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Full Length Research Paper

Effect of dietary *aloe vera* extract fed at larval stageon life-history traits of *Drosophila melanogaster* selected for faster pre-adult development

Short title: Effect of *Aloe vera* on *Drosophila melanogaster*

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Abstract

We investigated the effects of *Aloe vera* extract on the life-history traits of two *Drosophila melanogaster* populations, one of which was maintained on a 21 day egg-to-egg discrete generation cycle and hence presumed to be developmentally non-stressed, while the other was selected for faster per-adult development and hence presumed to be developmentally stressed. The two populations were reared on one of the four diets differing in the amount of *A. vera* extract (diet with 0 ml, 5 ml, 10 ml or 15 ml per liter of standard media). *A. vera* extract significantly increased the total body mass and adult-life span of developmentally stressed flies, and total body lipids of developmentally non-stressed flies.

Key words: Dietary *Aloe vera*; *Drosophila melanogaster*; extended longevity; lifetime fecundity; lipid content; phytochemicals.

INTRODUCTION

Drosophila melanogaster has contributed to our understanding of fundamental biological process and organismal biology (Beckingham et al. 2005). Single trait selection experiments have established the existence of trade-offs between important life-history traits (Prasad and Joshi 2003). Pre-adult development time is an important life-history trait that has a telling effect on adult life-history of the fly (Chippindale et al. 1993; Zwaan et al. 1995; Prasad et al. 2000; Handa et al. 2014). Selection for faster pre-adult development resulted in the reduction in the size and/or weight of the fly (Zwaan et al. 1995; Prasad et al. 2000; Jaya et al 2014). The size of the adult fly in turn determines important fitness traits such as reproductive success (Partridge et al. 1987; Blay and Yuval 1997; Yeniestti and Hegde 2003) and adult longevity, in that, smaller flies have shorter longevity (Chippindale et al. 2004; Piper et al. 2005) and lower reproductive success (Bangham et al. 2002; Prasad 2003). For an iteroparous organism prolonged reproductive longevity is an important fitness trait. Long-term selection of *D. melanogaster* for faster pre-adult development and early reproduction has given rise to flies that are significantly smaller in size (Prasad et al. 2000) live

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for significantly shorter duration (Prasad 2003), produce fewer numbers of eggs (A. Joshi pers. comm. and this study), and certain fraction of emerging flies had deformed legs and wings (M. Shakarad, pers. observ.). These observations suggest that the selected flies are under developmental stress and are aging faster than their corresponding ancestral population.

The search for anti-aging compound(s) has been the pre-occupation of mankind for time immemorial. Adult diet supplementation with chemical compounds of plant origin has shown to increase the longevity of both vertibrates (Valenzano et al. 2006) and invertebrates (Dubiley et al. 2010). *Aloe vera,* a cactus-like plant with dagger shaped leaves filled with viscous gel has been traditionally prescribed for many ailments. Although therapeutic effects have not been individually assigned to the well over 75 active ingredients identified from the *A. vera*, it has been used for many centuries for its curative and therapeutic properties (Hamman 2008). Danhof (1993) and West and Zhu (2003) reported anti photo aging properties of *A. vera* perhaps due to increased antioxidant absorption (Yagi et al. 2003; Botes et al. 2008;Chandrashekara and Shakarad, 2011). Further, addition of *A. vera* gel in the diet of patients with heart disease showed a reduction in the frequency of anginal attacks (Agarwal 1985). However, prolonged consumption of *A. vera* tea resulted in acute hepatitis and liver failure in a 26-year-old man (Curciarello et al. 2008) and topical application of crude gel extract resulted in dermatitis in a 72-year-old woman (Ferreira et al. 2007).

In this study we tested the effects of *A. vera* extract on the physiology and life-history traits of two *D. melanogaster* Populations, one of which has been selected for faster pre-adult development for more than 350 generations while the other is a matched ancestral population. Under standard laboratory conditions of 25 °C, 70-80% RH and 24:0 L:D cycle and adequate, nutritious food an average adult fly from ancestral population weighs about 250 µg, produces about 400 eggs and lives for 35-40 days. Under the same laboratory conditions, an average adult fly from the selected population weighs about 70 µg, produces about 35 eggs and lives for 11-14 days. In addition, a certain fraction of the adult flies from selected population are deformed. Overall, the flies from selected population seem to be aging faster than their ancestral flies. The question is, whether *A. vera* could ameliorate the fast aging symptoms of the selected population?

MATERIALS AND METHODS

Collection of Flies for Assays

Details of the maintenance protocol for the two *D. melanogaster* populations used in the study are as described by Prasad (2003). Briefly, the ancestral population was maintained on a 21 day egg-to-egg discrete generation cycle. Forty vials with ~60 eggs per 6 ml standard media per vial were incubated for 12 days at 25±1^{o}C, 75±5% relative humidity and 24:0 L:D cycle. All the flies that emerged by day 12 from egg collection day were transferred into Plexiglas cage with abundant food. Adults were maintained in population cage for further 9 days with food change every alternate day. Eggs for starting the subsequent generation were collected on ninth day after transfer to population cage. The selected population was derived from the ancestral population by transferring only the first 25% of the emerging flies from each of 120 vials to single breeding cage. The egg density per vial and the incubation conditions were identical to those of ancestral population. Eggs for initiating the subsequent generation were collected on day-3 post emergence. Selected and ancestral populations were maintained under common rearing conditions for one complete generation prior to assaying the populations for various life-history traits. Eggs were collected from the running cultures and dispensed into vials with about 6 ml of food at a density of ~ 60 eggs per vial. Forty such vials were set up per population. The vials were incubated for 12 days at 25±1^{o}C, 75±5% relative humidity and 24:0 L:D cycle. All the flies that emerged by day 12 from egg collection day were transferred into Plexiglas cages with abundant food supplemented with live yeast-acetic acid paste for two days (hence forth referred to as standardized flies). On day-3, eggs were collected from the standardized flies by providing fresh uncontaminated non-nutritive agar plate. The eggs were dispensed into 6 ml media vials containing 0 ml/l, 5 ml/l, 10 ml/l or 15ml/l *Aloe vera* extract that was added to standard fly media, before pouring into vials. Twenty vials with ~ 50 eggs per vial were set up for each treatment group per population. The vials with eggs were incubated at SLC. The emerging flies were either collected into pre-labeled dry vials or vials with ~ 6 ml standard fly media before being used in various assays.

Preparation of Standard and Aloe Vera Extract Supplemented Media

Procedure described by Chandashekara and Shakarad (2011) was followed for preparation of standard and *A. vera* treatment media. Banana (205g), jaggery (35g), yeast (36g) and barley (25g) ground to a fine paste in 180 ml water was added to Agar (12.4g) dissolved in 1000 ml lukewarm water and brought to a boil on low flame. The media was cooled to 37 ºC, and benzoate (2.4g) dissolved in ethanol (45ml) was added and mixed thoroughly. This is referred to as standard media. For *Aloe vera* treatment groups, either 5 ml, 10 ml or 15 ml extract was added to 1 liter ready media post addition of benzoate. For obtaining *A. vera* (crude) extract- a single freshly cut healthy leaf was

Table 1: Average trait values (mean ± s.e.) for the ancestral and selected populations.

	Ancestral population				Selected population			
Trait	**0ml**	**5ml**	**10ml**	**15ml**	**0ml**	**5ml**	**10ml**	**15ml**
Dry weight/fly (µg)	273.60 ± 6.56	282.32 ± 5.89	281.12 ± 3.14	282.72 ± 12.62	*71.08 ± 2.01*	*92.12 ± 4.05*	*118.59 ± 5.40*	*108.52 ± 4.12*
Total body lipid/fly (µg)	*53.04 ± 3.32*	*71.56 ± 4.30*	*69.96 ± 5.10*	*61.16 ± 3.69*	8.56 ± 2.13	8.44 ± 0.99	8.40 ± 1.68	3.88 ± 0.97
Adult longevity (days)	38.65 ± 3.49	39.70 ± 2.68	42.20 ± 3.36	40.80 ± 3.02	*14.20 ± 1.04*	*22.25 ± 2.13*	*15.25 ± 1.27*	*17.30 ± 1.87*
Lifetime fecundity	392.95 ± 34.23	439.20 ± 27.03	469.05 ± 39.73	458.35 ± 35.63	34.70 ± 4.08	52.90 ± 7.58	48.05 ± 6.15	49.35 ± 7.57

Trait values that were significantly altered by rearing flies during larval phase on media supplemented with *Aloe vera* extract have been *italicized*. In the developmentally non-stressed ancestral flies, total body lipids were increased ($F_{3,16} = 4.259$, $p = 0.022$), while in the developmentally stressed flies, adult dry weight ($F_{3,16} = 25.397$, $p < 0.01$) and adult longevity (Kaplan-Meier log-rank test, $\chi^2 = 14.451$, $df = 3$, * $p < 0.005$) were increased.

stored at for about 24 hours in the freezer chamber of a refrigerator, de-skinned using a sterile blade and clear liquid extracted by applying pressure (in a pre-sterilized pestle and mortar) on the clear pulp. The *A. vera* plants were maintained singly in pots containing sand, red soil and farm yard manure in 2:6:2 ratio. The potted plants were watered once in three days.

Adult Dry Weight and Lipid Assay

Flies collected into empty dry vials within 6 hours of emergence were freeze killed, females were sorted under the binocular microscope, distributed in groups of ten into clean dry vials, dried at 70 ºC for 36 hours and weighed to the nearest µg. Lipid content was estimated by de-fatting the flies following the method of Zwaan *et al.* (1995) with a minor modification as described in Chandrashekara and Shakarad (2011). After recording the dry weight, the flies were placed in 1.5 ml micro centrifuge tubes containing 1.3 ml diethyl ether. Lipid was extracted over a 36 hour period with gentle agitation on a gel-rocker set to 20 rpm. Ether was changed every 12 hours. At the end of 36 hours flies were removed from the ether, washed with 1 ml fresh ether, dried at 70 ºC for 2 hours, and weighed to obtain lipid free dry weights. The difference between dry weight 'before' and 'after' ether extraction was taken as the total lipid content. Five vials were set up per treatment per population.

Fecundity Assay

Freshly emerging flies from the above four treatments of both the developmentally non-stressed ancestral and developmentally stressed selected population were sexed under light CO_2 anesthesia and single pair (1 male + 1 female) were transferred to vial with ~ 3 ml of SM. Twenty such vials were set up per treatment per population. Flies were transferred without anesthesia to fresh standard media vials every 24 hours, and the eggs laid during the previous 24 hours were counted under binocular microscope and recorded. The daily egg counts were carried out till the death of the female fly in each test vial.

Longevity Assay

The longevity of reproducing females was assayed. One-day old flies were sorted into vials containing ~ 4 ml of standard media at a density of four mixed sex pairs (4 females + 4 males) per vial. Twenty vials were set up per treatment per population. In all 320 female flies were assayed for longevity. Mortality was recorded every 24h, the dead flies were removed from the vial and the live flies were transferred to fresh food every alternate day.

Statistical Analyses

The developmentally non-stressed ancestral and developmentally stressed selected populations have greatly divergent trait values for all traits studied (Table 1), hence, data from the two populations were analyzed separately. The significance of the difference between means was assessed using one way analysis of variance (ANOVA). The differences among treatments were compared by Tukey-Kramer Minimum Significant Difference ($MSD_{\alpha 0.05}$) Test (Sokal and Rohlf 1995). The significance of the difference between adult survival curves (standard *vs A. vera* $_{\alpha 0.05}$) supplemented media was analyzed using Kaplan-Meier log-rank test (Fisher and van Belle 1993).

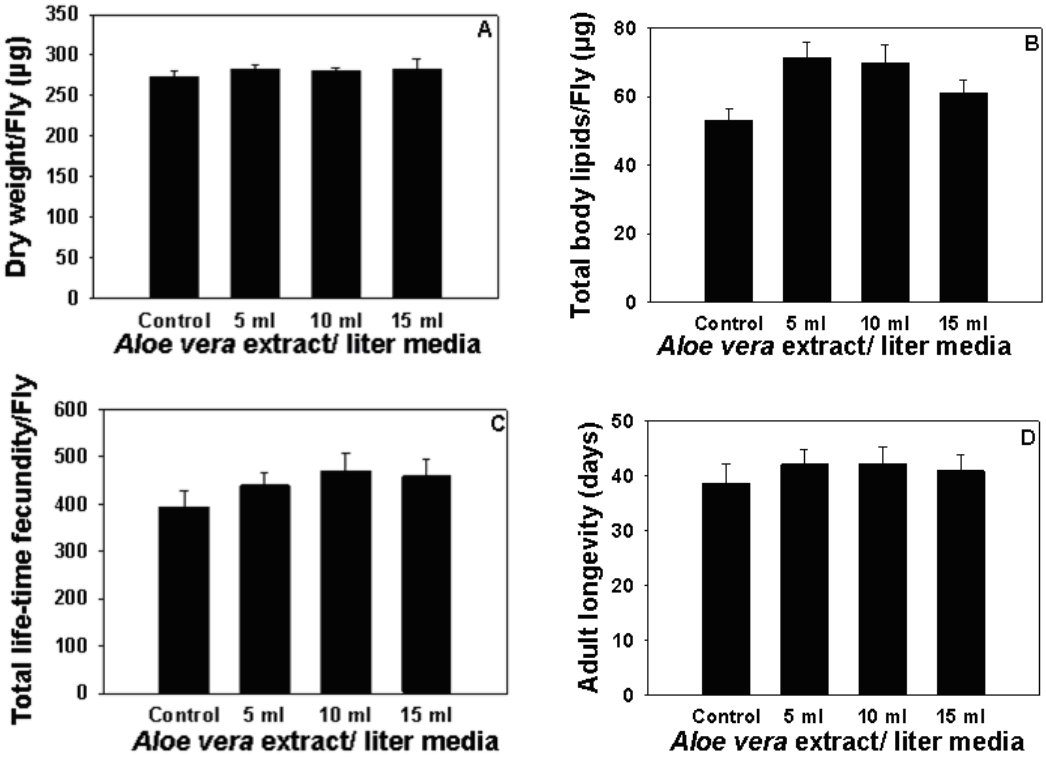

Figure 1: Mean (± s.e.) values of (A) dry weight (µg)/female fly, (B) total body lipids (µg)/female fly, (C) average life-time fecundity/female fly, and (D) Kaplan-Meier survival curves, for flies from developmentally non-stressed population reared on media supplemented with different concentrations of *A. vera* extract. Bars with different alphabets indicate significant difference. The survival curves were not significantly different.

RESULTS

The trait values are expressed as mean ± standard error of the mean (s.e.) (Table 1).

Developmentally Non-stressed Flies

Rearing developmentally non-stressed ancestral flies on *A. vera* extract (hence forth referred to as extract) supplemented media had no significant effect on dry weight ($F_{3,16}$ = 0.297, p = 0.827, Figure 1A), total life-time fecundity ($F_{3,76}$ = 0.953, p = 0.419, Figure 1C) and adult longevity ($F_{3,76}$ = 0.235, p = 0.872, Figure 1D) but significantly increased the total body lipids ($F_{3,16}$ = 4.259, p = 0.022, Figure 1B). Total body lipids of flies reared on media supplemented with 5ml and 10 ml extract were significantly higher ($MSD_{\alpha 0.05}$ = 16.668µg) than those reared on standard media.

Developmentally Stressed Flies

Adult Dry Weight and Lipid Content

Overall there was a significant effect of extract supplementation on the adult dry weight ($F_{3,16}$ = 25.397, p < 0.01, Figure 2A). The flies raised in standard media were significantly lighter than those reared in media supplemented with 5ml, 10 ml and 15 ml extract ($MSD_{\alpha 0.05}$ = 16.668µg), and the 5ml flies were significantly lighter than 10 ml flies. However, there was no significant effect of extract supplementation on the total body lipids ($F_{3,16}$ = 2.27, p = 0.12, Figure 2B). Interestingly, larvae that were reared in media supplemented with 15 ml extract had substantially lower body lipids (3.88µg) compared to their controls (8.56µg), as well as those that were reared in 5 ml (8.44µg) and 10 ml (8.40µg) extract supplemented media.

Fecundity

The total life-time fecundity of flies raised on extract supplemented diets were non significantly higher than of

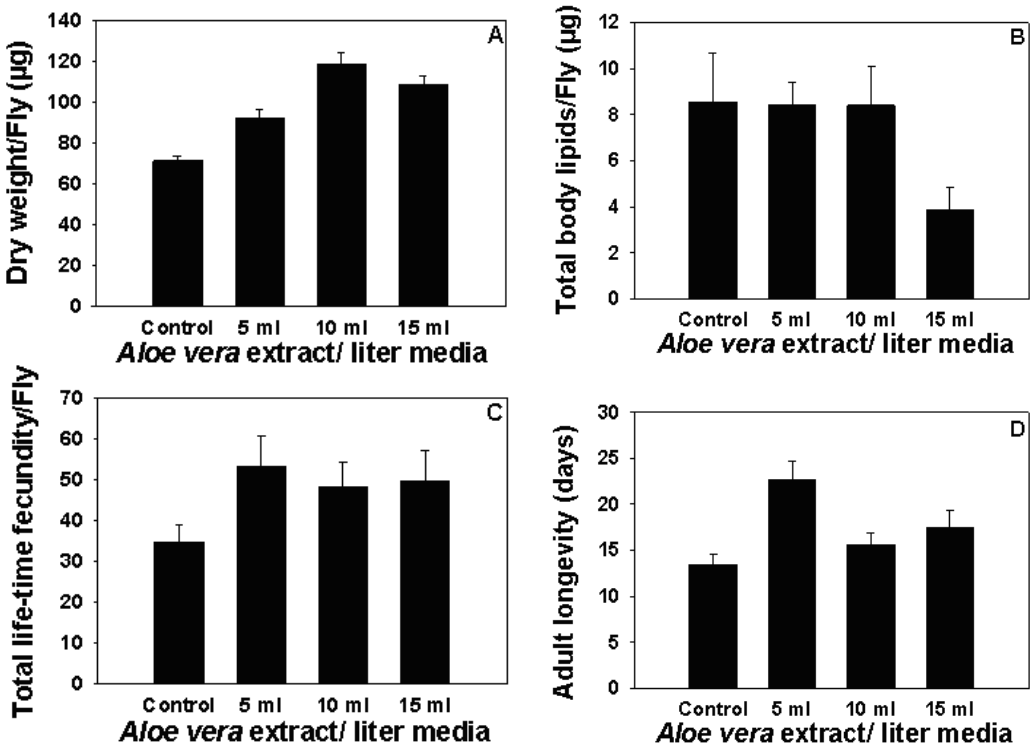

Figure 2: Mean (± s.e.) values of (A) dry weight (µg)/female fly, (B) total body lipids (µg)/female fly, and (C) average life-time fecundity/female fly for flies from developmentally stressed population reared on media supplemented with different concentrations of *A. vera* extract. Bars with different alphabets indicate significant difference. (D) Kaplan-Meier survival curves indicated that flies reared on 5ml extract per liter media lived significantly longer compared to other treatments (χ^2 = 14.451, *df* = 3, * *p* < 0.005).

those reared on standard media ($F_{3,76}$ = 1.5, *p* = 0.221, Figure 2C).

Longevity

There was a significant difference in the rate of death as indicated by the Kaplan-Meier survival curves (χ^2 = 14.451, *df* = 3, * *p* < 0.005). Overall there was a significant effect of extract supplementation on the adult longevity ($F_{3,76}$ = 4.394, *p* = 0.007) (Figure 2D). The post-hoc comparisons indicated that flies reared in media supplemented with 5 ml extract (22.25 days) lived significantly ($MSD_{\alpha 0.05}$ = 6.897) longer than those reared on standard media (14.2 days) and flies reared in media supplemented with 10 ml (15.25 days) extract. However, flies from 15 ml (17.3 days) extract supplemented media were not significantly different from the flies reared on standard media, 5 ml as well as 10 ml treatment flies.

DISCUSSION

The significant increase in the adult dry weight of developmentally stressed flies (Figure 2A) reared on diet supplemented with extract could have been due to an increase in the pre-adult development time that lead to acquiring and assimilating more resources. Earlier studies that selected populations for faster pre-adult development have shown a causal relation between development time and adult size/weight (Prasad et al. 2000; Chippindale et al. 1997; Nunney 1996). The increased body weight in turn could have given rise to an increase in the longevity of flies reared on extract supplemented diet as nutritional constituents in the culture media and feeding behavior are known to determine the longevity (Carvalho et al. 2005). Chippindale et al. (1998) and Rose and Bradley (1998) reported increased body weight and higher total body lipids for flies selected for extended adult longevity suggesting the role of lipid in regulating longevity. However, in this study, *A. vera* supplementation did not increase the total body lipids in the developmentally stressed flies (Figure 2B). Alternatively, the longevity could have increased at the cost of reproduction as longevity and reproduction are known to tradeoff, especially under resource constraints (Djawdan et al. 2004). Interestingly, the flies reared on 5 ml extract supplementation had marginally higher egg production

compared to their controls, suggesting that some of the minerals, vitamins and other compounds at lower concentration (Massie et al. 1993; Bahodarani et al. 2008) could have improved the overall health of flies as indicated by increased weight at emergence (Figure 2A) and non appearance of deformed flies in the *A. vera* treatment groups (Mallikarjun Shakarad pers. observ.).

Further, the increased longevity could also be due to increased antioxidant activity (Botes et al. 2008) that lead to improvement in general health of flies. Another interesting finding from this study is that, the reduction in the total body lipids of flies reared in media laced with 15 ml extract had no detrimental effect on either longevity or total life-time fecundity. This suggests that the flies perhaps had reduced metabolic rates resulting in production of fewer free-radicals (Dubiley et al. 2010). The reduction in the frequency of anginal attacks of heart patients provided with *A. vera* gel mixed diet (Agarwal 1985) could have been due to the clearing of the arteries of their lipid lining as indicated by the significant reduction in the total lipids of flies reared on media laced with 15 ml extract. Our results suggest *A. vera* extract perhaps modulates the lipid metabolic pathway. More importantly, our results indicate that the link between longevity and fecundity is weak at best and hence the trade-off between the two is conditional (Marden et al. 2003; Partridge et al. 2005) and that lipids are not the only energy currency regulating the two traits.

In conclusion, we show that dietary supplementation with *A. vera* extract does not improve the performance of already healthy flies but plays a significant role in improving the health of developmentally stressed flies. The data also suggests the role of *A. vera* extract in lipid biosynthesis and metabolism. Further studies are necessary to ascertain the exact mechanism(s).

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