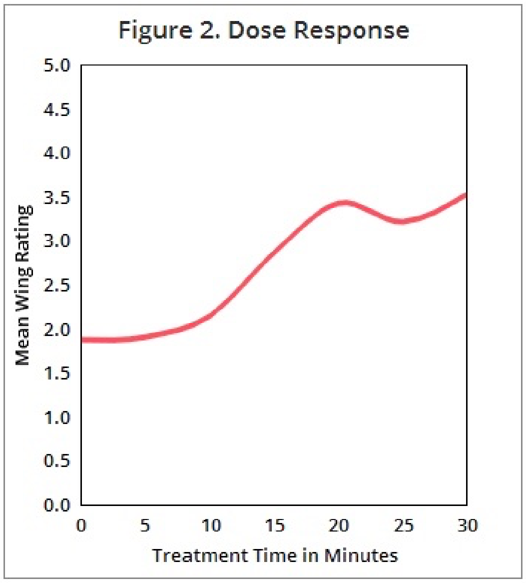

Figure 2. Dose Response

Table 1. Comparison of mean wing rating in dumpy-oblique mutants with Control (no treatment) and Heat Shock at 5 and 26.5 hours of pupal development. Values in parentheses are number of flies rated.

dumpy allele		Control (mwr)	5 Hour (mwr)	26.5 hour (mwr)
ov1	female	1.85 (255)	0.52 (126)	3.32 (244)
	male	1.90 (228)	0.42 (108)	3.55 (183)
ov7b	female	1.05 (64)	0.44 (34)	1.82 (66)
	male	0.60 (58)	0.10 (20)	1.52 (50)
ov56a	female	1.75 (149)	0.89 (35)	2.49 (126)
	male	1.45 (128)	0.60 (25)	2.06 (111)
ovA12	female	4.70 (146)	3.63 (52)	4.74 (77)
	male	4.74 (115)	3.67 (56)	4.46 (65)

(3) Comparative Response: Table 1 shows that all of the *dumpy-oblique* mutants tested have similar responses at both early (suppression) and late (enhancement) developmental ages as we have illustrated with dp^{ov1}. The exception to this conclusion being that 26.5 hour treatments with *dpovA12* do not show enhancement, but the phenotype of this mutant is so extreme, average mwr of 4.72, that it would be difficult to determine an enhancement effect with our method of scoring wing phenotype. This mutant does follow the pattern of suppression at 5 hours of development. Dose response studies at 5 hours of development with this extreme mutant follow the same pattern as seen with dp^{ov1} except that at high doses of heat shock, above 25 minutes, there is a reduction in viability.

This pattern of early suppression and later enhancement was also observed with the *crossveinless-like* studies of the 1960's (Thompson, 1967) with minor differences in timing. Whether this pattern applies to other wing mutations is unknown with the exception of an initial attempt to induce early suppression with *vestigial* which did not respond to 20 minute heat shock. It may be that moderate wing morphology mutations, for example *miniature*, and venation mutations such as *cubitus interruptus* and *abrupt* could be better candidates to respond to heat shock than the more extreme morphology changes seen with *vestigial*.

Acknowledgments: This research was funded by the Department of Molecular Biology and Genetics of Cornell University to RJM and the Department of Biology of Ithaca College to SRT.

References: Altenburg, E. and H.J. Muller 1920, Genetics 5: 1-59; Carmon, A., F. Topbas, M. Balon, and R.J. MacIntyre Fly 4.2: 117-127; Carmon, A., M.J. Guertin, O. Grushko, B. Marshall, and R. MacIntyre 2010, PLoS One 5: e12319; Thompson, S.R., 1967, Genetics 56: 13-22; Waddington, C.H., 1940, J Genet. 41: 75-139.

Evaluation of the neuroprotective potential of aged garlic extract and grape flour in *Drosophila melanogaster*.

Bizzo, L.E.M.[1], B.N. Ludwig[1], M.A.S. Ferreira[1], and S.D. Conceição[1]. Centro Universitário - Católica de Santa Catarina, Joinville, SC, Brazil. Corresponding author: luis.bizzo@catolicasc.org.br

Abstract

Oxidative stress is considered the main cause of aging and diverse neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's. To decrease the number of these diseases, the antioxidant effects of many compounds have been explored. In this research, lines of *Drosophila melanogaster* were exposed to

Aged Garlic Extract and Grape Flour and the Rapid iterative negative geotaxis (RING) test was used to evaluate their aging. Additionally, the Lethal Dose 50 test was performed to evaluate the toxicity of the extract to flies. There was a decrease in the motor performance of the tested organisms from the 14 days, but a tendency to stabilize the aging process of the flies exposed to the medium with the aged garlic extract in the 5× concentration from 21 days. At the end of the experiment, the number of individuals at 28 days was higher in medium containing the highest concentrations of garlic extract and grape in relation to the control. In the LD50 test, it was not observed a significant number of deaths so that the lethal dose of the garlic extract could not be stipulated. Key-words: Antioxidants, aging, RING test, oxidative stress.

Introduction

In the last decades, the population's life expectancy has been increasing and the frequency of neurodegenerative diseases is also expected to increase. In the United States of America, the number of elders with Alzheimer's disease is expected to double in the next decade (Thies and Bleiler, 2013). Although age is a determinant factor for the development of some diseases, the influence of other factors has been estimated. There is growing evidence that aging can be accelerated through oxidative damage to cells. Free radicals (reactive oxygen species and reactive nitrogen species) may break DNA strands, damage desoxyribose or modify purine and pyrimidine bases (Lobo *et al.,* 2010).

With the recognition of the oxidation process of DNA, proteins and lipids as a central mechanism in the development of neuropathies and aging, antioxidant functional foods that might complement the defense mechanism of the body have been exploited to fight them (REF). Many compounds are indicated as antioxidants, and phenolics have received special attention. Aged Garlic Extract (AGE) is an important antioxidant, but was studied only in cell cultures or in mouse lines (Ray, Chauhan, and Lahiri, 2011; Kohda *et al.,* 2013; Morihara, 2016).

For the production of AGE, garlic is stored in ethanol solution for about two years. This long term extraction confers its aged characteristic, altering its antioxidant properties through molecular changes. During this process, the production of cystein (S-allylcysteine, SAC) is intensified, which confers a higher antioxidant activity in relation to *in natura* garlic (Ide and Lau, 1997; Imai *et al.*, 1994; Borek, 2001). Among the role of AGE in protecting against aging-related cognitive impairment, the inhibition of lipid peroxidation, and Low Density Lipoprotein (LDL) cholesterol oxidation (Ide and Lau, 1997) can be highlighted. In this way, it can decrease the risks of degenerative diseases such as Alzheimer's. The AGE's antioxidant effect has already been tested *in vitro* in mouse and *in vivo* cells in transgenic mice with familial Alzheimer's disease and demonstrated important neuroprotective activity (Chauhan and Sandoval, 2007; Ray *et al*., 2011).

Another important functional food is grape. These have high antioxidant content, with phenolic compounds as resveratrol and anthocyanins (Xia *et al.*, 2010). In this way, Grape Flour (GF) has the main functional antioxidant properties of fresh grapes. A previous study with GF added to *Drosophila* culture medium influenced the longevity of flies. Female longevity increased 127% and male longevity increased 50% (Fernandes, 2006), an indication that functional foods might have an important role on fighting against aging.

Many of the common age-related behavioral changes in humans are also observed in *Drosophila*, including motor decline, olfactory, memory, and circadian rhythmic ability (Jones and Grotewiel, 2011). *Drosophila* has been used as a study model in the neurotoxicology of cisplatin and acrylamide (Podratz *et al*., 2011; Prasad and Mudalihara, 2012). Therefore, *Drosophila* might be an important model organisms in aging studies.

In the present study, *Drosophila* were maintained on culture media enriched with phenolic compounds (AGE and GF), and their motor activity was evaluated weekly. Flies submitted to the culture medium enriched with AGE and Grape Flour in higher concentrations had their populations conserved, such as better motor performances with the aging process.

Material and Methods

In this experiment, a Canton-S *Drosophila melanogaster* line was used. Flies were raised on standard corn culture medium and virgin adults were transferred and maintained on enriched medium with Grape Flour or Aged Garlic Extract (powdered) in two concentrations, 1,200 mg/kg of medium and 3,000 mg/kg (corresponding to two and five times the dose recommended for daily human consumption, from now on called the 2× and 5× treatments). In addition, raw garlic powder at concentration of 600 mg/kg was used for preliminary tests. Virgin flies maintained on corn medium as negative controls. Five replicates of each treatment (AGE and GF) were established.

To evaluate aging of flies, the Rapid iterative negative geotaxis (RING) test, proposed by Gargano *et al*. (2005), was used. In this test, the motor ability of flies is measured as the high flies ascend after being tapped, and was inspected weekly (7, 14, 21, and 28 days after hatching). Following the protocol of Nichols *et al*. (2012), about twenty flies were inserted into each of the falcon tubes of the device. For each trial, two photographs were taken and one was used for analysis.

The evaluation of the lethal dose of AGE through LD50 test was performed in groups with about 80 flies reared in standard culture medium, 7-10 days after hatching. Flies were exposed to different concentrations of AGE diluted in 5% sucrose solution and applied 100 μL to pieces of 2×2 cm filter paper. The survival of the flies was evaluated by the number of individuals at the end of 24 hours and after 48 hours of exposure to the extract.

According to previous published works, the initial concentration in the LD50 test would be 600 mg/kg (solution); flies were maintained in vials with 10× that concentration, 150× and 300× the indicated concentration, besides the control group (without addition of age). In total, 321 flies were used in this test, performed in cylindrical glassware with added pink dye to the solution for better visualization. 24 hours after the start of the test, the filter papers were changed to avoid the lack of solution, maintaining the same concentrations, and fly survival was again registered 24 hs later.

In the RING test, differences in fly heights were evaluated by Univariate Analysis of Variance (ANOVA), using treatments as a predictor variable. As the result was statistically significant, Tukey's multiple comparisons test was performed in order to verify which comparisons were significant. In all tests, $p < 0.05$ was used. All analyses were performed in the Statistica 10 program (StatSoft).

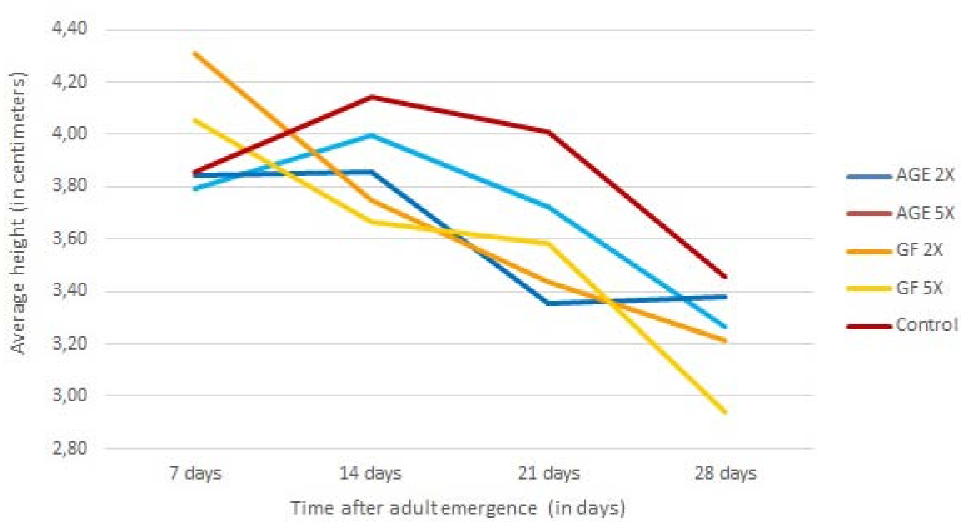

Figure 1. Average heights in RING Test analysis of the different treatments in *Drosophila melanogaster*. Flies were maintained on media enriched with AGE - aged garlic extract, or GF - grape flower, or non-enriched (control).

Results and Discussion

About 6800 observations were listed with RING test between the control groups and treatments. In general, there was a decrease in fly motor performance after 14 days from hatching (Figure 1). This effect was expected due to the consequences of aging, as Gargano (2005) demonstrated in his protocol and recorded in other studies (Jones, 2010; Simon *et al.*, 2006; Grotewiel *et al.*, 2005). An increase in average heights at the beginning of the experiment was not expected, although similar behavior was seen in other works, such as in Piazza (2009).

It was possible to trace a motor development line due to aging in each of the treatments used in *Drosophila*. In the animals exposed to the medium with the 5× garlic extract concentration, there is a tendency to stabilize the heights from the 21 day period, showing an apparent and possible relation with its neuroprotective potential.

A comparison of the heights between the two treatments and the control group was performed separately (Table 1).

Table 1. Univariate analysis of variance (ANOVA) between the control group and the treatments. AGE - Aged Garlic Extract; GF - Grape Flour.

	7 days		14 days		21 days		28 days	
	F test	p	F test	p	F test	p	F test	p
AGE	1.90	ns	1.11	ns	5.48	**	0.35	ns
GF	3.41	*	4.92	**	5.03	**	1.46	ns

* $p < 0.05$ and ** $p < 0.01$ and ns: non-significant.

There was no difference between treatments when compared to their concentrations (2× to 5×). Differences found were restricted to the relationship between the control group and one of the treatments.

Flies maintained on 5× AGE medium presented lower locomotor activity at 21 days, which was not maintained at 28 days. However, the treatments that presented lower performance in the averages of the heights were those that presented a better survival rate at the end of the experiment (5× Grape Flour and 5× AGE concentrations).

Flies maintained on 2× GF medium presented higher locomotor activity at 7 days after hatching, and lower activity at 14 and 21 days. Regarding the 5× GF treatment, at 14 days the height was also lower than the control.

After the tests, a qualitative analysis of the flies survival was carried out (Table 2). Both 5× AGE and 5× GF presented higher survival rates after 28 days.

Table 2. Population survival rate (%) after seven to 28 days after hatching. AGE - aged garlic extract; GF -grape flour.

	7 days	*14 days*	*21 days*	*28 days*
Control	100	68.36	28.77	11.81
2x AGE	100	64.83	37.93	16.23
5x AGE	100	81.36	47.31	36.16
2x GF	100	47.90	41.08	26.43
5x GF	100	67.91	53.56	38.15
Control	100	68.36	28.77	11.81

The decay rate of the populations was slower from 21 to 28 days, at which point the control presented higher contrast due to the onset of aging. These groups stand out from the others, which can be attributed to the higher concentration of antioxidant compounds present in the culture medium. The 2× AGE treatment presented survival rates similar to the control, which might be explained by a dose-dependent effect.

Borek (2001) reports the power of garlic extract in increasing the longevity of mice studied. In our research, we did indeed find evidence that AGE treatment had similar effects, increasing the survival rate of the exposed flies in relation to the control, at least in high concentrations.

The longevity of the *Drosophila* lineages in this study may be associated with the neuroprotective potential of AGE, as well as may be related to the tendency of stabilization of the heights of the treated group in higher concentration. Thus, the AGE study becomes suggestive about the use of antioxidants from the diet to improve quality of life.

In the LD50 test, it was not observed a significant number of deaths in order to estimate the lethal dose of AGE. The 300× AGE solution presented only 6% deaths after 48 hs. This is strong evidence for AGE safety and an indication that their antioxidant compounds might not act as pro-oxidants in high concentrations, as found for ascorbic acid (Carr and Frei, 1999).

In our tests, we found some unforeseen side-effects. AGE, odorless in regular concentrations (2× and 5×), presented a strong and characteristic odor in solutions with higher concentrations (150 and 300×). Studies with *Drosophila* show that these are influenced by odors, which act as stimulants, and in this way may increase the activity of these flies or even provoke environmental disapproval (Budick, 2006). This attitude of aversion could be observed in the preliminary tests with culture medium added in garlic powder *in natura*, which for having more prominent odor did not succeed in egg laying and establishment of the lineage. This type of behavior is not observed in other model study organisms due to the influence of body weight and different olfactory sensitivities.

It was also observed that in the LD50 trials, glasses containing the highest concentrations of AGE presented higher amounts of residues. This may be due to a possible increase in the metabolic activity of the flies, which had slightly altered behaviors regarding agitation and excretion. This alteration in metabolism, somehow, may have masked the antioxidant action of garlic extract, due to its increase being related to the anticipation of aging.

One weakness of the present study design is that we could not determine the daily dose of antioxidant compounds ingested by the flies, as there was no control of how much of the extract the flies were fed from the culture medium. Future studies that directly administer food compounds to the individuals might overcome this limitation.

Conclusion

The data obtained in this research show that there was an increase in the life time of the flies treated with AGE and GF. Nutraceuticals may increase the longevity of individuals, as can be seen in Soh *et al*. (2012) and also in studies with trans-resveratrol (Fernandes, 2006) and D-chiro-inositol (HADA, 2012). The difference in relation to the survival rate may be associated with the antioxidant potential of the treatments, which presents dose-dependent effects. We found no evidence of toxicity of AGE on *Drosophila*, even when flies were submitted to high doses. We suggest that further research should be done at higher concentrations of AGE, and might use *Drosophila* lines that are more susceptible to oxidative stress.

Acknowledgments: The authors would particularly like to thank the professors Daniela de Toni and Norma Silva, from Federal University of Santa Catarina (UFSC), who kindly donated the *Drosophila melanogaster* Canton-S lines used in this research. The authors also thank Mr. Yukihiro Kodera, senior researcher from Drug Discovery Laboratory (Wakunaga Pharmaceutical Co., Ltd., Hiroshima, Japan) for donation of the Aged Garlic Extract.

References: Borek, C., 2001, The Journal of Nutrition 131: 1010-1015; Budick, S.A., and M.H. Dickinson 2006, Journal of Experimental Biology 209: 3000-3017; Carr, A., and B. Frei 1999, The FASEB Journal 13: 1007-1024; Chauhan, N.B., and J. Sandoval 2007, Phytotherapy research 21: 629-640; Fernandes, F.A., 2006, Efeito do trans-resveratrol sobre a longevidade e o metabolismo de *Drosophila melanogaster* em diferentes idades. (Unpublished theses); Gargano, J.W., I. Martin, P. Bhandari, and M.S. Grotewiel 2005, Experimental Gerontology 40: 386–395; Grotewiel, M.S., I. Martin, P. Bhandari, and E. Cook-Wiens 2005, Ageing Research Reviews 4: 372–397; Hada, B., M. Yoo, K.M. Seong, Y. Jim, H. Myeong, and K. Min 2012, The Journal of Gerontology 63: 226-234; Ide, N., and B.H.S. Lau 1997, Journal of Pharmacy and Pharmacology 49: 908-911; Imai, J., N. Ide, S. Nagae, T. Moriguchi, H. Matsuura, and Y. Itakura 1994, Planta Med. 60: 417-420; Jones, M.A., and M. Grotewiel 2011, Experimental Gerontology 46: 320-325; Kohda, K., H. Goda, K. Itoh, K. Samejima, and T. Fukuuchi 2013, Pharmacology & Pharmacy 4: 31-40; Lobo, V., A. Patil, A. Phatak, and N. Chandra 2010, Pharmacognosy Review 4: 118-126; Morihara, N., A. Hino, T. Yamaguchi, and J. Suzuki 2016, The Journal of Nutrition 146: 460-463; Nichols, C.D., J. Becnel, and U.D. Pandey 2012, Journal of Visualized Experiments 61: 1-5; Piazza, N., B. Gosangi, S. Devilla, R. Arking, and R. Wessells 2009, Plos One 4: 1-11; Podratz, J.L, N.P. Staff, D. Froemel, A. Wallner, F. Wabnig, A.J. Bieber, A. Tang, and A.J. Windebank 2011, Neurobiology of Disease 43: 330-337; Prasad, S.N., and

H.R. Muralidhara 2012, Neurotoxicology 33: 1254-1264; Ray, B., N.B Chauhan, and D.K. Lahiri 2011, Journal of Neurochemistry 117: 388–402; Simon, A.F., D.T. Liang, and D.E. Krantz 2006, Mechanisms of Ageing and Development 127: 647-651; Soh, J., N. Marowsky, T.J. Nichols, A.M. Rahman, T. Miah, P. Sarao, R. Khasawneh, A. Unnikrishnan, A.R. Heydari, R.B. Silver, and R. Arking 2013, Experimental Gerontology 48: 229-239.

First record of *Drosophila incompta* Wheeler 1962, *flavopilosa* group, in the Brazilian state of Minas Gerais.

Moreira, Henrique da Rocha[1], Francine Cenzi de Ré[2], Elgion Lucio da Silva Loreto[2], and Lizandra Jaqueline Robe[1,2*]. [1]Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Rio Grande, RS, Brazil; [2]Programa de Pós-Graduação em Biodiversidade Animal, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil; *Corresponding author: lizbiogen@gmail.com

Drosophila incompta belongs to the *flavopilosa* group, which comprises 17 species (Bächli, 2017), distinguished mainly by their internal male genitalia morphology (Wheeler *et al*., 1962). This group presents a remarkable characteristic, being ecologically specialized and totally dependent on flowers of *Cestrum sp* (Solanaceae) for both feeding and reproduction (Brncic, 1966). In this sense, these species developed several adaptations to their host, including their small to medium size, the yellowish color (cryptic to *Cestrum* flowers), and the presence of strong spines on the outer region of the female genitalia (Brncic, 1983; Ludwig *et al*., 2002).

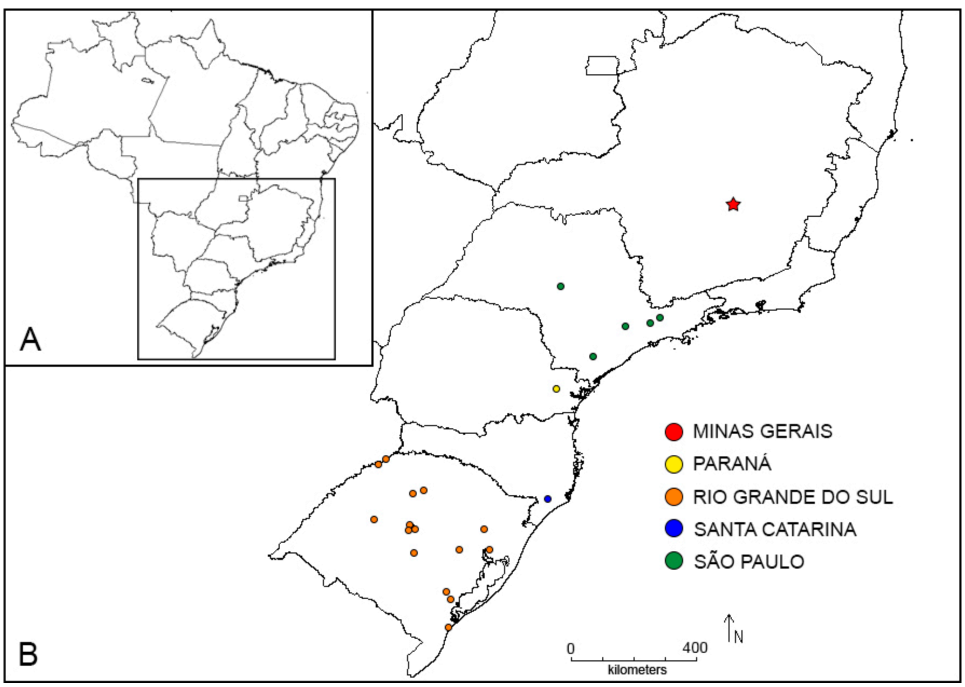

Figure 1. Known geographical distribution of *D. incompta* in Brazil, according to Bächli (2017). Orange, blue, yellow, and green circles correspond to sample locations in the states of Rio Grande do Sul, Santa Catarina, Paraná, and São Paulo, respectively. The red star corresponds to the first occurrence location of *D. incompta* in the state of Minas Gerais.

Due to their restricted ecology, the members of the *flavopilosa* group are geographically restricted to areas with *Cestrum* sp., which seems to be highly abundant in the Neotropics (Robe *et al*., 2013). Even so, most species are specialized to a small array of *Cestrum* species (Santos and Vilela, 2005) and are widely endemic to a restricted area (Robe *et al*., 2013). *Drosophila incompta* appears to have the widest distribution among the members of this group, being found from Mexico to northern Argentina (Bächli, 2017). Nonetheless, across this area, the distribution of the species is predominantly scattered, with registers encountered only for Antilles (1 record), Colombia (2 records), and Panama (2 records), besides Mexico (2 records), Brazil (22 records,