturbation of insulin and insulin-like growth factor signaling (IIS) has been shown to extend lifespan (Clancy et al. 2001; Tu et al. 2002; Fontana et al. 2010). However, not surprisingly, improved insulin sensitivity is also associated with increased lifespan and health (Barzilai et al. 2012). As a result, we hypothesized that if cinnamon did indeed improve insulin signaling in humans, it may also do so in experimental animals, and improve lifespan. This was previously found to be the case in *C. elegans*, where cinnamon extended lifespan with an apparent dependence on IIS (Yu et al. 2010). Here, we examined the effect of cinnamon on lifespan and its potential mechanism of action in a different invertebrate model, *Drosophila melanogaster*. We found that the extract increased lifespan in both sexes and in two types of flies, a commonly used inbred strain *w¹¹¹⁸*, and an out-bred population, JIV (Fig 1), demonstrating that its effect on lifespan is not limited to *C. elegans*, and not specific to any sex or genotype.

In order to determine whether cinnamon works directly through IIS, we used flies that lacked the insulin receptor substrate, *chico*. Our finding that cinnamon was not able to extend lifespan in *chico¹/chico¹* males, demonstrated a requirement for the insulin receptor substrate, and supports an action through IIS in males. However, in *chico¹/chico¹* females, cinnamon was still able to extend lifespan, though not to the same magnitude as in the control flies (9% increase in *chico¹/chico¹* flies vs. 23% in control flies (Table 1)). This suggests a possible IIS-independent or partial IIS-independent mechanism for cinnamon in females. The lower magnitude of lifespan extension by cinnamon in *chico¹/chico¹* females may be due to a lower feeding rate as has been suggested (Clancy et al. 2001; Wong et al. 2009), and the amount of cinnamon consumed by *chico¹/chico¹* females may be simply less than the *w¹¹¹⁸* controls. An IIS-independent action of cinnamon in flies would not be too surprising, as it is known that perturbation of IIS has different effects between males and females in flies (Clancy et al. 2001; Tu et al. 2002). For example, *chico¹/+* heterozygotes are longer lived than wild types in both sexes. Whereas complete loss of *chico* extends lifespan in females, but has no effect, or can even shorten lifespan in male flies (Clancy et al. 2001; Tu et al. 2002). Thus, it appears that there may be an alternate pathway for IIS present in females that permits them to bypass, to some extent, the loss of *chico*, though, this may in part be due to the lower feeding rate as suggested above. Nevertheless, our results in males are consistent with the repression of IIS being important in the action of cinnamon. Interestingly, we found no effect of cinnamon on the expression levels of 3 ageing-related *Drosophila* insulin-like peptides (Dilps). Therefore, cinnamon is unlikely to act on Dilp producing cells, and combined with the *chico* data, suggests that cinnamon may act directly on the insulin receptor itself. The work of others have shown cinnamon to have a complex relationship with insulin signaling where it activates insulin-like growth factor 1 (IGF1) signaling in fibroblasts, but down-regulates insulin signaling in adipocytes (Takasao et al.

2012; Cao et al. 2010). It seems that cinnamon does have an effect on IIS, though, its precise mode of action is not clear yet.

A surprising effect of cinnamon in our study was that it had limited and sex-specific effects against stress. Extended lifespans in laboratory organisms are often associated with increased tolerances to various types of stresses (Kirkwood and Austad 2000; Vermeulen and Loeschcke 2007). This observation has even been used as the basis for selecting for genetic variants with increased lifespans (Lin et al. 1998; Rose et al. 1992). In the particular case of botanicals, many are thought to mediate their beneficial effects through the antioxidant activity of polyphenols (Osawa 1999; Pandey and Rizvi 2009; Landete 2012; Liu 2013). Therefore, it was somewhat curious that cinnamon exhibited no antioxidant effect (Fig. 5A, C, E) in males, and little antioxidant effect in females (Fig. 5B), despite its ability to extend lifespan. In addition, the extract actually sensitized males to desiccation, starvation, and heat. A slightly different picture was present in females, where cinnamon afforded a modest protective effect against paraquat, sensitized them to iron (Fig. 5B, D, F), and had no effect on survival when challenged to starvation and desiccation. The notable commonality between the sexes, though, was that cinnamon sensitized females to heat, as it did in males, and protected both against cold. When we examined the action of cinnamon on the expression levels of HSP22 and HSP70, we found no effect on HSP22, but found HSP70 to be up-regulated. While the relationship of HSPs to aging isn't exactly clear, increased HSP70 expression levels, and moderate levels of heat stress have resulted in increased lifespans in flies (Tatar et al. 1997; Khazaeli et al. 1997; Le Bourg et al. 2001; Hercus et al. 2003). Therefore, one possible mode of action of cinnamon is that it might effectively mimic heat stress. In popular culture, cinnamon is considered to be a "warming spice" and thought to have the ability to raise body temperature (Inokawa et al. 2006). Our findings here, that cinnamon protects flies against cold, sensitizes them to heat, and elevates HSP70 expression, lends some credibility to this idea.

Cinnamon as a plant extract contains many different compounds, which have been shown to exhibit varied activities. We examined the effect on lifespan of two of the better-known, but potentially toxic, compounds, coumarin and cinnamaldehyde (Cohen 1979; Abraham et al. 2010; Bickers et al. 2005). Cinnamaldehyde was particularly interesting, as it has been shown to elevate the expression levels of peroxisome proliferator-activated receptor γ (PPAR γ) and activate AMP kinase (AMPK) (Sheng et al. 2008; Huang et al. 2011). Both PPAR γ and AMPK can elevate metabolism in part by activating mitochondrial biogenesis (Alaynick 2008; Haemmerle et al. 2011; Hardie 2011). Cinnamon, cinnamaldehyde, and eugenol, another constituent compound, have been shown to directly modulate mitochondrial physiology (Usta et al. 2002). A possible explanation of the "heat" effect of cinnamon could be due to increased mitochondrial content or function. This could also explain the increased physical performance seen in cinnamon flies relative to controls (Fig. 3B). However, we found no effect of cinnamon on mitochondrial content, respiration rates, or on the expression of mitochondrial uncoupling proteins (Fig. 8A, B, C, and Table 3). Thus, it is unlikely that cinnamon modulates mitochondrial function in our flies. Whatever compound in cinnamon is responsible for extending lifespan; it is unlikely to be coumarin or cinnamaldehyde, as neither could extend lifespan alone (Fig. 2). We plan to systematically examine a range of compounds in the near future.

One of the most robust treatments for improving lifespan in laboratory organisms is dietary restriction (DR), which is a decreased caloric intake without malnutrition. One of the dangers of using a natural product, or a drug, is that the treatment may prove distasteful or have an offending odor. In such a situation, an increased lifespan would result, not from a molecular action of our treatment, but simply from the flies eating less food. There are several ways to test for such an effect. In flies, DR has been shown to decrease total body weights, fecundity, and soluble protein levels, and elevate fat content (Hwangbo et al. 2004; Magwere et al. 2006; Bai et al. 2012; Schriner et al. 2013). At the cinnamon dose used for this study, 25 mg/mL, we found no effect on fecundity, or on protein and fat contents in either sex (Fig. 7A, B, C), arguing against any DR effect. We did find that cinnamon decreased female body weights by about 9%, while male weights were unaffected (Fig. 7D). While females would be expected to be more sensitive to a DR effect due to their reproductive systems, we have previously shown that male weights are also decreased in response to DR (Schriner et al. 2013). At the higher dose of 75 mg/mL there was a moderate decrease in fecundity consistent with a DR effect (Fig. 3A). Thus, we can't completely rule a DR-like effect of cinnamon in females, due to their lowered weights, however, the absence of the elevated fat, decreased soluble protein levels and decreased body weights in males all argue against a simple DR effect.

It is also possible that cinnamon may act by directly inhibiting reproduction. While we found no negative effect on fecundity at the principal dose used in this study, 25 mg/mL, we cannot rule some other effect of cinnamon on reproductive fitness, such as courtship, mating, or sperm production. Any of which could possibly confer a lifespan extension. We have already found such a relationship in green tea, where lifespan extension is associated with decreased fertility in males (Lopez et al. 2014). We are currently examining the effect of cinnamon and other botanicals on these other aspects of reproduction.

Here we have shown evidence that cinnamon can extend lifespan of the fruit fly. In male flies, cinnamon requires the insulin receptor substrate, CHICO, to extend lifespan. This supports a plausible action on insulin signaling in humans and lends credibility for its suggested anti-diabetic activity. However, in females cinnamon doesn't require CHICO, suggesting that it extends lifespan through other pathways possibly independent from IIS. Our future work will be to examine the action of cinnamon on additional known ageing pathways, such as the target of rapamycin (TOR) and cyclic-AMP signaling (Kapahi et al. 2004; Fontana et al. 2010; Tong et al. 2007), and to identify the molecule or molecules responsible for cinnamon lifespan-extending property in flies. It is also important to mention that it was recently reported that cinnamon, along with several other natural products, was unable to extend lifespan in laboratory mice (Spindler et al. 2013). However, this result doesn't rule a positive effect of cinnamon, or the other extracts, on human health. The mice used in this study were F1 hybrids, and were fed a slightly reduced calorie diet. Thus, these animals were genetically and nutritionally healthy. If cinnamon were able to improve health in diabetic animals, for example, we may very well expect no effect of the extract in this study. By contrast, in our study here, we show that cinnamon does extend lifespan, even in animals that are nutritionally and genetically healthy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IIS	insulin and insulin-like growth factor signaling
H₂O₂	hydrogen peroxide

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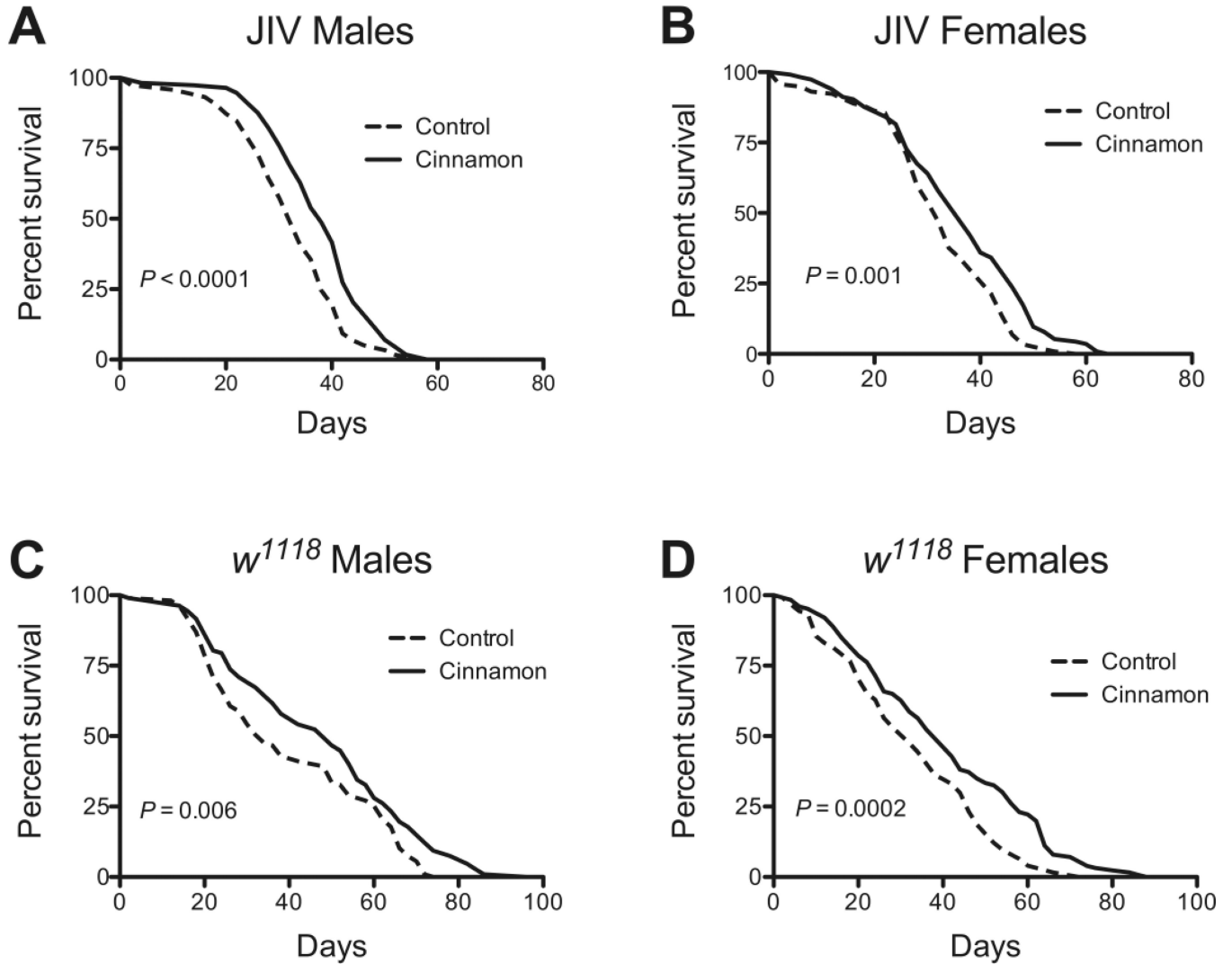
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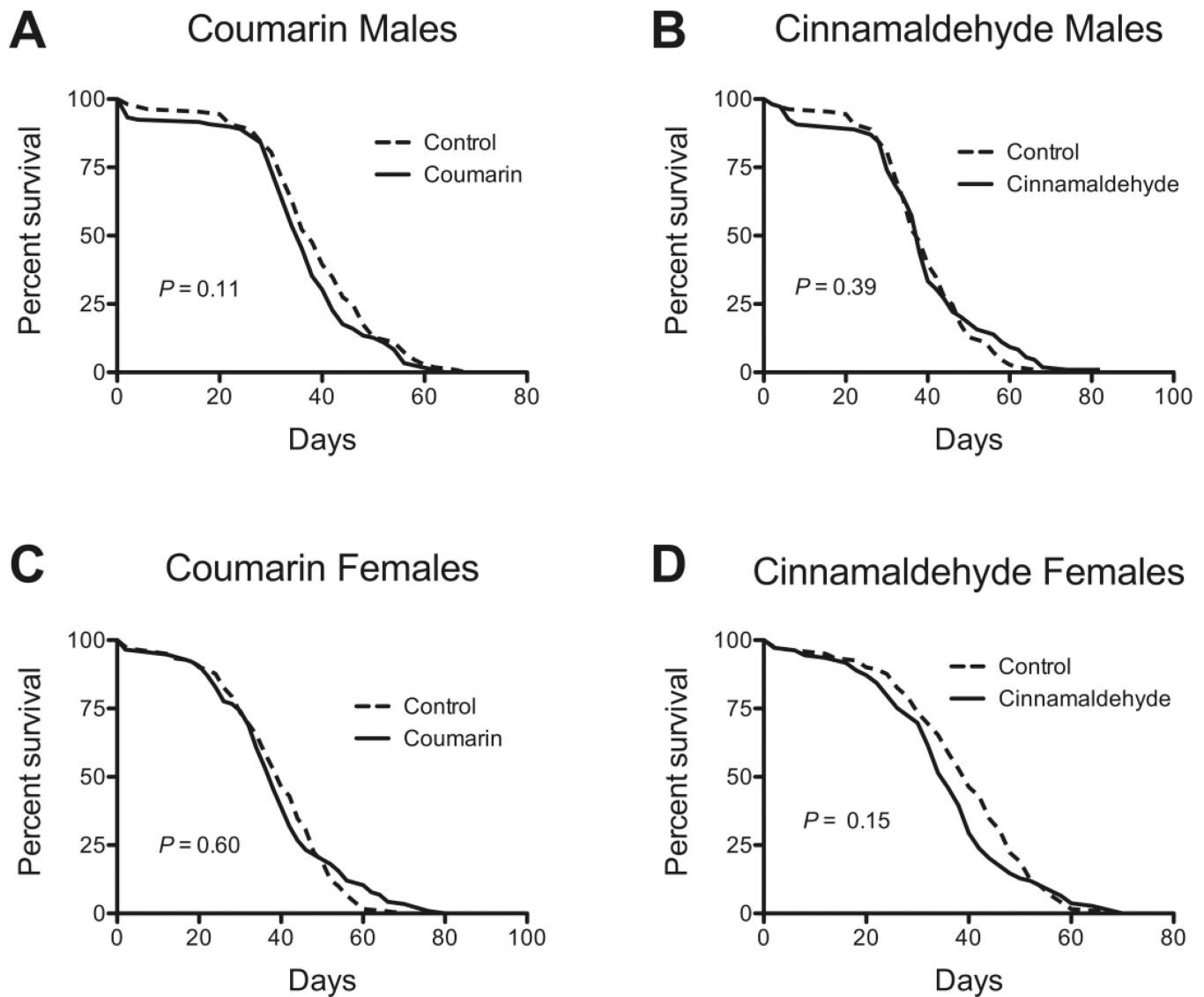
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Highlights

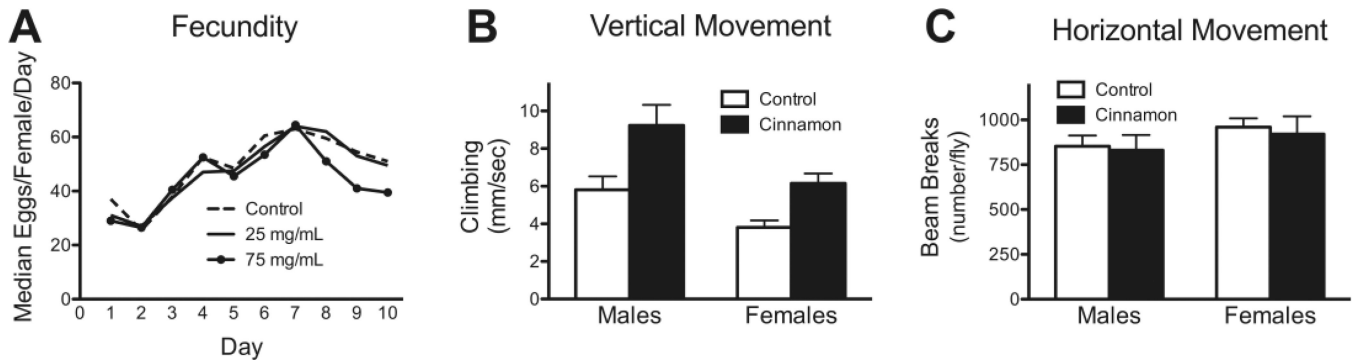
- Cinnamon has been suggested to lower blood glucose levels in diabetics
- Cinnamon extended lifespan in the fruit fly, *Drosophila melanogaster*
- Cinnamon required the insulin receptor substrate in male flies, but not in female flies, to extend lifespan.
- Cinnamon also protected flies against cold, sensitized them to heat, and elevated HSP70 expression levels

**Fig. 1.**

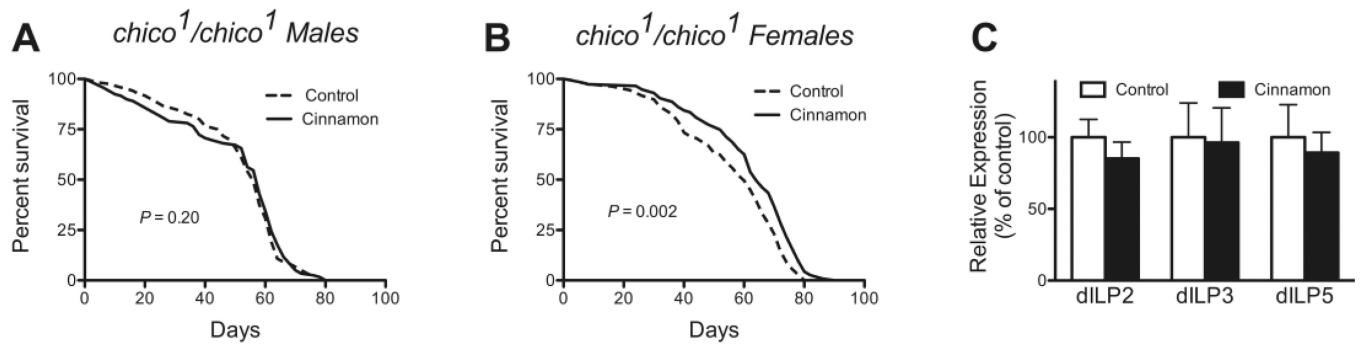
Cinnamon increases survival in the fruit fly, *Drosophila melanogaster*. Cinnamon increased survival in both (A) male, $P < 0.0001$, and (B) female, $P = 0.001$, JIV flies, and in (C) male, $P = 0.006$, and (D) female, $P = 0.0002$, w^{1118} flies. The increases in mean lifespan due to cinnamon were 17%, 12%, 18%, and 24%, respectively. P -values were calculated with the Mantel-Cox log-rank test. Sample sizes for the control groups and treated groups respectively were as follows: JIV males: 118, 113; JIV females: 117, 114; w^{1118} males: 107, 107; w^{1118} females: 124, 126.

**Fig 2.**

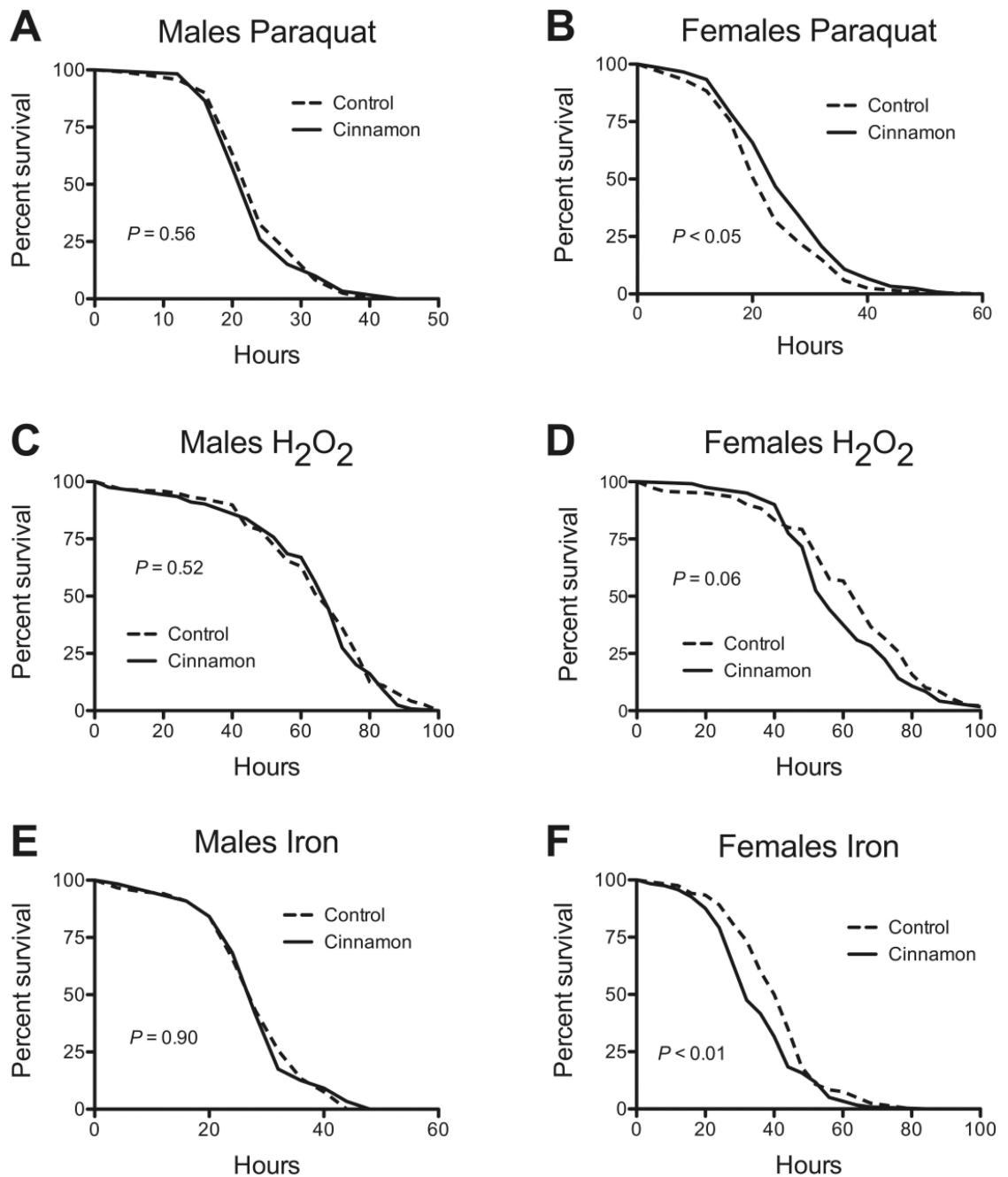
The effect of coumarin and cinnamaldehyde on *w¹¹¹⁸* lifespan. Coumarin at a 5 mg/mL had no effect on survival in (A) male or (B) female *w¹¹¹⁸* flies. Neither did 5 μ L /mL cinnamaldehyde in (C) male flies. In (D) females cinnamaldehyde also had no effect on overall survival via the Mantel-Cox log test, but appeared to actually decrease their mean lifespan ($P < 0.05$). *P*-values were calculated with the Mantel-Cox Log-Rank test and Man-Whitney nonparametric test. Sample sizes for the control groups and treated groups respectively were as follows: coumarin males: 109, 119; coumarin females: 121, 116; cinnamaldehyde males: 109, 108; cinnamaldehyde females: 121, 109.

**Fig. 3.**

The effect of cinnamon on reproduction and locomotion in w^{1118} flies. (A) At 25 mg/mL cinnamon had no effect on fecundity, however, a dose of 75 mg/mL did have a negative effect after day 7. $P < 0.05$, repeated measures ANOVA. $P > 0.05$ for control vs. 25 mg/mL, and $P < 0.05$ for control vs. 75 mg/mL, Dunnett's multiple comparison test, $n = 20$ pairs for each group. (B) Cinnamon improved vertical climbing ability in both males and females. $P < 0.0001$, 2-Way ANOVA for diet, $P < 0.01$ for each sex, control vs cinnamon, Bonferroni posttest, $n = 23$ male controls, 26 males fed cinnamon, 39 female controls, 40 females fed cinnamon. (C) Cinnamon had no effect on horizontal locomotion. $P = 0.72$ for diet, 2-way ANOVA, $n = 13$ male controls, 19 males fed cinnamon, 13 female controls, 18 females fed cinnamon.

**Fig 4.**

The dependence of cinnamon on the insulin receptor substrate *chico*, and its effect on the expression levels of 3 *Drosophila*-like insulin peptides. Cinnamon was not able to extend lifespan in (A) *chico* male flies, but could do so in (B) *chico* female flies. *P*-values were calculated with the Mantel-Cox Log-Rank test. Sample sizes for the control groups and treated groups respectively were as follows: *chico* males: 120, 119; *chico* females: 109, 115. (C) Cinnamon had no effect on the expression levels of the 3 aging-related dilps in male flies. *P* = 0.54 for diet, 2-way ANOVA, n = 6 groups of 10 flies.

**Fig 5.**

The effect of cinnamon on the tolerance of *w¹¹¹⁸* flies to oxidative stress and iron toxicity. Cinnamon had no ability to improve tolerance to (A) 12.5 mM paraquat, (C) 5% H₂O₂, or (E) or 80 mM Fe-NTA in male flies. In females, cinnamon had a marginal protective effect against (B) paraquat, had a borderline negative effect against (D) H₂O₂, and sensitized females to (F) iron. *P*-values were calculated with the Mantel-Cox Log-Rank test. For all groups, *n* = 120.

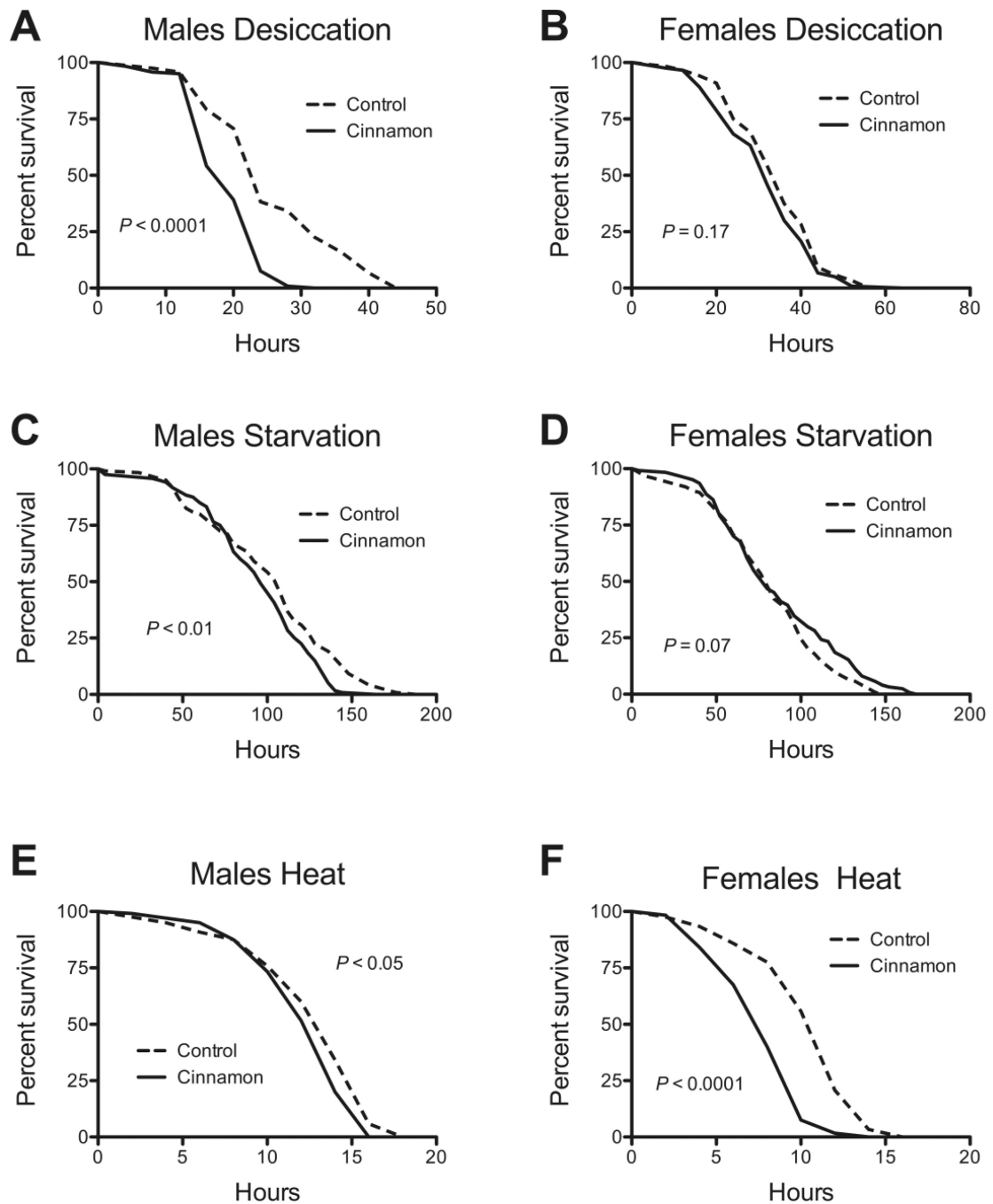


Fig 6. The effect of cinnamon on the tolerance of *w¹¹¹⁸* flies to desiccation, starvation, and heat. Cinnamon sensitized male flies to (A) desiccation, (C) starvation, and (E) heat. In females, cinnamon had no effect on (B) desiccation, or (D) starvation, but sensitized females to (F) heat. *P*-values were calculated with the Mantel-Cox Log-Rank test. For all groups, $n = 120$.

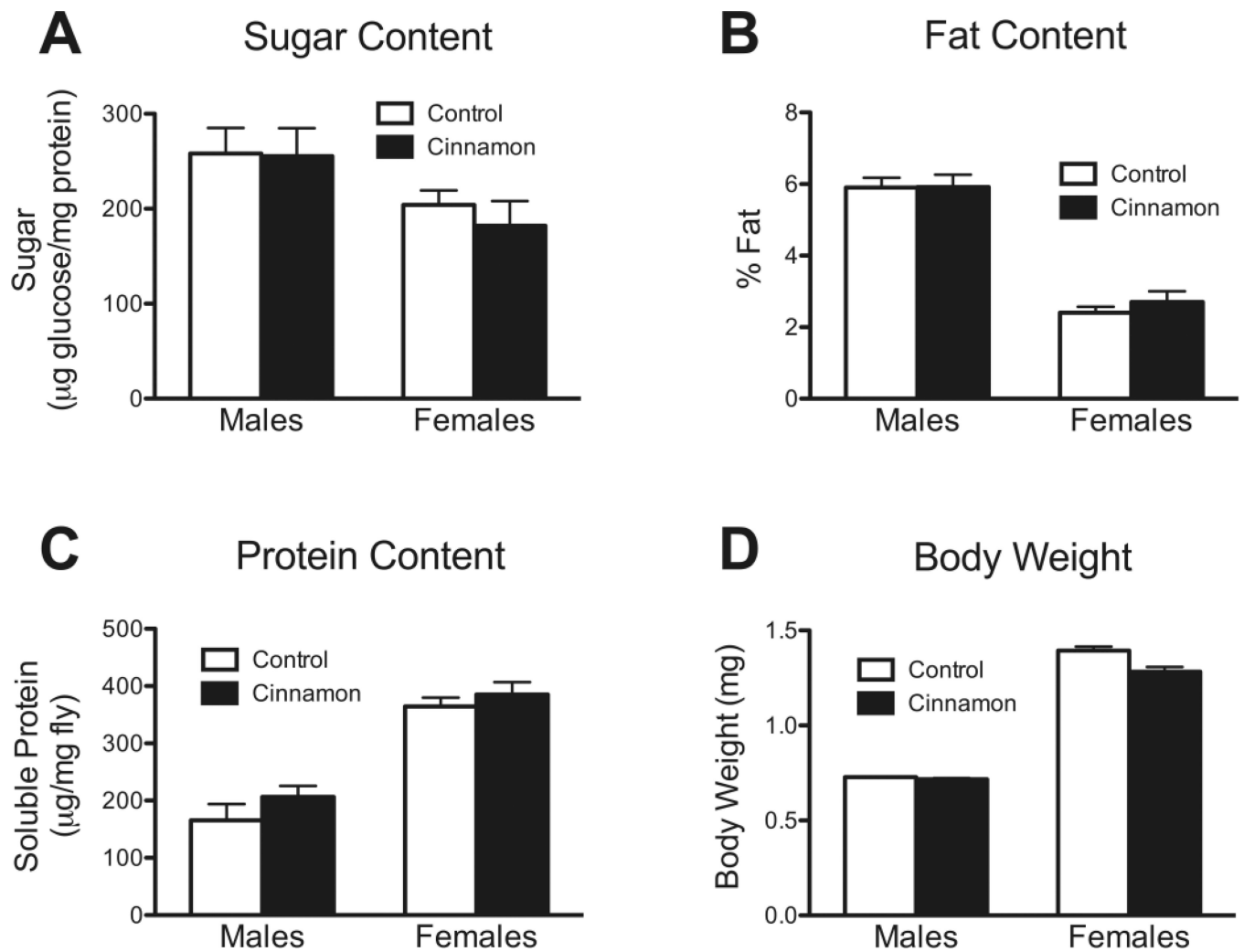
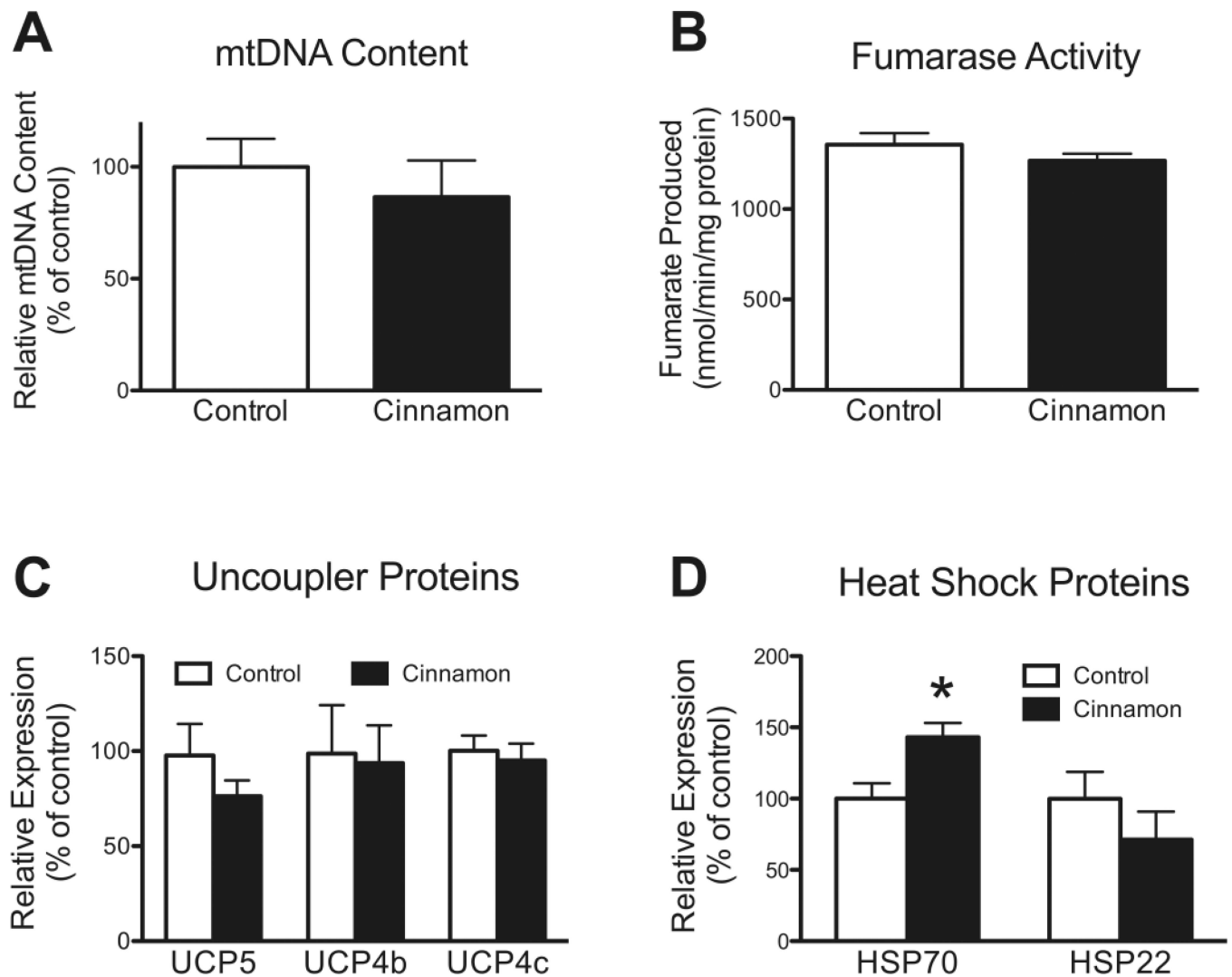


Fig 7. The effect of cinnamon on sugar, fat, and protein content, and on weight in w^{1118} flies. Cinnamon had no effect on (A) sugar content, $P = 0.62$, (B) fat content, $P = 0.58$, or (C) soluble protein content, $P = 0.17$, in either males or females. P-values were calculated by 2-way ANOVA, $n = 6$. (D) Cinnamon also had no effect on male body weights, but it decreased female body weights, $P = 0.0004$, 2-way ANOVA, $P > 0.05$ for males, and $P < 0.001$ for females, Bonferroni posttest, $n = 18$.

**Fig 8.**

The effect of cinnamon on mitochondrial content and expression of heat and uncoupler proteins in *w¹¹¹⁸* flies. Cinnamon had no effect on mitochondrial content by measures of either (A) mtDNA copy number, $P = 0.51$, or (B) fumarase activity, $P = 0.27$, unpaired t test, $n = 10$ and 6 , respectively. (C) Cinnamon had no effect on the expression levels of three uncoupler proteins. $P > 0.05$ for each UCP, unpaired t test, $n = 5$ or 6 . (D) Cinnamon increased the expression levels of HSP70, $*P < 0.05$, but had no effect on HSP22, $P = 0.32$, unpaired t test, $n = 5$ or 6 .

Table 1

Mean life span values for Cinnamon-fed and control diet flies

Strain	Sex	Dose (mg/mL)	mean	% increase	P
<i>w¹¹¹⁸</i>	males	0	32.1 ± 0.9		
<i>w¹¹¹⁸</i>	males	2.5	34.6 ± 0.9	8	< 0.05
<i>w¹¹¹⁸</i>	females	0	31.8 ± 1.1		
<i>w¹¹¹⁸</i>	females	2.5	33.9 ± 1.2	7	0.22
<i>w¹¹¹⁸</i>	males	0	32.1 ± 0.9		
<i>w¹¹¹⁸</i>	males	25	37.5 ± 0.9	17	< 0.0001
<i>w¹¹¹⁸</i>	females	0	32.6 ± 1.4		
<i>w¹¹¹⁸</i>	females	25	40.0 ± 1.6	23	< 0.0001
<i>w¹¹¹⁸</i>	males	0	40.7 ± 1.6		
<i>w¹¹¹⁸</i>	males	75	45.9 ± 2.1	13	0.07
<i>w¹¹¹⁸</i>	females	0	32.6 ± 1.4		
<i>w¹¹¹⁸</i>	females	75	44.6 ± 2.3	37	< 0.0001
JIV	males	0	32.1 ± 0.9		
JIV	males	25	37.5 ± 0.9	17	< 0.0001
JIV	females	0	31.9 ± 1.1		
JIV	females	25	35.7 ± 1.3	12	< 0.05
<i>chico¹</i>	males	0	50.4 ± 1.6		
<i>chico¹</i>	males	25	49.7 ± 1.8	0	0.62
<i>chico¹</i>	females	0	55.3 ± 1.7		
<i>chico¹</i>	females	25	61.1 ± 1.6	9	< 0.01

Values are means ± sem. Units are days. The genotype of *chico¹* flies are *w¹¹¹⁸, chico¹/chico¹*. P-values were calculated by Mann-Whitney analysis, control vs. cinnamon.

Table 2

Protective effect of cinnamon against cold

	Control	Cinnamon-fed
Males alive	89	104
Males dead	24	3
Females alive	77	105
Females dead	35	13

$P < 0.0001$ for males, $P = 0.0002$ for females, Fisher's exact test.

Table 3

Mitochondrial respiratory parameters in Cinnamon-fed and control flies.

Diet	State 3	State 4	RCR	uncoupled
Control	162 ± 11	32 ± 2	5.1 ± 0.3	284 ± 22
Cinnamon	131 ± 12	31 ± 5	4.6 ± 0.4	235 ± 29
<i>P</i> -values	0.07	0.77	0.28	0.19

Rates are mean ± sem. Units are nmol O₂/min/mg protein except for RCR, which is a ratio of state 3/state 4. *P*-values were calculated by Student's *t* test, *n* = 10.