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Effects of *Stevia rebaudiana* Pure and Crude Leaf Powder on the Pupation Site Preference of *Drosophila melanogaster*

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Abstract

Preference to pupate on the appropriate substratum by a larvae enhances its survivability, it also depends on the energy acquired during its feeding period. Pupation site preference is a critical developmental event in *Drosophila*. Choosing and moving a long distance during its last instar is challenge to all insect and influenced by variety of extrinsic and intrinsic factors. Percentage of larvae preferred to pupate on different site and the height travelled by the larvae to pupate has taken as a measure of the pupation site preferences in order to choose an appropriate place to pupate. In the present study, we use two different type of natural sugars for their efficacy to acquire energy to travel greater heights has been observed in the flies treated with stevia pure powder and dried stevia leaf powder (crude extract and Pure extract) of the plant *Stevia rebaudiana*. We measured the height preferred by pupate on media (<1 cm), wall of the bottle (> 5cm) and on the neck (> 10 cm) of the bottle. Interestingly, except for the control all the treated groups have traveled the greater height of > 5 cm. Compared to stevia powder the energy acquired in the stevia leaf powder is greater than control group have showed 40% pupation in the neck of the bottle (>10cm) height. The data is analyzed and discussed.

Keywords: *D. melanogaster,* pupation site preference, pupation height, stevia, stevia dried leaf

Introduction

Since stevia leaves are known to offer more advantageous functional and sensory qualities than many other high-potency sweeteners, stevia is expected to play a significant role in the future development of the expanding natural food sector. While stevia is beneficial to all people, some groups are more likely to gain from its exceptional sweetening properties. These comprise kids, people with diabetes, and people who want to cut back on their caloric intake. Since ancient times, people have utilized stevia, a tiny perennial shrub, as a bio-sweetener and for several medical purposes, including lowering blood sugar. The natural herbal sweetener with no calories, stevioside, is a white crystalline molecule that is nearly 300 times sweeter than table sugar.

Research on behavior is a fascinating area of study that has been done on a range of species, including *Drosophila*. As a holometabolous insect, it passes through different stages of life cycle such as egg, larva, pupa, and adult. The capacity of third-instar *Drosophila* larvae to pupate on an appropriate substrate is a crucial part of their life cycle, as pupae are exposed to desiccation, illness, and predators while stationary (Manning and Markow, 1981) [20].

Larval behaviors include foraging, digging, skipping, and choosing a preferred pupation site. A crucial part of the *Drosophila* life cycle is the larva's selection of an appropriate pupation site, which involves habitat selection. Because the location that the larvae choose can have a significant impact on their survival as pupae, the pupation site preference (PSP) is a crucial developmental event in *Drosophila* PSP is often examined using two criteria. One is Pupation Site Preference, which measures the percentage of larvae that pupate on various surfaces, such as cotton, glass, or media, and the other is Pupation Height, which measures how far the larvae must travel upward to pupate from the surface of food, that depend on the energy of the larvae acquired during its feeding.

Numerous *Drosophila* species studied by many authors on pupation height have revealed (Fogleman and Markow, 1982; Bauer and Sokolowski, 1989; Casares and Carracedo 1986, 1987; Singh and Pandey, 1991; Carracedo, 1986) [20, 37, 5, 33]. A variety of extrinsic factors, including light (Manning and Markow, 1981) [20], gravity (Markow, 1979) [21], larval density (Singh & Pandey, 1993) [34] and larval developmental time (Markow, 1979) [21], are known to cause distinct reactions in third instar larvae when they migrate to pupation sites. Numerous abiotic factors have been shown to influence both intra-and interspecific differences in pupal behavior (Markow, 1979; Sokolowski and Hansell, 1983) [21, 36]. Research on the glue proteins, the secretions from the salivary glands of larvae reveals a link between PSP and these proteins. It is discovered that larvae have a tendency to migrate away from the media's

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surface in proportion to the quantity of adhesive protein secreted by the salivary glands (Shivanna *et al*., 1996) [32].

Plethora of research has demonstrated that plants and their components include vital nutrients that are mostly advantageous and necessary for an organism (Okwu and Morah, 2004; Okwu and Ohuko, 2001) [27, 25]. One such plant is *Stevia rebaudiana*. It is currently unknown what exactly makes up the Stevia species chemically. Still, the chemical makeup of some Stevia species has been investigated. The leaves on this shrub are its most valuable feature. Only eighteen of the 110 species that were evaluated for sweetness were found to have it (Soejarto *et al*. 1982) [35]. The sweet taste impression is produced by eight ent-kaurene glycosides, which are dulcoside A, rebaudiosides AE, steviolbioside, and stevioside (Kinghorn *et al*. 1984) [16]. The majority of these glycosides are made up of steviol, a diterpene derivative (Shibata *et al*. 1995) [31]. The sweetest species, *S. rebaudiana Bertoni*, has all eight ent-kaurene glycosides in its leaves (Kinghorn *et al*. 1984)[16], with the main component being stevioside (~8% of the dried leaf weight) (Melis 1992) [23].

Stevia is utilized in a variety of food products, such as beverages, jams, sauces, confections, and other dental products, and it exhibits resistance to high temperatures. One kilogram of sugar can be substituted with fifty grams of stevia leaf, and cooking with stevioside does not cause it to turn brown. Numerous significant phytochemicals found in stevia, including as steviol, stevioside, and rebaudioside, have the ability to lower blood sugar levels. Due to its strong anti-hyperglycemic properties, it can replace saccharose in diabetics' diets. It improves anti-diabetic qualities and raises insulin levels in pancreatic tissue, which are favorable activities. It also reduces oxidative stress and inflammation, which aids in keeping blood sugar levels within normal ranges. It is a significant supplier of high-potency sweeteners to the expanding natural food industry. In addition to giving the body the best nutrition possible, functional teas, jellys, and efficient stevia strains can also aid in the management of diabetes. If regular consumption of it provides long-term benefits for humans, more research is required to make that determination. The current study has been conducted to ascertain the effect of stevia supplemented diet, on pupation site preference of *Drosophila melanogaster.*

Materials and Methodology

Establishment of Stock

The experimental stock of *Drosophila melanogaster* was obtained from *Drosophila* stock center, Manasagangotri, University of Mysore. *D. melanogaster* is one of the most widely used and one of the most understood of all model organism. The flies obtained were redistributed and raised in different culture bottles containing wheat cream agar media (100g of jagery, 100g of wheat powder, 10g of agar agar was boiled in 1000ml of double distilled water. 7.5ml of propionic acid was added at last). Twenty flies (10 males and 10 females) were introduced into culture bottles and maintained at a temperature of 22ºC ± 1ºC with a relative humidity of 70% in 12 hours dark: 12 hours light cycle. The virgin flies were isolated in pupa stage and cultured in test media. The test media containing 1%, 2% and 3% natural sweetener, stevia powder (pure extract) based media and the flies grown in normal wheat agar media were used as control. In the same way, the test media containing 1%, 2% and 3% dried stevia leaves powder based media and the flies grown in normal wheat agar media were used. The results of both the products ie stevia extract and dried stevia leaves were compared and were discussed. Five day old flies were isolated from the culture and raised in control, stevia extract media and dried stevia leaves media. It was maintained in the conditions mentioned above. They were allowed for five days in the corresponding media, control and test and then used for studying different parameters.

Pupation Site Preference

Adult male and female flies were collected and 10 male and 10 female flies were placed in culture bottles containing wheat-cream agar media, different concentration of pure stevia and dried stevia leaf (1%, 2% and 3%). The flies were allowed to mate for one day and the females were allowed to lay eggs. These parental flies were removed from the culture bottle. The eggs were allowed to hatch, form larvae and pupae. The number of pupae produced were counted and recorded. The culture bottle was divided into 3 regions ie on media, wall of bottle and neck of the bottle. The number of pupae in each region were counted and recorded. The data was subjected to statistics and the graph of pupation site preference was plotted.

Statistical Analysis

The data obtained were analyzed using IBM SPSS version 29.0. Mean, standard error, one way ANOVA, and Tukey's Post-Hoc test and two way ANOVA were carried out for the data obtained for pupation site preference. A graph of site preference v/s number of pupae was plotted for both stevia pure extract and stevia dried leaf extract. The graph of the two was compared.

Results

Fig.1 represents the effect of normal wheat agar media on the pupation site preference of *D.melanogaster* treated with Stevia powder. From the graph it is observed that the pupation site preference of the larvae is more on the wall of the bottle than on media and neck region which is significant with df = 2, F=57.105 and $p<0.001$. Fig.2 represents the effect of 1% stevia on the pupation site preference of *D.melanogaster.* The pupation site preference is significantly high on the wall of the bottle with df= 2, F = 836.736 and $p< 0.001$. Fig.3 reveals the effect of 2% stevia on the pupation site preference of *D.melanogaster.* Similar to 1% and 2% pupae prefer to pupate on the wall of the bottle which is significant with df= 2, F = 255.825 and $p< 0.001$. The effect of 3% stevia (Fig. 4) on the pupation site preference of *D.melanogaster* pupation preferences observed more on the wall of the bottle and it is significant with df= 2, F= 1992.548 and $p< 0.001$. Interestingly, Fig.5 represents the comparative effect of pure stevia on the pupation site preference of *D.melanogaster* compared to control pupation occurred at the wall of the bottle in all three concentration which is significant with $p< 0.001$. The value of F=41.706 between media, F=612.369 between site preference and F= 36.184 between media and site both one way and two way analysis of variance found to be greatly significant between media with different concentration of stevia powder and occurrence of the pupae at the different part of the bottle. Greater distances were covered by the larvae to pupate at the > 10cm height in stevia media compared to control. Fig.6 represents the effect of 1% dried stevia leaf on the pupation site preference of *D.melanogaster.* The pupation found to be maximum of 60% on the wall of the bottle which is significant with df= 2, F= 386.926 and $p< 0.001$. whereas fig.7 (2%) and 8 (3%) dried stevia leaf also preferred to pupate on the wall of the bottle which is significant with df= 2, F= 120.458 (2% stevia leaf powder) and with df= 2, F= 63.685 and p< 0.001(3%) respectively. Fig.9 is the combined effect of dried stevia leaf on the pupation site preference mostly on the wall of the bottle in all three concentrations compared to control which is highly significant with p< 0.001, F=23.407 between media, F=288.374 between site preference and F=9.687 between media and site. Fig.10 represents the comparative effect of both pure stevia and dries stevia leaf extract on the pupation site preference of *D.melanogaster, where the* pupation is high on the wall of the bottle in all the concentrations of both pure stevia and dried stevia leaf but comparatively they are significant. Very less pupation observed on the media (shorter distance) and the control group represented highest pupation preferences at the neck when compared to the stevia treated groups.3% of stevia powder group represents very least pupation on the neck of the bottle, whereas dried stevia leaf treated groups preferred to pupate on the wall but 35% of the larvae preferred the neck region of the bottle for their pupation showing the energy acquired during the feeding is more, As the increase in concentrations increased pupation on the wall was observed both in stevia pure powder and stevia leaf powder.

Fig 1: Pure stevia extract

Fig 2: Dried stevia leaf extract

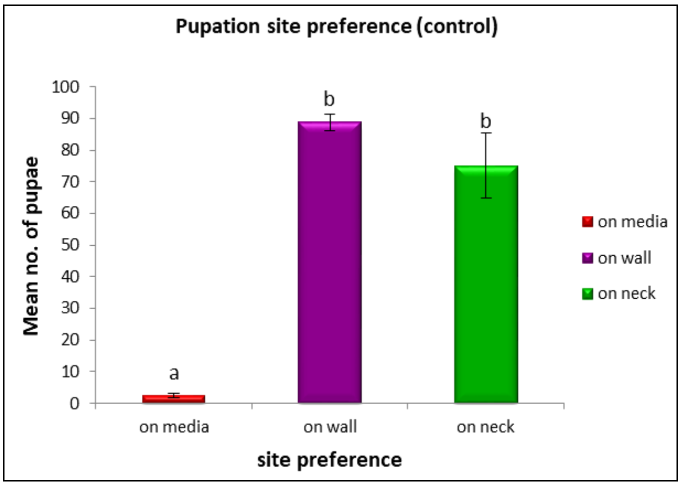

Fig 3: Effect of normal media on the pupation site preference of *D.melanogaster.*

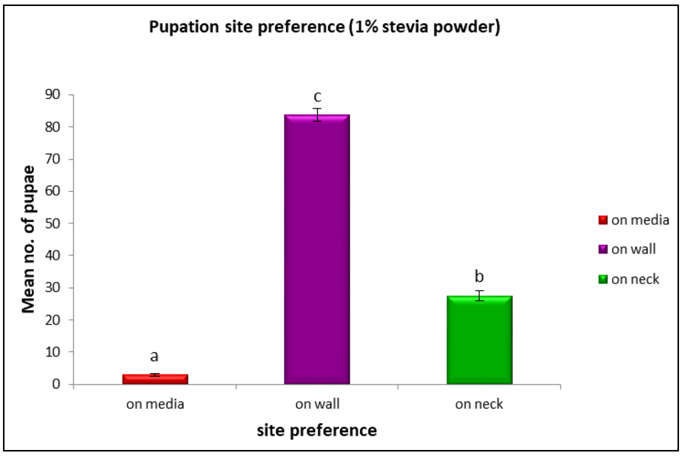

Fig 4: Effect of 1% stevia on the pupation site preference of *D.melanogaster.*

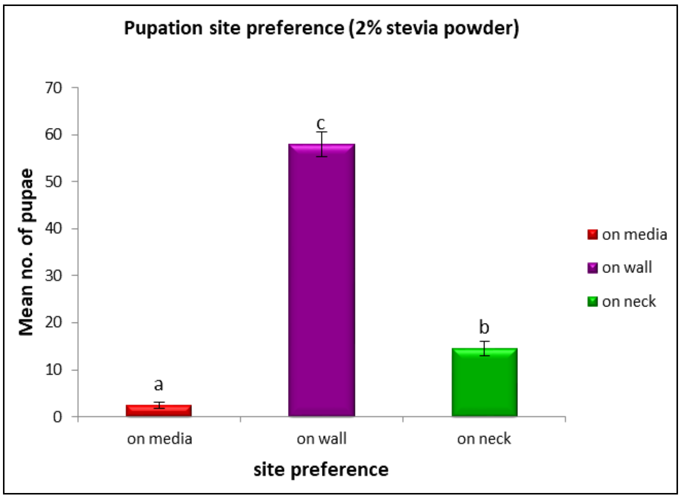

Fig 5: Effect of 2% stevia on the pupation site preference of *D.melanogaster.*

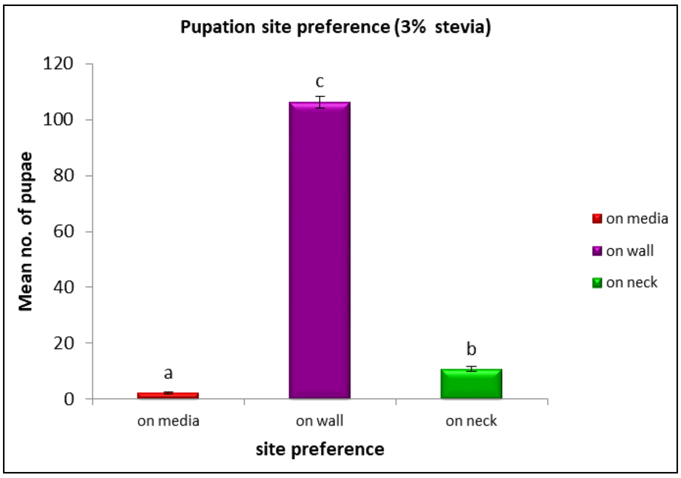

Fig 6: Effect of 3% stevia on the pupation site preference of *D.melanogaster.*

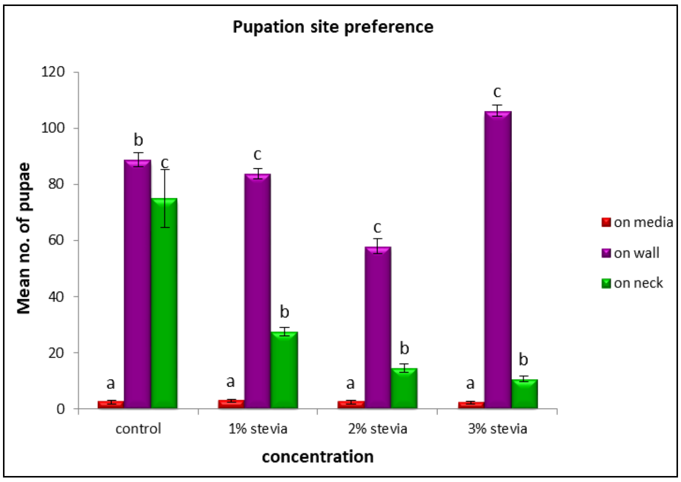

Fig 7: Comparison of effect of pure stevia on the pupation site preference of *D.melanogaster.*

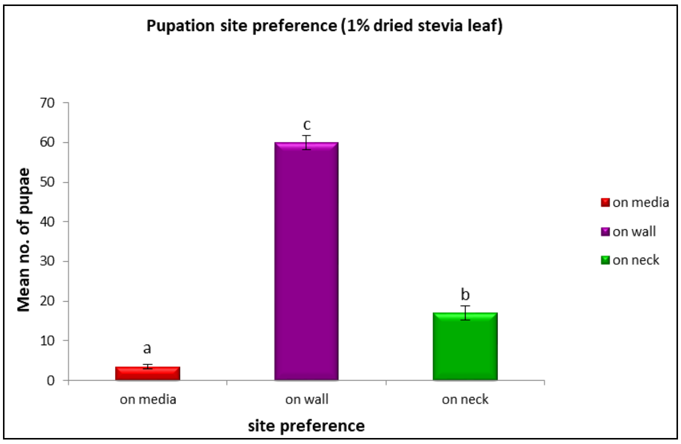

Fig 8: Effect of 1% dried stevia leaf on the pupation site preference of *D.melanogaster.*

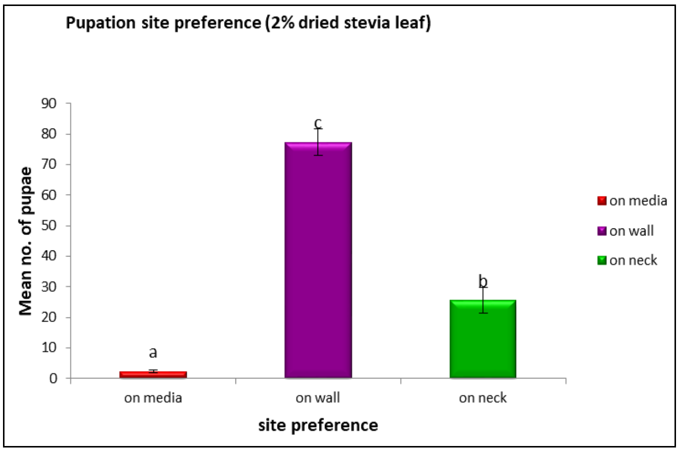

Fig 9: Effect of 2% dried stevia leaf on the pupation site preference of *D.melanogaster.*

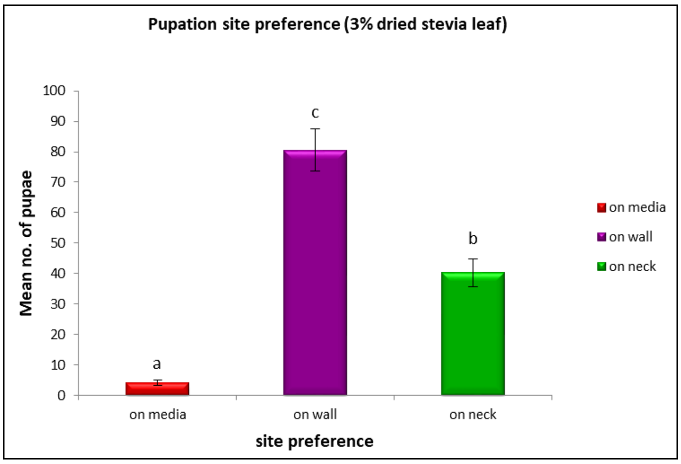

Fig 10: Effect of 3% dried stevia leaf on the pupation site preference of *D.melanogaster.*

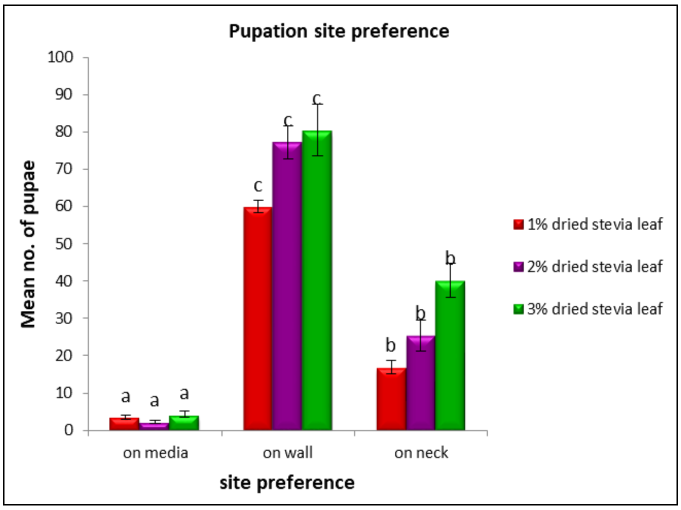

Fig 11: Comparison of effect of dried stevia leaf on the pupation site preference of *D.melanogaster.*

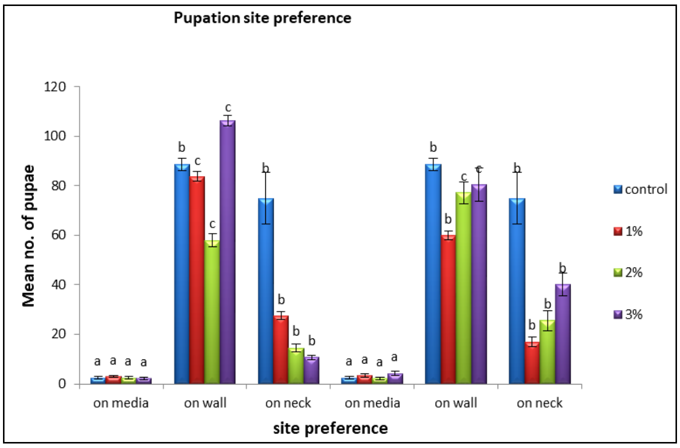

Fig 12: Effect of pure stevia and dried stevia leaf on the pupation site preference of *D.melanogaster.*

Discussion

Behavioral differentiation in pupation site selection of various Drosophila species concerning temperature, gravity, or light has been documented previously. Sokal *et al*. (1960) [36] and Markow (1979) [21] found that temperature, moisture content of the medium and larval density influenced pupation site selection in *D. melanogaster*. In the temperature range of 22 to 25°C, peripheral pupation was preferred over central sites.

While the genetic basis for pupation height has been established, larvae likely respond to several variables when moving upward to pupate. Sameoto and Miller (1968) and Barker (1971) [3] reported that *Drosophila* larvae move to higher pupation sites in response to increasing water content in the medium. Additionally, *D. mettleri* larvae, which live in the soil, face a severe directional constraint as they must pupate at the soil surface to successfully emerge as adults. Natural selection may have led to a geonegative pupation behavior in *D. mettleri*.

The current study demonstrates that both pure and dried stevia significantly affect the pupation site preference of *Drosophila melanogaster*. Higher concentrations of stevia lead to increased energy acquisition, allowing larvae to pupate at greater heights. These findings highlight the potential of natural sweeteners like stevia in influencing developmental behaviors in insects.

The current study's findings align with previous research showing that environmental factors significantly influence pupation site preference. The observed higher pupation sites in stevia-treated groups could be attributed to enhanced energy acquisition from the stevia, enabling larvae to travel greater distances. This supports the hypothesis that nutritional quality and environmental conditions are critical determinants of pupation site preference.

The addition of 1% stevia slightly altered pupation preference, decreasing the neck and media pupation compared to the control. This suggests an influence of stevia on energy acquisition and pupation site choice. Whereas pure stevia led to significant pupation on the wall across all concentrations, with very low media and neck pupation. Pure stevia consistently influenced larvae to pupate at higher elevations, suggesting a strong link between pure stevia and increased energy for larvae movement. The different concentration stevia leaf powder reveals the pupation differences such as 1%: 60% wall, 17% neck, 3.5% media, 2%: 77.25% wall, 25.5% neck, 2.25% media. 3%: 80.5% wall, 40.25% neck, 4.25% media. Dried stevia leaf also influenced higher pupation sites but with a noticeable difference in neck preference, especially at higher concentrations. This suggests a different energy release or nutrient profile compared to pure stevia.

The consistency of higher pupation on the walls and neck regions in the presence of stevia mirrors findings from other studies where larvae preferred elevated pupation sites under optimal environmental conditions. These results underscore the multifaceted nature of pupation behavior, influenced by both genetic and environmental factors.

These experiments on pupation site preference allow for several comparisons of adult and preadult behavior. Markow and Fogleman (1981) [7] investigated adult geotaxis and phototaxis in two *Drosophila* species. Their results indicated that *D. nigrospiracula* adults were geoneutral to slightly geopositive, while *D. mettleri* adults were strongly geonegative. In contrast, pupation site preference (PSP) experiments showed strong photopositive tendencies. Similarly, *D. melanogaster* strains that display strong photopositive behavior as adults are highly photonegative in terms of PSP (Markow, 1981) [20]. Rizki and Davis (1953) observed that *D. melanogaster* and *D. willistoni* differed in their PSP regarding light, suggesting that light intensity is an environmental factor influencing interspecific competition in *Drosophila*.

Environmental and Genetic Influences on Pupation Site Preference

Previous studies have revealed that pupation site preference (PSP) in various *Drosophila* species is affected by both biotic factors (such as sex, density, locomotory path length, developmental time, and digging behavior) and abiotic factors (such as temperature, humidity, moisture, light, darkness, and pH) (Sokal *et al*., 1960; de Souza *et al*., 1970; Fogleman and Markow, 1982; Pandey and Singh, 1993) [36, 6, 8, 28]. Furthermore, genetic factors also control pupation behavior in various *Drosophila* species (de Souza *et al*., 1970; Bauer and Sokolowski, 1985, 1989; Garcia-Florez *et al*., 1989) [6, 37, 9]. For example, Joshi and Muller (1993)[13] reported that pupation height is a polygenic trait that responds effectively to bidirectional selection. Additionally, de Souza *et al*. (1970) [6] found that the preference of larvae to pupate on food or at the bottom of culture bottles is influenced by a single gene.

At higher temperatures (30°C), larvae pupated on food, whereas at lower temperatures (21°C), pupation occurred on the cotton used to plug the culture bottles. The variation in PSP may be attributed to the synthesis of glue protein. Higher temperatures (30°C) induce the synthesis of large quantities of glue protein, helping the pupae adhere to the food for pupation. In contrast, at lower temperatures (21°C), reduced synthesis of glue protein allows larvae to move to the sides of the container to pupate on cotton.

Effects of Larval Niches and Competition

D. nigrospiracula larvae typically utilize rotting cactus tissue, while *D. mettleri* larvae live in soil inundated with rot juices (Heed, 1977, 1978). Despite the spatial separation of larval niches, both species' larvae occasionally coexist in the same cactus rot (Mangan, 1978) [19], or at the interface of cactus and soil substrates. Under certain circumstances, significant larval niche overlap and interspecific competition can occur during pre-imaginal stages. The site at which larvae pupate can significantly impact their survival, especially in extreme desert conditions. Habitat selection may reduce competition, and behavioral differentiation in pupation site preference (PSP) might serve this purpose for these species. Markow and Fogleman (1981) [7] reported on adult responses to light and gravity. Here, we present an investigation of the effects of light, temperature, and gravity on the pupation site preferences of late third-instar larvae of these two species.

Conclusion

The comparative analysis reinforces the notion that pupation site preference in *Drosophila melanogaster* is influenced by various factors, including diet, environmental conditions, and genetic predisposition. The enhanced energy from stevia appears to facilitate higher pupation sites, aligning with the broader literature on the topic. Future research could further explore these interactions to provide deeper insights into the adaptive significance of pupation behaviors. The present study show evidences of the nutritional influence of PSP behavior by a larvae.

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