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A survey of the protective effects of some commercially available antioxidant supplements in genetically and chemically induced models of oxidative stress in *Drosophila melanogaster*

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ABSTRACT

Oxidative stress remains one of the most well studied, albeit somewhat contentious, causes of age-related changes in humans. Consequently, a large number of putative antioxidant compounds are freely available in myriad formulations that are often not tested for their efficacy or regulated for quality control. Following the development of a *Drosophila* model of oxidative-stress dependent aging (p38 MAP K (p38K) mutants) in our laboratory, we attempted to test the protective effect of some of these commonly available formulations against oxidative stress, in the p38K model. As environmental exposure to oxidizing toxins has been linked to a variety of human diseases, we also tested the efficacy of these supplements on chemically-induced models of oxidative stress (paraquat and hydrogen peroxide exposure). Our results suggest that when added as a dietary supplement, some of these over-the-counter compounds, notably containing açai extracts, confer significant protection for both the p38K-dependent genetic model as well as the toxin-induced model. These products were also remarkably effective at dampening stress-induced expression of the detoxifying enzyme GSTD1 and eliminating paraquat induced circadian rhythm deficits. Overall, our results suggest potential benefits of dietary supplementation with some of these compounds, especially under conditions of elevated oxidative stress. These findings should be assessed in the context of other studies that seek to identify active principles in these extracts, determine their effective dosage for human consumption and evaluate the safety of long-term prophylactic applications.

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1. Introduction

Oxidative stress has been implicated in a number of diseases including Alzheimer's disease, cancer and retinitis pigmentosa (Yan et al., 1996; Benz and Yau, 2008; Tuson et al., 2009; Clark et al., 2010). Though how oxidative stress relates to these disorders is not always fully understood, it is thought that oxidative stress results in increasing cellular damage and eventual cell death if not properly managed. Furthermore, animals either exposed to oxidizing agents,

such as the paraquat and rotenone models of Parkinson's disease, or harboring mutations in oxidative stress response genes generally have a shortened life span and progressive locomotor behavior defects (Betarbet et al., 2000; Melov et al., 2001; Blander et al., 2003; Holzenberger et al., 2003; Kieran et al., 2004; Brys et al., 2007; Chaudhuri et al., 2007; Paul et al., 2007; Bernard et al., 2011; Vrailas-Mortimer et al., 2011). Therefore, a great deal of interest has been generated in the effectiveness of antioxidants to treat oxidative stress related diseases. For instance, melatonin (Cardinali et al., 2010), diets rich in polyphenols (Morillas-Ruiz et al., 2010), vitamin E (Sano et al., 1997) and vitamin C (Engelhart et al., 2002; Morris et al., 2002) have all been shown to have ameliorating effects for Alzheimer's disease patients, whereas, the antioxidants derived from *Ginkgo biloba* (DeKosky et al., 2008) and curcumin (Baum et al., 2008) were not found to be as effective. Similarly, the relationship between antioxidant supplements and cancer has also been largely inconclusive with a few trials indicating minor protective effects (Goodman et al., 2011). In

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order to utilize the full potential of the multitude of antioxidant supplements available, a greater understanding of the efficacy, safety and mode of action of these compounds is necessary. Given the large number of compounds with potential antioxidant properties (as measured by chemical assays), rapid testing in animal models has emerged as a viable screening platform.

The fruit fly *Drosophila melanogaster* has proven to be a rapid and powerful genetic platform for assessing the properties of various antioxidant supplements. For example, dietary supplementation of vitamin C (ascorbic acid, Bahadorani et al., 2008), and apple phenols (Peng et al., 2011) have been shown to extend adult life span in wild type flies. Interestingly, other dietary constituents may also play an important role in life span extension as resveratrol is able to extend life span in wild type animals on a rich diet (Bauer et al., 2004; Wood et al., 2004), but not when the amount of sugar and yeast is reduced (Wood et al., 2004). Similarly, dietary supplementation of resveratrol during larval developmental rescues short-lived *Drosophila* lines (Chandrashekar and Shakarad, 2011), suggesting that resveratrol may only extend life span for specific genetic backgrounds. Further evidence for the role of the base diet is that açai pulp extends life span in wild type as well as oxidatively stressed SOD1 mutant flies only on a high fat diet (Sun et al., 2010).

Studies using *Drosophila* may further reveal how different antioxidants have protective effects against specific sources of oxidative stress. For example, vitamin E is protective against chemically induced lipid peroxidation (Miquel et al., 1982) but is ineffective against exposure to the oxidizing agent paraquat (Driver and Georgeou, 2003), whereas a variety of other antioxidant compounds including vitamin C (ascorbic acid) and melatonin are effective treatments against paraquat toxicity (Bonilla et al., 2006). Genetic models of oxidative stress have also demonstrated that particular antioxidants can extend life span and reduce reactive oxygen species (ROS) in specific genetic backgrounds. As an example, loss of the detoxifying enzyme SOD1 (Cu/Zn SOD) results in a shortened life span, which is ameliorated by dietary supplementation with either vitamin E (α -tocopherol) or vitamin A (retinol) dietary supplementation (Bahadorani et al., 2008). Additionally, supplementation with a resveratrol complex was able to rescue the locomotor behavior defects and reduced life span of the alpha-synuclein induced *Drosophila* Parkinson's disease model (Long et al., 2009), but not paraquat induced models of PD (Bagatini et al., 2011).

Although antioxidants have become increasingly popular in commercial health and beauty products, the effectiveness of these compounds is often not assessed *in vivo*. In an effort to test the efficacy of some of these commercially available antioxidant compounds in an intact animal, we have utilized *Drosophila* models of both genetically- and chemically-induced oxidative stress in the current study. We recently reported a new *Drosophila* model of oxidative stress and aging, in which reduced levels of p38 MAP Kinase result in a reduced lifespan, age-dependent locomotor behavior defects, and increase sensitivity to the oxidizing agents hydrogen peroxide and paraquat. Furthermore, p38K mutants also have elevated levels of endogenous oxidative stress and a reduction in the expression of the antioxidant enzyme SOD2 (MnSOD). In addition, over-expression of p38K specifically in the muscle extends lifespan and increases resistance to oxidizing agents. We have found that p38K functions in the muscle to regulate the expression of SOD2 through the transcription factor Mef2. Overexpressing SOD2 in the p38K mutant background rescues the mutant lifespan defect, suggesting that oxidative stress contributes to p38K aging phenotypes (Vrailas-Mortimer et al., 2011). Thus the p38K model is an ideal system in which to test the efficacy of antioxidant supplements against genetically induced oxidative stress. In addition to the p38K model, we also tested two chemical exposure models of oxidative stress, exposure to hydrogen peroxide and paraquat to determine the protective effect of commercially available antioxidant supplements containing açai and/or resveratrol against oxidative stress.

2. Methods

2.1. *Drosophila* strains, husbandry and genetics

Oregon R was used as the wild type control strain. p38b^{Δ45} (Vrailas-Mortimer et al., 2011), GST-GFP (gift of D. Bohmann) and GST-GFP, p38b^{Δ45} (Vrailas-Mortimer et al., 2011) have been generated previously. Flies were raised at 25 °C on standard media until eclosion.

2.2. Media preparation

Oregon R or p38b^{Δ45} virgin females were raised on standard media (yeast (3.2%, Lynside Lesaffre)), cornmeal (8%, LabScientific), agar (2.6%, Genesee Scientific), molasses (8%, Golden Barrel), propionic acid (0.6%, Acros Organics) and Tegosept (1.6%, 10% in ethanol, Apex Bioresearch Products), aged 1 day and then starved for 6 1/2 h before being transferred to test food sources to remove standard media from the digestive tract. Sucrose food consisted of 1% sucrose (Fisher Scientific), 1.3% agarose alone or supplemented with one of the following supplements: super fruits (açai, goji, pomegranate, mangosteen, noni, vegetable cellulose, microcrystalline cellulose, natural colors, stearic acid, magnesium stearate, silica, and purified water, Nature's Plus), açai (açai, microcrystalline cellulose, stearic acid, magnesium stearate, silica, Activessence (cellulose, pectinase, hemicellulose and xylanase), natural color, vegetable cellulose, and purified water, Nature's Plus), resveratrol (resveratrol, cellulose, vegetable cellulose capsule, silica, maltodextrin, and magnesium stearate, Solray), ginger (ginger, silica, di-calcium phosphate, vegetable cellulose, and purified water, Nature's Plus) and açai and resveratrol (açai, resveratrol, modified cellulose gum, silica, vegetable stearate, and hydroxypropyl methylcellulose, Michael's Naturopathic Programs). Complex food consisted of dextrose (5.2%, Fisher Scientific), sucrose (2.6%, Sigma), yeast (1.5%, Lynside Lesaffre), cornmeal (8.58%, LabScientific), agar (0.465%, Genesee Scientific) and Tegosept (1.6%, 10% in ethanol, Apex Bioresearch Products). Concentrations for all supplements were equalized to the amount of açai (50 mg/ml, 25 mg/ml, or 12.5 mg/ml) or resveratrol (14.3 mg/ml, 7.18 mg/ml or 3.59 mg/ml). Ginger was used at the concentrations of 50 mg/ml, 25 mg/ml, or 12.5 mg/ml to control for nutritional content. As far as possible, we tried to account for variation by using the formulations that belonged to the same lot number from the same company. Survival was assessed daily. Flies were transferred to fresh food every 2–3 days.

2.3. Hydrogen peroxide treatment

Oregon R flies were raised on standard media. Virgin females were aged 1 day and then starved for 6 1/2 h before being transferred to sucrose food and 1% H₂O₂ (mixed in the food, Fisher Scientific) alone or with one of the following supplements: super fruits (Nature's Plus), açai (Nature's Plus), resveratrol (Solray), ginger (Nature's Plus) and açai and resveratrol (Nature's Plus). Concentrations for all supplements were equalized to the amount of açai (50 mg/ml, 25 mg/ml, or 12.5 mg/ml) or resveratrol (14.3 mg/ml, 7.18 mg/ml or 3.59 mg/ml). Ginger was used at the concentrations of 50 mg/ml, 25 mg/ml, or 12.5 mg/ml to control for nutritional content. Survival was assessed daily. Flies were transferred to fresh food every 2–3 days.

For pre-treatment experiments virgin Oregon R flies were aged 1 day and starved for 6 1/2 h before being transferred to sucrose food that contained 1% H₂O₂ mixed within the food for 24 h. Flies were then transferred to sucrose food alone or supplemented with either super fruits (50 mg/ml açai) or açai (50 mg/ml). Flies were transferred to fresh food every 2–3 days.

2.4. Paraquat treatment

Oregon R flies were raised on standard media. Virgin females were aged 1 day and starved for 6 1/2 h before being transferred to sucrose

food mixed with 3 mM, 5 mM or 10 mM paraquat (Sigma) with either super fruits (50 mg/ml) or açai (50 mg/ml). Alternatively, virgin females or males were aged 1 day and starved for 6 1/2 h before being transferred to complex food mixed with 5 mM paraquat with either super fruits (50 mg/ml) or açai (50 mg/ml). Flies were transferred to fresh food every 2–3 days.

2.5. Western blot analysis

GST–GFP, *p38b*^{Δ45} virgin females were exposed to sucrose, super fruits (50 mg/ml) or açai (50 mg/ml) for 3 days. Three heads and thoraces were homogenized in 1 × laemmli buffer. 5 μl of each sample was loaded on an SDS–PAGE gel. For paraquat exposure, GST–GFP flies were exposed to sucrose, super fruits (50 mg/ml) or açai (50 mg/ml) with and without 5 mM paraquat. Flies were exposed for 2 days. Three heads and thoraces were homogenized in 1 × laemmli buffer. 5 μl of each sample was loaded on an SDS–PAGE gel. Antibodies: rabbit anti–GFP 1:1000 (Molecular Probes), mouse anti–actin 1:50,000 (Millipore), goat–anti–rabbit–HRP (Jackson Labs), and goat–anti–mouse–HRP (Jackson Labs).

2.6. *Drosophila* activity measurements

Oregon R virgin females were aged 1 day, starved 6 1/2 h and then exposed to Sucrose, super fruits (50 mg/ml) or açai (50 mg/ml) with and without 5 mM paraquat in the *Drosophila* Activity Monitor (TriKinetics, Inc.) for a 12:12 h light:dark cycle for 6 days. Food was changed after 6 days. To determine if longer exposure may affect circadian rhythm further, Oregon R virgin females were aged 1 day, starved 6 1/2 h and then exposed to sucrose, super fruits (50 mg/ml) or açai (50 mg/ml) with and without 5 mM paraquat in standard vials for 6 days. The animals were then placed in the *Drosophila* Activity Monitor for 6 days on the same paraquat containing food. The number of animals tested is as follows young sucrose, 30; young sucrose plus paraquat, 24; young super fruits, 32; young super fruits plus paraquat, 32; young açai, 29; young açai plus paraquat, 26; aged super fruits, 40; aged super fruits plus paraquat, 36; aged açai, 31; and aged açai plus paraquat, 36. Locomotor behavior was analyzed using DAMFileScan102 × in 30 minute bins. Actograms were generated using the ImageJ (rsbweb.nih.gov/ij/) plugin ActogramJ (actogramj.neurofly.de).

2.7. Statistical analysis of *Drosophila* Activity Monitor data

Data acquired from the *Drosophila* Activity Monitor were analyzed for the length of the period and strength of rhythmicity. Maximum entropy spectral analysis (Levine et al., 2002; Dowse, 2009) was employed to determine the length of the period (Tau). The presence of significant rhythmicity was determined by autocorrelation analysis (Levine et al., 2002; Dowse, 2009) taking multiple peaks that exceed the 95% confidence interval that match the spectral peak as evidence of a robust rhythm. The strength of the rhythm is assessed by further interpretation of the autocorrelogram. The height of the third peak, counting the one at lag zero as #1 is the rhythmicity index. Since many of the fly rhythms were bimodal, we looked only at the peaks occurring at approximately 24-h intervals to establish RI. This was not possible in the case of the flies consuming sucrose with 5 mM paraquat. By these criteria, only 2 animals would have had significant rhythmicity. To make comparisons possible, we relaxed the criterion demanding a peak in the 48-h range and took RIs from the third peak wherever it fell for this group. This is justified, as it allows flies with clear rhythmicity to be scored even if they do not live long enough to generate RIs by the more stringent criterion. Tau and RI were compared among groups using nonparametric analysis (Kruskal–Wallis χ^2 , R statistics package).

3. Results

3.1. Açai containing formulations extend life span in *p38* MAP Kinase mutants on minimal food

Adult *p38Kb* null animals were grown on controlled sucrose minimal food alone or supplemented with either açai, resveratrol, açai + resveratrol or super fruits (a combination advertised to contain açai, goji, noni, pomegranate, and mangosteen, without resveratrol; SF). Ginger was used as a reasonable control for “inert” compounds that might otherwise impact the life span of *p38Kb* mutant flies. Compounds that contained açai were administered such that the concentration of açai was maintained uniformly across vials (see Section 2.2). Our results clearly indicated a beneficial effect of adding açai to the food (Fig. 1) as either SF or açai alone increased life span (average 26.5 and 26.8 days respectively, as compared to 8 days for sucrose). Resveratrol alone resulted in a mild extension of life span (average life span 13 days), whereas a combination of açai and resveratrol conferred significant life span extension (average life span of 16 days). As predicted, ginger did not prove beneficial for *p38Kb* mutant animals (average life span of 6 days). Overall, in spite of the potential for non-standardized formulations in these commercial products, our results seemed to suggest a clear beneficial effect of dietary açai supplementation. In fact, it is possible that açai, in combination with other fruit extracts might provide added benefits as seen from the effect of SF on life span extension (Fig. 1). We further tested different doses of these compounds and found that all concentrations of açai containing compounds were protective (Table 1).

3.2. “Super fruits” and açai can protect against hydrogen peroxide induced oxidative stress

Açai and SF can alleviate reduced life span phenotypes due to a genetic source of oxidative insult (*p38K* mutants). To further confirm that this protection is due to the ability of these compounds to antagonize oxidative stress, we directly tested açai and SF for their protective potential against hydrogen peroxide insult. These experiments would more directly document an oxidative stress-related role for açai and SF since it is formally possible that life span rescue observed in *p38Kb* mutants could be due to factors other than oxidative stress. When adult wild type flies are treated with 1% hydrogen peroxide concurrently with or without antioxidants in sucrose food they display a mean life span of 4 days. As shown in Fig. 2A, both açai and SF significantly extend the life span of animals that are challenged

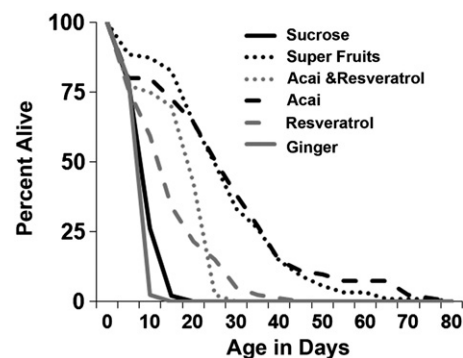


Fig. 1. Açai containing compounds extend life span of *p38Kb* mutants. *p38Kb* mutant animals were continuously reared on a variety of antioxidant formulations. All compounds are normalized for either açai (50 mg/ml of food) or resveratrol (14.3 mg/ml of food) concentration. The non-antioxidant compound ginger was used as control and had no effect on life span.

Table 1Life span parameters when *p38Kb* mutant flies are reared on different concentrations of putative antioxidant supplements and control compounds.

Compound	Average lifespan in days	Median lifespan in days	Oldest living fly	Logrank test	n
Sucrose	8.21	8	19	N/A	145
Super fruits high^a	26.51	25	76	146.5453*	87
Super fruits medium ^b	23.41	25.5	62	43.2002*	34
Super fruits low ^c	26.40	27	53	83.4848*	29
Açai and resveratrol high^{a,d}	16.30	20	28	63.42*	40
Açai and resveratrol medium ^{b,e}	20.24	23	51	69.101*	39
Açai and resveratrol low ^{c,f}	20.17	20	61	66.6204*	38
Açai high^a	26.83	26	78	111.1801*	47
Açai medium ^c	20.90	26	40	54.2644*	40
Açai low ^b	17.22	18	41	49.234*	41
Resveratrol high ^d	14.32	14.5	44	54.2897*	77
Resveratrol medium^e	13.35	13	43	39.254*	79
Resveratrol low ^f	9.00	9.5	17	3.0658	30
Ginger high	6.03	7	11	27.5854*	39
Ginger medium	8.66	9	19	0.0964	38
Ginger low	7.47	7	15	2.9005	36

Bold in Table 1 indicates data represented in Fig. 1.

* Denotes *p* value < 0.001.^a 50 mg/ml of açai.^b 25 mg/ml of açai.^c 12.5 mg/ml.^d 4.3 mg/ml resveratrol.^e 7.18 mg/ml resveratrol.^f 3.59 mg/ml resveratrol.

with peroxide (6 days and 12 days respectively, Table 2). This result is consistent with the idea that açai and SF can protect against oxidative stress. To test whether açai and SF can confer protection to animals that have already undergone insult from oxidative stress, we pretreated wild type animals with hydrogen peroxide in the absence

of supplements. Flies were exposed to 1% hydrogen peroxide for 24 h and were then transferred to food containing sucrose alone, açai or SF. As compared to control sucrose-only food, both açai and SF were capable of ameliorating the negative impact of hydrogen peroxide on life span (Fig. 2B, Table 3). Next, we tested whether pre-feeding with açai and SF could preemptively protect against oxidative stress. We reared wild type flies on food containing açai or SF for 24 h, followed by transfer to food containing 1% hydrogen peroxide. In this case, we observed no significant rescue of life span suggesting that the beneficial effects of these supplements do not last much beyond the feeding period (data not shown). Taken together, results described in this section support a positive role for açai and SF in combating the effects of oxidative stress and also suggest a temporal contingency between the intake of these supplements and stress.

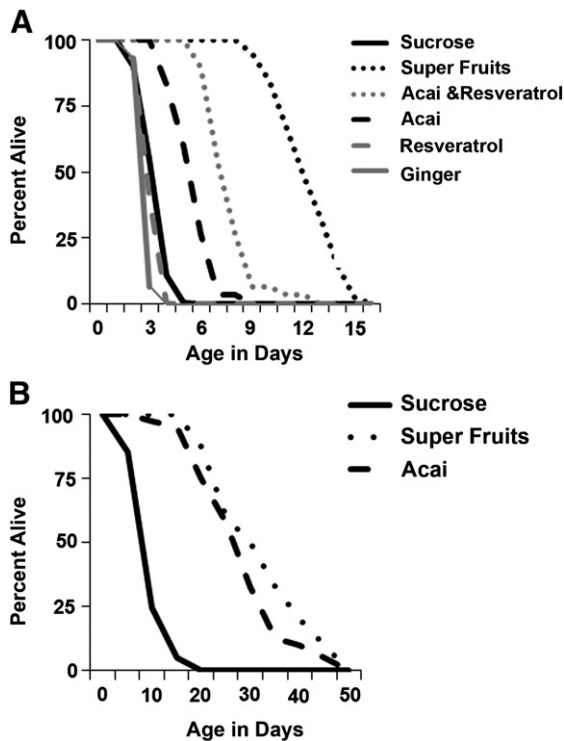


Fig. 2. Açai containing antioxidant compounds are protective against hydrogen peroxide. Wild type flies were exposed to hydrogen peroxide (A) concurrently with antioxidant supplements or (B) pre-exposed to hydrogen peroxide followed by antioxidant feeding. Life spans were measured and plotted to evaluate the effect of hydrogen peroxide and antioxidant compounds.

3.3. “Super fruits” and açai in minimal media protect against paraquat induced stress in wild type *Drosophila*

Once we had confirmed that açai and SF confer life span extension to hydrogen peroxide treatment, we were curious to see if these compounds could protect against paraquat induced stress, which results in an excess of superoxide radicals. Paraquat is a widely used herbicide and the toxicity of this environmental xenobiotic has been demonstrated in many studies (Bismuth et al., 1990; Suntres, 2002) including several in which paraquat has been linked to the loss of nigral dopaminergic neurons in the mammalian brain and therefore, to the incidence of sporadic Parkinson's disease (Thiruchelvam et al., 2000; Dinis-Oliveira et al., 2006; Berry et al., 2010; Cannon and Greenamyre, 2010; Nistico et al., 2011; Jackson-Lewis et al., 2012). Wild type flies were treated with 3 mM, 5 mM or 10 mM paraquat together with sucrose, açai, or SF (Table 4). Açai and SF were both protective against paraquat induced oxidative stress at all concentrations. Feeding 5 mM paraquat reduced the mean life span to 4 days as compared to control animals that have a mean life span of 8 days. If açai and SF protect against paraquat, we should observe significant rescue of this life span reduction. As shown in Fig. 3A and D, both açai and SF showed dramatic protection against paraquat. When 50% of the animals were dead following paraquat treatment, 94% of the animals were alive with açai and 100% with SF. These

Table 2
Life span measurements for wild type flies in response to hydrogen peroxide exposure concurrent with different concentrations of antioxidant supplements.

Compound	Average lifespan in days	Median lifespan in days	Oldest living fly	Logrank test	n
Sucrose + hydrogen peroxide	3.56	4	6	N/A	167
Super fruits high^a + hydrogen peroxide	12.46	12	16	134.8432*	40
Super fruits medium ^b + hydrogen peroxide	10.95	11	15	130.2839*	38
Super fruits low ^c + hydrogen peroxide	9.08	9	15	136.5059*	41
Açai and resveratrol high^{a,d} + hydrogen peroxide	3.61	4	5	0.2039	31
Açai and resveratrol medium ^{b,e} + hydrogen peroxide	7.90	8	13	112.4486*	31
Açai and resveratrol low ^{c,f} + hydrogen peroxide	5.73	6	8	70.3814*	31
Açai high^a + hydrogen peroxide	5.77	6	9	74.6008*	31
Açai medium ^b + hydrogen peroxide	3.87	4	5	2.5537	31
Açai low ^c + hydrogen peroxide	3.57	3.5	5	0.0836	29
Resveratrol high ^d + hydrogen peroxide	3.33	3	4	3.8174	40
Resveratrol medium^e + hydrogen peroxide	3.26	3	4	6.0690*	39
Resveratrol low ^f + hydrogen peroxide	3.29	3	4	4.7020*	39
Ginger high + hydrogen peroxide	3.00	3	4	17.7847*	30
Ginger medium + hydrogen peroxide	3.53	4	4	0.3995	31
Ginger low + hydrogen peroxide	2.87	3	3	25.9502*	31

Bold in Table 2 indicates data represented in Fig. 2.

* Denotes *p* value <0.05.

^a 50 mg/ml of açai.

^b 25 mg/ml of açai.

^c 12.5 mg/ml.

^d 14.3 mg/ml resveratrol.

^e 7.18 mg/ml resveratrol.

^f 3.59 mg/ml resveratrol.

Table 3
Life span measurements for wild type flies in response to hydrogen peroxide pretreatment followed by exposure to 50 mg/ml of açai per antioxidant supplements.

Compound	Average lifespan in days	Median lifespan in days	Oldest living fly	Logrank test	n
Sucrose	8.81	9	18	N/A	81
Super fruits	30.88	30.5	48	108.7226*	40
Açai	27.40	27	50	99.3480*	40

* Denotes *p* value <0.0001.

results, together with life span extension seen in *p38Kb* mutants, strongly suggest that açai and SF can neutralize oxidative stress in *Drosophila* resulting in a significant improvement in life span.

3.4. Açai containing formulations in a complete media protect against paraquat induced oxidative stress in wild type *Drosophila*

As açai and SF increased survival in response to paraquat in the simple sucrose media, we next tested if these supplements are also effective in a nutritionally complete media (complex food). Animals were reared on a standard cornmeal–molasses media and then transferred to the better defined and less variable complex food described

in the Methods section alone or supplemented with either açai or SF. Concurrent with antioxidant treatment, animals were exposed to 5 mM paraquat. As observed with the simple sucrose media, both açai and SF supplementation in complex food were protective against paraquat induced oxidative stress (Fig. 3B, C and E, F). We and others have previously described that males and females respond to paraquat toxicity differently, with males having increased sensitivity (Chaudhuri et al., 2007; Vrailas-Mortimer et al., 2011). Therefore, we tested if treatment with antioxidants differentially affects males and females in regard to paraquat sensitivity. Açai and SF were equally protective in females with an average lifespan of 17 and 15 days, respectively, compared to 7 days for complex food alone (Fig. 3C and F, Table 5). Strikingly in males, SF is significantly more protective than açai against paraquat with SF treated animals surviving for 22 days as compared to 11 days for açai and 3 days for complex food alone (Fig. 3B and E, Table 5).

Furthermore, sex differences were also observed when animals were fed a complex diet supplemented with antioxidants in the absence of any oxidizing agents. In wild type males, açai extends lifespan from 21 days for complex food alone to 40 days (Table 5), whereas, SF has a negligible effect on lifespan. Though açai also extends lifespan in wild type females, this effect is not as pronounced as that seen in males (30 days for complex food versus 34 days for

Table 4
Life span measurements for wild type flies in response to paraquat treatment in sucrose food with simultaneous exposure to different concentrations of antioxidant supplements.

Compound	Average lifespan in days	Median lifespan in days	Oldest living fly	Logrank test	n
Sucrose 0 mM paraquat	7.80	7	18	N/A	172
Sucrose 3 mM paraquat	2.73	2	7	203.1465*	102
Sucrose 5 mM paraquat	3.55	3	7	162.8590*	107
Sucrose 10 mM paraquat	2.73	2	7	203.1465*	102
Super fruits 0 mM paraquat	32.08	31	98	N/A	182
Super fruits 3 mM paraquat	24.98	23	35	20.7336*	57
Super fruits 5 mM paraquat	17.88	18	33	116.1874*	136
Super fruits 10 mM paraquat	19.08	22	30	53.9028*	59
Açai 0 mM paraquat	39.61	35	115	N/A	166
Açai 3 mM paraquat	11.20	12	16	244.1654*	40
Açai 5 mM paraquat	9.13	9	15	326.5166*	107
Açai 10 mM paraquat	4.23	5	9	274.1095*	39

Bold in Table 4 indicates data represented in Fig. 4.

* Denotes *p* value <0.0001.

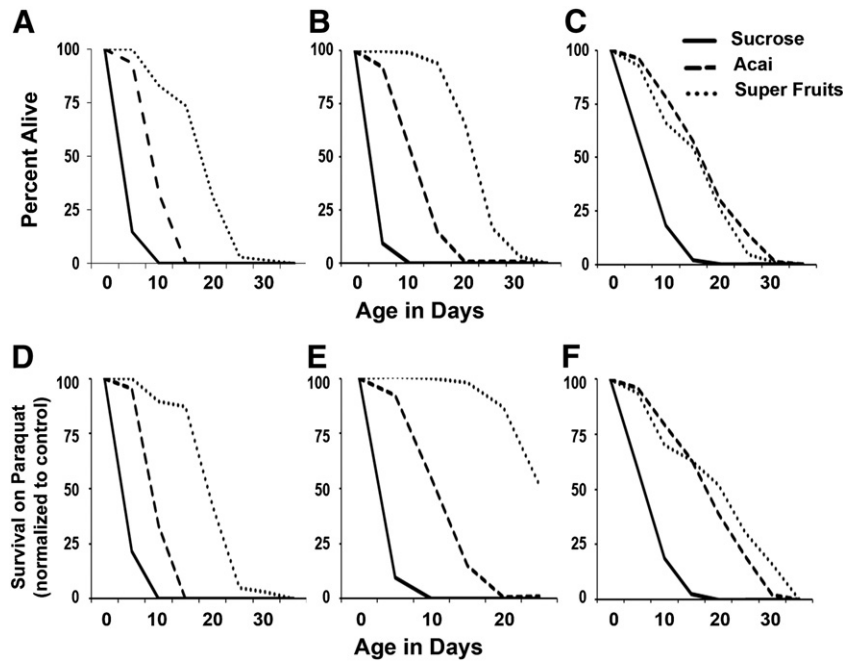


Fig. 3. Paraquat toxicity is ameliorated by açai containing antioxidant formulas. Wild type animals were exposed to 5 mM paraquat concurrently with antioxidant supplements in minimal media (A, D) or in complex media with females (C, F) or males (B and E). Super fruits has a greater protective effect than açai alone when comparing animals treated with 5 mM paraquat (A–C) or when treated animals were normalized to untreated controls (D–F).

açai, Table 5). Interestingly, resveratrol also extends the lifespan of wild type females to 37 days even though this compound had little protective effect against oxidative stress.

3.5. “Super fruits” and açai lower oxidative stress induced expression of GST

Another useful measure of oxidative stress relies on quantifying the native antioxidant response *in vivo*. To this end, transgenic flies have been generated in the Bohmann laboratory that reports transcription of the GSTD1 enzyme using a promoter fusion technique (Sykiotis and Bohmann, 2008; Vrailas-Mortimer et al., 2011). The GST–GFP transgenic carries antioxidant response elements (AREs) from the GSTD1 promoter placed upstream of the coding region for GFP. Following oxidative stress, transcription is activated at the AREs leading to increased GFP expression. Our previous results have shown increased GFP expression in *p38Kb* mutants as well as in paraquat treated wild type animals (Vrailas-Mortimer et al., 2011).

Therefore, we used this system to evaluate the effect of açai and SF on oxidative stress. Both açai and SF effectively reduce basal GFP expression from the GSTD1 based reporter in a *p38Kb* mutant background (Fig. 4A and B). However, only SF and not açai, significantly reduce GFP expression in response to 5 mM paraquat (Fig. 4C and D). While these results molecularly confirm the antioxidant potential of açai and SF, they also reveal the limitations of açai in these paradigms. It is interesting to note that consistent with this result, SF performed better than açai alone in all assays tested in this study. Given that we have calibrated dosage keeping açai concentrations constant, these results further suggest that other extracts present in SF might potentiate the beneficial effects of açai.

3.6. Paraquat induced destabilization of circadian rhythm is rescued by “Super fruits” and açai

The beneficial effects of açai and SF on life span and molecular markers of oxidative stress are encouraging. However, a simple

Table 5
Life span measurements for wild type flies in response to paraquat treatment in complex food with simultaneous exposure to antioxidant supplements.

Compound	Average lifespan in days	Median lifespan in days	Oldest living fly	Logrank test exposed	Logrank test unexposed	n
<i>Males</i>						
Complex	20.59	17	65	N/A	N/A	122
Complex 5 mM paraquat	3.49	3	9	284.9600**	N/A	126
Super fruits	23.03	24	30	N/A	8.2224*	123
Super fruits 5 mM paraquat	21.93	22	33	3.6444	N/A	122
Açai	39.87	35	77	N/A	103.9616**	126
Açai 5 mM paraquat	10.93	11	35	275.2173**	N/A	129
<i>Females</i>						
Complex	30.05	23	99	N/A	N/A	321
Complex 5 mM paraquat	6.81	6	19	490.7614**	N/A	160
Super fruits	20.48	20	36	N/A	64.6175**	250
Super fruits 5 mM paraquat	15.30	17	32	40.4173**	N/A	156
Açai	33.62	34	66	N/A	0.5414	140
Açai 5 mM paraquat	16.65	16	31	127.6946**	N/A	78
Resveratrol	37.43	40	75	N/A	6.3692*	136

** Denotes *p* value < .0001.
* Denotes *p* value < 0.01.

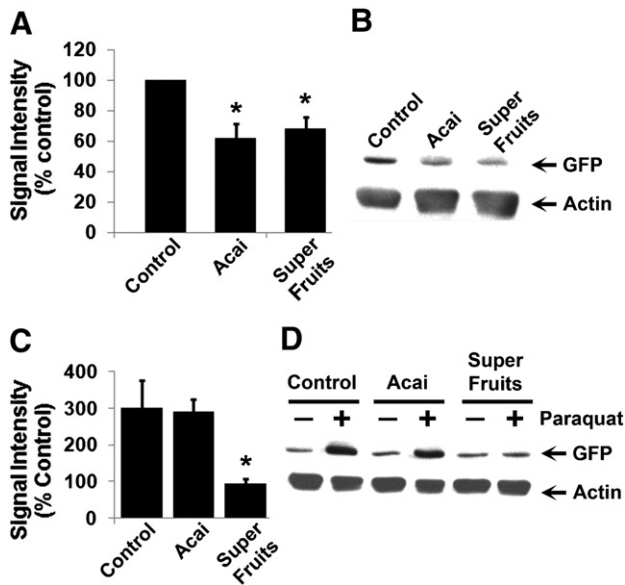


Fig. 4. Antioxidant supplementation reduces expression of oxidative stress response reporters. GFP is a readout of GST-D1 reporter expression in *p38Kb* mutant animals (A, B) and in wild type animals exposed to 5 mM paraquat (C, D). Antioxidant exposure with either açai or super fruits reduces reporter expression in *p38Kb* mutants whereas only super fruits was effective at reducing elevated reporter expression in response to paraquat exposure. Representative western blots from adult head and thorax protein lysates are shown in B and D. Quantitative measurements and statistical significance are depicted in A and C. Asterisks denote $p < 0.05$. Actin is used as a loading control.

extension of life span does not reveal any useful effect on normal physiological functioning or “health span.” To directly test if antioxidant supplementation improves health span, we turned to a previously

reported phenotype in flies that is the outcome of paraquat exposure. Flies, like most organisms, possess a robust light-entrained circadian rhythm that is best seen by following their locomotor activity over 24 h light:dark cycles (Rosbash and Hall, 1989). Upon chronic treatment with paraquat, this locomotor rhythm degenerates rapidly (Koh et al., 2006) with a reduction in both the amplitude and rhythmicity of movement until animals eventually die (Fig. 5 A and B). When flies are treated with paraquat in the presence of açai or SF, a remarkable improvement in their circadian rhythm is noticed in addition to the previously documented effect on life span (Fig. 5C–F). Autocorrelation analysis, which can be used to measure rhythmicity indices, confirms that the rescue of circadian rhythm is dramatic. While the normal ~24 hour rhythm is completely abolished by paraquat, açai and SF restore this 24 hour rhythm (Fig. 6 shows the maximum entropy spectra for the various experimental and control groups). Even the amplitude of locomotion during a circadian day is near normal. To further test the efficacy of açai and SF, we asked if these extracts are protective against prolonged exposure to paraquat. Indeed, animals treated with SF did not develop any defects in rhythm or amplitude over a longer period of exposure (Fig. 7). However, açai treatment was not as effective over the longer term as rhythm amplitude decreased with exposure time (Fig. 7). These results provide additional evidence in support of the notion that açai and SF not only reduce the deleterious effects of oxidative stress on life span, but also are quite effective at restoring normal physiological function in the presence of toxic levels of paraquat. Thus açai, particularly in combination with other fruit extracts in SF, is capable of normalizing both the life span and health span of *Drosophila* in the face of increased oxidative stress.

4. Discussion

Since the harmful consequences of high oxidative stress have deeply permeated the public psyche, a large number of antioxidant

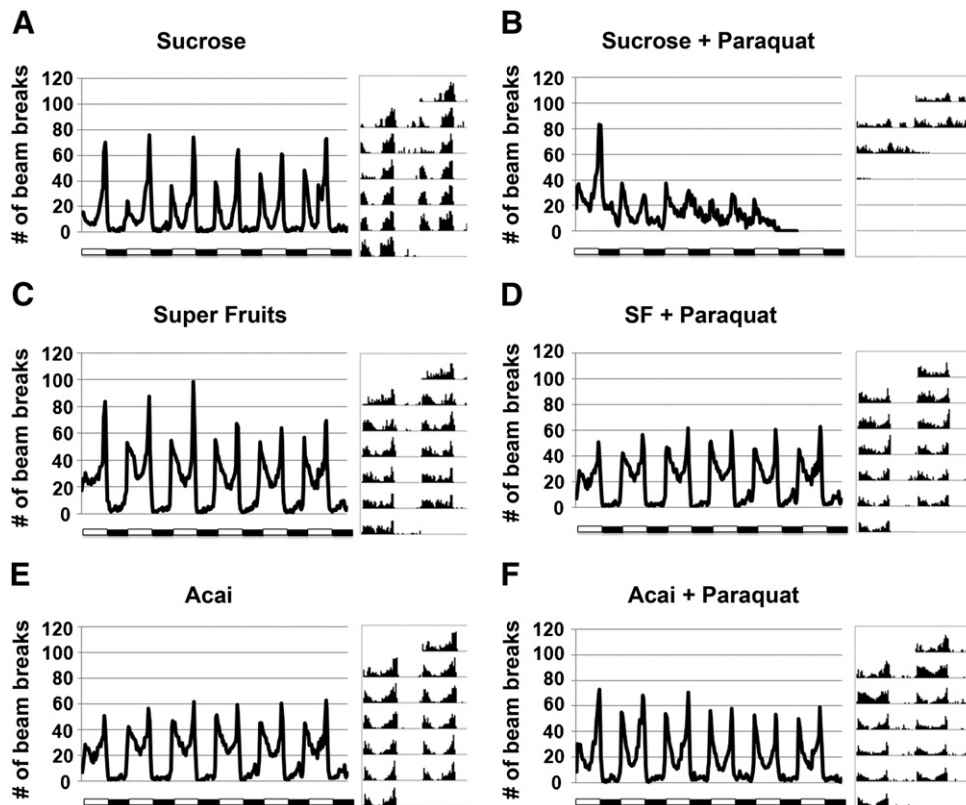


Fig. 5. Antioxidant supplements protect against deterioration of circadian rhythms in response to paraquat. (A, B) Cumulative activity of flies over a 6 day light:dark cycle when flies are reared on sucrose medium or exposed to 5 mM paraquat. Representative actograms next to each graph show the circadian periodicity in activity patterns. (C, D) Activity of flies reared on sucrose medium with “super fruits” supplement or exposed to both “super fruits” and 5 mM paraquat. (E, F) Activity of flies reared on sucrose medium with açai supplements or exposed to both açai and 5 mM paraquat.

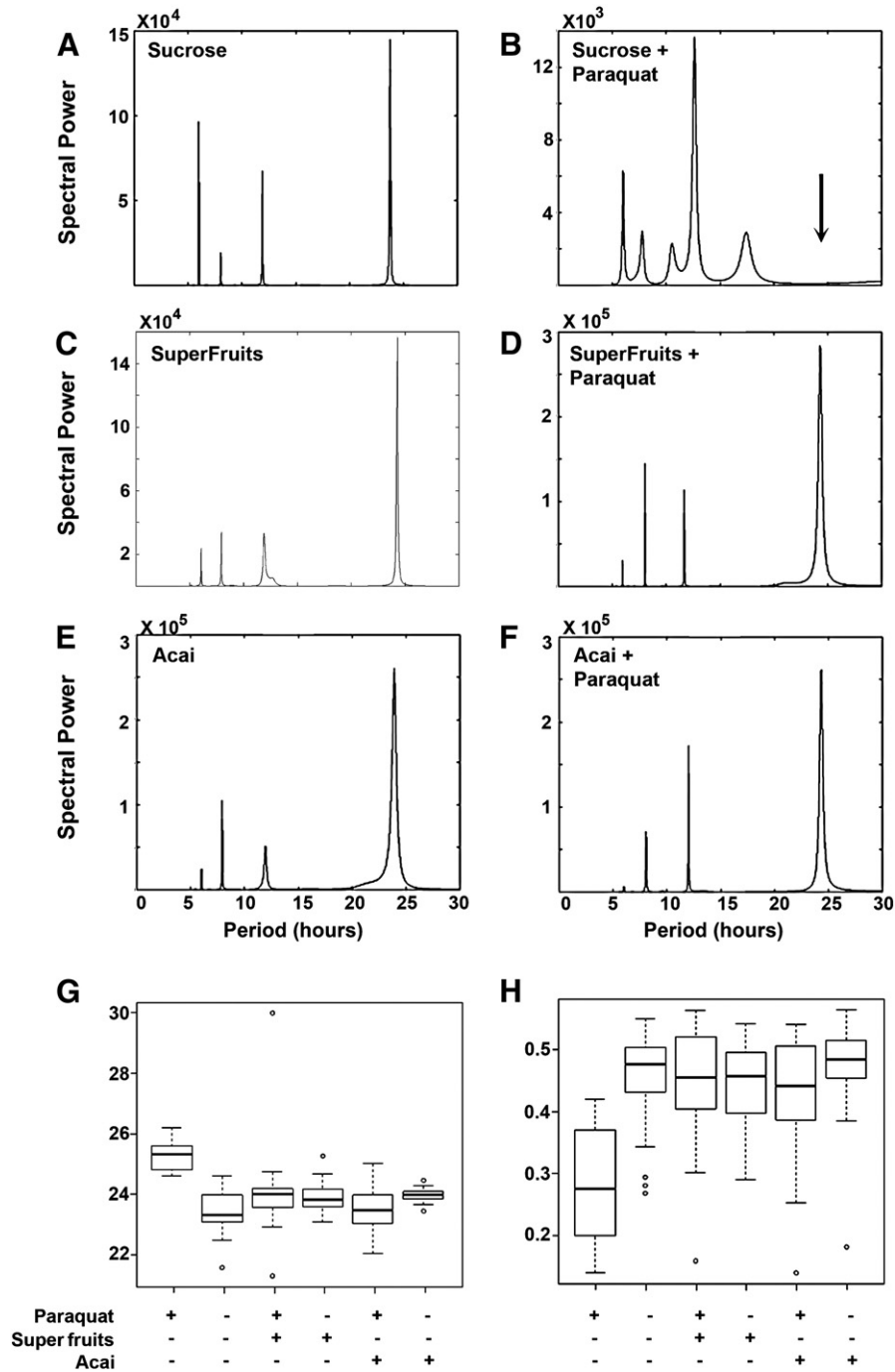


Fig. 6. Super fruits and açai restore normal circadian rhythm periodicity and rhythmicity in paraquat treated animals. (A–F) MESA spectra from typical records of fly activity in each test group. A: sucrose alone; B: sucrose with 5 mM paraquat. The spectrum from the control animal is simple with a major peak at about 24 h (Tau) with a rhythmicity index (RI) of 0.54. In B, the fly given 5 mM paraquat in sucrose has a spectrum with multiple peaks. Using the convention making comparisons of these very ill flies (assuming bimodality) the dominant peak at 12.6 h was doubled to yield 25.2. RI was only 0.32. C: flies fed super fruits had a clean spectrum with Tau = 24.3 h, RI = 0.52. D: flies given super fruits and 5 mM paraquat has a good clean spectrum with Tau = 24.3 h and RI = 0.42. E: flies given açai, Tau = 23.9 h and RI = 0.52. F: flies given açai and 5 mM paraquat has a clean spectrum with Tau = 24.3 h and RI = 0.50. (G) Boxplot of Tau for all results, dark lines are the medians, the boxes are \pm the first quartiles and the whiskers are \pm the largest and the smallest within the normal range. Small circles are outliers. Treatments are shown below. (H) Boxplot, as in G, for RI.

formulations have been presented by numerous “health food” companies as panacea. It is generally true that most of these compounds have antioxidant potential at least as measured by chemical assays that directly assess the redox properties of a test substance (Juranic and Zizak, 2005; Kaur and Geetha, 2006). However, their efficacy *in vivo* remains a matter of debate. Animal models have been widely used to test a number of antioxidant compounds, and though these studies have been relatively promising, clinical trials with antioxidant compounds have not always lived up to expectations (Schwedhelm

et al., 2003; Kamat et al., 2008; Klein, 2009; Wojcik et al., 2010; Floyd et al., 2011). One reason why antioxidants have performed poorly in clinical trials might have to do with the fact that these compounds may only be effective in a narrow temporal window in relation to oxidative damage. Once serious oxidative damage has occurred, further treatment with antioxidants may not be able to reverse that damage. Similarly, antioxidants delivered as prophylactic may be insufficient to combat subsequent oxidative stress. Indeed, our experiments suggest that adult fruit flies that are fed antioxidants

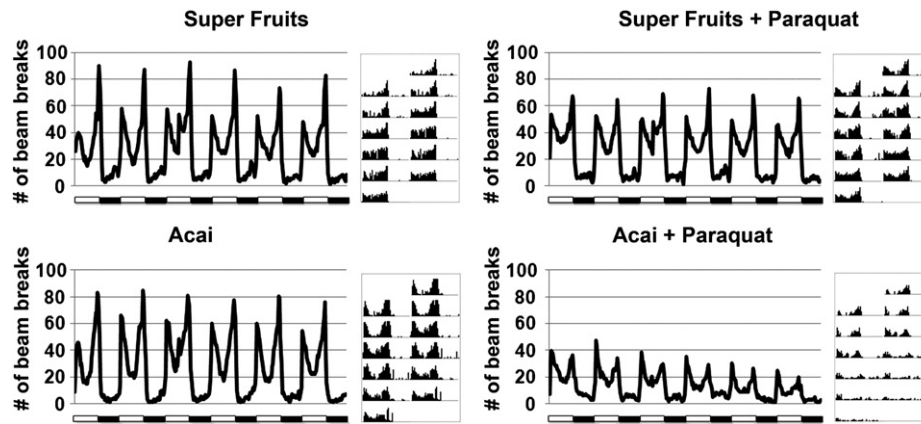


Fig. 7. Super fruits, but not açai, protects against prolonged paraquat exposure. Activity rhythms are shown from extended (2 week) recordings made from animals that are treated with 5 mM paraquat and are fed either açai or super fruits. The last 6 days of recording are shown. SF supplementation maintains a robust circadian rhythm while açai is incapable of alleviating the effects of paraquat treatment over this time period. Representative actograms are shown next to the cumulative activity profiles.

prior to oxidative damage do not perform better than control animals. To investigate these possibilities systematically and to continue to screen for effective antioxidant compounds, animal models still offer the most rapid and useful platform. *Drosophila* with its powerful genetic tools, short generation times and ease of experimental manipulation lends itself admirably to these demands.

The most widely available antioxidant formulations available to the general public are numerous non-FDA tested over-the-counter products sold in health food stores. We asked whether some of these products are actually effective at combating oxidative stress in a *Drosophila* model. To narrow down the myriad compounds available, we focused on açai, either by itself or in combination with other potential antioxidant extracts. As experimental models we chose either a p38 MAP Kinase mutant (shown previously by us to have a reduced lifespan and increased oxidative stress) or a more conventional paradigm in which wild type flies are challenged with either hydrogen peroxide or the herbicide paraquat, which results in a shortened lifespan (Reveillaud et al., 1992; Chaudhuri et al., 2007; Vrailas-Mortimer et al., 2011) and circadian rhythm defects (Koh et al., 2006). Overall, our results suggest that açai, in particular, is quite effective at prolonging life span in the face of oxidative challenge. Açai is similarly effective at normalizing GSTD1 expression and is protective against the deleterious effect of oxidative stress on circadian rhythm in wild type animals. We further noted that while resveratrol did not potentiate these beneficial effects of açai, one or more compounds present in a mixture (super fruits) containing açai, did augment açai-dependent benefits significantly in both simple and complex media. Furthermore, we observed that açai has differentially protective effects against oxidative stress in males and females. This sex difference may be due to the increased sensitivity of males to oxidative stress or possible differences in metabolic rates between males and females. Intriguingly, açai also had beneficial effects for wild type animals, particularly males, even in the absence of paraquat, suggesting that açai may either provide a protective effect against the endogenous mitochondrially produced oxidative stress in the body or is involved in some as yet unidentified process that promotes the health of the animal. Interestingly, super fruits, which often performs better than açai alone in promoting resistance to oxidative stress does not provide any beneficial effect to unexposed wild type animals, and is even detrimental for females. It may be that super fruits reduces endogenous levels of oxidative stress in untreated animals to a degree in which oxygen radical mediated cellular signaling is impaired. Another key observation is that both açai and SF are more effective against paraquat than hydrogen peroxide induced oxidative stress (compare 25 days for 3 mM paraquat and 12 days for 1% hydrogen peroxide in response to SF treatment).

As these oxidizing agents produce different forms of oxidative stress (O_2^- and H_2O_2 respectively), it is possible that the antioxidant properties of açai are more efficient at detoxifying superoxide radicals and hence the animals survive longer than when exposed to hydrogen peroxide. Thus, our data suggest that in multiple tests, these readily available compounds did in fact protect against the adverse effects of oxidative stress and improved both the life span and health span of these animals. This study also highlights the need to understand the mode of action of commercially available antioxidant supplements and how they interact in the body in stressful and non-stressful conditions.

In general, it seems that at least in the formulations tested, there are active principles present. Both ginger and resveratrol were ineffective. Thus, it is unlikely that inert compounds present in the mixture were responsible for the phenotypes observed. Given that one of the main goals of this study was to assess widely available supplements, we note that these relatively crude extracts might retain some of the advertised beneficial effects of açai (and potentially some other compounds). Additionally, dietary supplementation is sufficient for the observed benefits. It may be argued that antioxidants present in these mixtures neutralize oxidants such as hydrogen peroxide in the media, thus reducing the load outside the animal. However, we find that these antioxidants protect p38K mutants and also that they are able to protect animals when delivered immediately after hydrogen peroxide exposure. These results would suggest that their antioxidant activity occurs *in vivo*. Both basal and stress induced GSTD1 transcription, as measured by an anti-oxidant response element (ARE) based reporter transgene, are reduced by these antioxidants. Interestingly, this contrasts with earlier findings where açai feeding increased GSTD1 transcription (Sun et al., 2010). While we do not know the precise reason for this apparent discrepancy, we speculate that since animals in our experiments are in a state of increased oxidative stress, GSTD1 transcription is already augmented. Under these conditions, açai probably leads to a reduction in oxidative stress followed by a lowering of GSTD1 transcription. Whether açai directly promotes GSTD1 transcription remains unclear.

Antioxidants used in this study not only rescue life span deficits seen in mutants and oxidant treated animals, but they are also able to promote the maintenance of normal circadian locomotor patterns in the continued presence of paraquat. This observation is promising since it is often unclear (especially in invertebrate experiments) whether life span extension occurs together with an improvement in health span. Finally, it is important to reiterate that our antioxidant treatments have proved effective in a sensitized background. The effect of açai on normal lifespan has already been tested previously and these experiments suggest that açai is incapable of extending

the life span of wild type flies that are reared under normal laboratory conditions on classical media. However, when flies are reared on a high fat diet, açai is able to rescue the negative effect of high fat on overall lifespan (Sun et al., 2010). Consistent with these results, our data indicate that açai is protective under certain conditions of increased oxidative stress and might not offer any benefits under naïve conditions. The extent to which these findings translate to mammalian models and to humans, will likely form a topic for future studies. At the very least our study highlights the utility of multiple assays that include lifespan measurement, biochemical readouts of oxidative stress and age-dependent motor performance that can be easily combined when using *Drosophila* as a screening platform.

5. Conclusions

We have found that commercially available antioxidant supplements containing açai are protective against both genetically and chemically induced models of oxidative stress. Dietary supplementation with super fruits or açai was sufficient to rescue the lifespan defect observed in p38K mutants as well as in wild type animals treated with the oxidizing agents hydrogen peroxide or paraquat. Furthermore, both super fruits and açai also resulted in a reduction of GSTD1 reporter expression, suggesting that these supplements are able to reduce the oxidative stress generated in both p38K mutants and paraquat exposed animals. Finally, paraquat-induced circadian rhythm destabilization is also rescued by dietary supplementation of either super fruits or açai. Though the results reported here are not exhaustive analyses of all commercial products or meant to substitute for future analyses of purified principles from these plant sources, this study suggests that açai containing antioxidant supplements may be effective against multiple sources of oxidative stress.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper. AVM was supported by NIH T32 ES012870 training grants administered by the PD-CERC, Emory University. SS thanks funding from the Cell Biology Department and the University Research Council at Emory University.

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