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The Effect of the Ensure® Nutrition Supplement on Pupation Site Preference in *Drosophila melanogaster*

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Abstract

Pupation site preference is a crucial phase in the pre-adult behavioral development of *Drosophila* because the location that the larva chose might have a significant impact on its chances of surviving as a pupa. PSP was studied by counting the number of larvae pupated on different sites such as towards the cotton, on the wall and on the surface of the media. The variation in pupation site preference across different media was an adaptive character to escape from desiccation, disease and predators. In the present study, larvae of *D. melanogaster* grown in test (Ensure®) media preferred to pupate in a greater percentage near/on the cotton plug, followed by on the wall of the media bottle and less percentage on the surface of the media. On the other hand, larvae grown in wheat-cream agar media and mixed (wheat-cream agar + Ensure®) media preferred to pupate in a greater percentage on the surface of the media, followed by on the wall of media bottle and less percentage near/on the cotton plug. This suggests that the variation in pupation site preference was mainly influenced by the quantity and quality of nutrients present in the different media.

Keywords: *Drosophila melanogaster*, Pupation site preference, glue protein, Ensure®

Introduction

Behavior analysis is a fascinating topic of research that has been applied to many other organisms, including *Drosophila*. As a holometabolous insect, *Drosophila* undergo four life stages: egg, larva, pupa, and adult. The capacity of third-instar larvae to pupate on an appropriate substrate is a crucial part of the *Drosophila* life cycle, as puparia are susceptible to desiccation, disease, and predators when it is immobile (Manning and Markow, 1981) [15].

Larval behavior include feeding, digging, skipping, and choosing a preferred pupation location. An essential part of the *Drosophila* life cycle is the larva's selection of an appropriate pupation location, which includes habitat choice. 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* (Someoto and Miller, 1968) [19]. PSP is often assessed based on two parameters. 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 have to move upward to pupate away from the surface of food.

Studies on PSP and pupation height have been conducted on a number of *Drosophila* species (Cleoan and Krishna, 2018; Seema and Girish, 2019; Shivanna *et al.,* 1996; Vandal *et al.,* 2008; Singh and Pandey, 1991; Casares and Carracedo, 1986, 1987; Bauer and Sokolowski, 1989; Folgleman and Markow, 1982) [6, 20, 21, 25, 9].

A variety of extrinsic factors, including light (Manning and Markow, 1981; Rizki and Davis, 1953) [15], moisture (Sameoto and Miller, 1968) [19], gravity (Markow, 1979) [16], larval density (Singh and Pandey, 1993) [22], larval developmental time (Markow, 1979) [16], and the presence of other species (Rizki and Davis, 1953), are known to cause various responses in third instar larvae when they migrate to pupation sites. Numerous abiotic variables have been shown to influence both intra and interspecific differences in pupal behavior (Markow, 1979; Sokolowski and Hansell, 1983) [16]. Even biotic factors like sex, density, locomotory path length, developmental time and digging behavior plays important role in PSP (Beltramí *et al.,* 2010; Sokal *et al.,* 1960; Medina-Munoz *et al.,* 2005) [4, 23].

Genetic factors have also been found to control pupation behavior in a variety of *Drosophila* species (Bauer and Sokolowski, 1985, 1988; Garcia-Florelz *et al.,* 1989) [3, 10]. For instance, Joshi and Mueller (1993) reported that pupation height is a polygenic trait that responds effectively to bidirectional selection. Similarly, preference of pupation height (in shell vials) in *D. ananassae* was found to be under polygenic control and most of the variances were additive in nature (Pandey and Singh 1993) [17]. In a recent study, researchers discovered that loci on chromosomes X and II control differences in interspecific pupation behavior of

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Drosophila sechellia and *Drosophila simulans*, while intraspecific variation is highly polygenic, with loci on chromosome III (Erezyilmaz and Stern, 2013) [8].

There is a link between PSP and glue proteins, which are generated by the salivary glands in larvae. Studies on glue proteins demonstrate that the more glue proteins secreted by the salivary glands, the more the larvae tend to migrate away from the surface of the media (Shivanna *et al.,* 1996) [21].

Ensure® is a comprehensive, well balanced diet that includes HMB, macronutrients (high quality protein, fat and carbohydrates) and micronutrients (vitamins and minerals). It is the world's most popular nutrition supplement drink that enhances bone and muscular development, immunological function, and overall health. Ensure® provides thirty-two nutrients, including high quality protein, calcium, zinc, vitamin C, vitamin D and iron. It also contains a unique substance called HMB. HMB, or β-Hydroxy-β-Methyl butyrate, is an amino acid that promotes and preserves muscle growth. Although it includes eleven immune boosting nutrients-vitamins A, C, E, B6, iron, D, Folate, Zinc, and copper-Ensure® is a high protein nutrient, the peoples of all ages take this drink (Aishwarya *et al.,* 2024) [1].

There is a published data available on the effect of Ensure® nutrition supplement on starvation resistance in *Drosophila melanogaster* (Aishwarya *et al.,* 2024) [1]. A number of studies are available, on the effect of temperature, humidity, light, intra and interspecific competition, pH, glue protein on the PSP in different *Drosophila* species (Seema and Girish, 2019; Divya singh *et al.,* 2022; Manning and Markow, 1981; Bezerra Da Silva *et al.,* 2019; Hodge and Simon, 2001; Vandal *et al.,* 2008; Shivanna *et al.,* 1996) [20, 77 15, 5, 11, 25, 21]. However, the effect of the Ensure® nutrition supplement on pupation site preference behavior has not been carried out in any other animal model. Hence present study has been undertaken to study the effect of the Ensure® nutrition supplement on pupation site preference in *Drosophila melanogaster.*

Materials and Methods

The Ensure® nutrition supplement powder was purchased from Medplus pharmacy shop, Sriramapura, Mysuru, Karnataka, India. This Ensure® nutrition supplement powder was used to prepare the experimental media.

Establishment of Stock

Experimental Oregon K strain of *Drosophila melanogaster* used in the study was collected from *Drosophila* stock centre. Department of studies in Zoology, University of Mysore, Mysuru and this stock was cultured in bottles containing wheat cream agar media (100g of jaggery, 100g of wheat cream rava, 10g of Agar was boiled in 1000ml distilled water and 7.5ml of propionic acid was added). Each with 20 flies (10 males and 10 females). These flies were maintained in laboratory conditions such as humidity of 70% and 12 hours dark: 12 hours light cycles and temperature 22° ± 1°c.

Effect of Ensure® Nutrition Supplement on Pupation Site Preference in *D. melanogaster.*

First instar larvae were collected using Delcour procedure (1969) and subjected to culture bottles containing wheat-cream agar media: Wheat cream agar media was prepared from 100g of jaggery, 100g of wheat cream rava powder, 10g of agar boiled in 1000ml distilled water and 7.5ml of propionic acid added to it, test media (referred as Ensure®): Ensure® media was prepared from 100g of jaggery, 100g of Ensure® nutrition supplement powder, 10g of agar boiled in 1000ml of distilled water and 7.5ml of propionic acid added to it; Mixed media (Wheat cream agar + Ensure®): Mixed media is prepared from 100g of jaggery, 50g of wheat cream powder and 50g of Ensure® nutrition supplement powder, 10g of agar boiled in 1000ml of distilled water and 7.5ml of propionic acid added to it. The obtained pupa was used to study the pupation site preference. Total forty larvae were observed for pupation site preference in each of the culture media. Experiments were carried out separately and simultaneously for all the three diets studied.

Result and Discussion

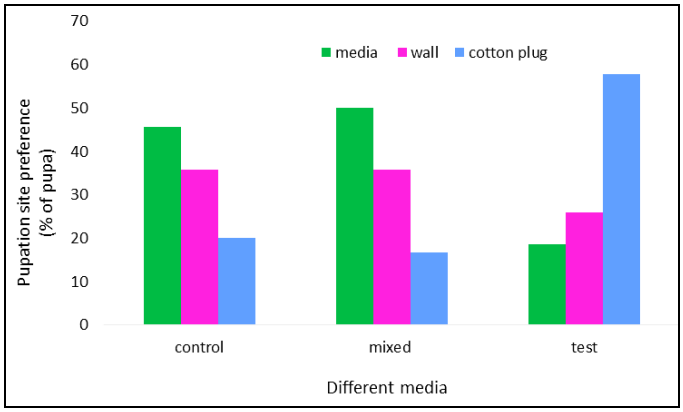

Fig 1: Effect of Ensure® nutrition supplement on the pupation site preference of the *D. melanogaster*.

Figure-1, represents the pupation site preference of larvae in different media-control media, mixed media (Wheat cream agar+ Ensure®) and test media (Ensure®). The surface of media, the glass wall of the media bottle, and the site nearby/on the cotton plug were the preferred locations by the larva to pupate. The above graph was built by taking percentage of larva pupated on the surface of media, the glass wall of the media bottle, and the site nearby/on the cotton plug.

From the figure 1, it was revealed that in different media the larvae prefer different sites for pupation. In control media, more percent of pupae found on the surface of media, followed by on the wall of the glass and a smaller percent of pupa found nearby/on the cotton plug. Similar observation was seen in the mixed media. Whereas in test media, a greater percent of pupas was found nearby/on the cotton, followed by on the wall of the glass and less number on the surface of the media. This variation in PSP was mainly influenced by concentration of protein in diet, in our experiment quantity of the nutrients varies in different media. High protein content can be seen in test media compared to control and mixed media. Our study supports the findings of Krittika *et al.,* 2019 [14], according to their research low protein concentration decreases pupation height and high concentration of protein increases pupation height in *D. melanogaster*.

In the *Drosophila* development, pupal behaviour plays a crucial role, as the location that the larvae choose might have a negative impact on their chances of surviving as pupae (Someoto and Miller, 1968) [19]. PSP is majorly influenced by many external factors like light, temperature, humidity, pH, larval density, interspecies and internal factors like amount of glue protein secreted in the salivary gland, locomotory path length, sex, digging behaviour, developmental time and genetic factors.

In our experiment, light had no effect on the variance in pupation site preference, since equal amounts of light and dark phases were maintained consistently. Contrast to our result, some studies shows that if PSP was influenced by the light, the *Drosophila melanogaster* prefers dark region to pupate because it is photopositive, whereas its sibling species *D. simulans*, it is a photoneutral prefers the light region to pupate, this behaviour aid to reduce competition between two species (Manning and Markow, 1981) [15].

Studies on PSP in *D. jambulina* showed that, at higher temperature (30°c) larva tends to pupate on food and at a lower temperature (21°c) it pupates on cotton plug. According to Seema and Girish (2019) [20], lower temperature caused less glue protein to be created, which allowed larvae to migrate along the edges of the container for pupation on cotton and higher temperature worked as an inducer for the production of large quantities of glue protein, that helps the pupa to attach food for pupation. On the other hand, consistent laboratory temperatures were maintained during our research. Therefore, variations in temperature were not the cause of the variance in PSP.

The number of studies examining the relationship between pH and pupation distance reveals that larvae prefer to pupate near the lowest pH resource as opposed to the highest pH resource. This suggests that acidic resources have an impact on development time and that larvae would travel greater distance to become more fit (Hodge *et al.,* 1996; Vandal *et al.,* 2008) [25]. In our research we maintained constant pH it could not be a reason for differences in a pupation site preference.

High pupation site preference is correlated with increased larval density (Singh and Pandey, 1993) [22]. Increased larval density leads to intraspecific competition and demands for food. The crowd and food deprived condition lead to high prone for larval cannibalism in *D. melanogaster* and migration to the high pupation site, it is one of the anticannibalistic strategy (Bezerra Da Silva *et al.,* 2019) [5]. But in our experiment, larval density was less, therefore more percent of pupa were present on the surface of media in control and mixed media. The food-selected pupae have no competition and keep all the food for themselves (Seema and Girish, 2019) [20]. Increased larval density also leads to high levels of toxic nitrogenous metabolic wastes such as urea, uric acid and ammonia were released into the media. Female fertility, adult growth, and larval survival are all known to be reduced by larval biotic residues (Vandal *et al.,* 2009).

The PSP was also influenced by humidity. According to Divya singh *et al.,* (2022) [7], in wet conditions larva pupate on the wall and nearby/on the cotton because surrounding moisture may cause challenges to construct pupal chamber. During dry conditions, larva pupates on media where high-water content is present. our experiment supports above statement, that the test (Ensure®) media was viscose, hence greater percent of pupa can be observed nearby/on the cotton and followed by on the wall of the media bottle to protect from the moisture.

The Ensure® nutrition supplement contains a unique substance called HMB. HMB, or β-Hydroxy-β Methyl butyrate, is an amino acid that promotes and preserves muscle growth (Holeček, 2017) [12]. Due to the presence of HMB substance in the test media, it prompts muscle growth in the larva because of that most of the larva climbed and pupate nearby/on the cotton and followed by on the wall of the bottle.

Another factor contributing to higher pupation locations is a larger feeding area and reduced digging activity (Sokolowski and Hansell, 1982). In our experiment, the foraging area was the same for all larvae in all the different types of media. There was enough space for each larva to find food, and digging was simple. Therefore, this factor was not the cause for variation in PSP in our experiment.

The release of a salivary gland protein known as glue protein is another significant element that influences pupation site preference. The pupation site is determined by the quantity of glue proteins released (Shivanna *et al.,* 1996) [21]. They state that larvae that secrete more glue protein often pupate on cotton, larvae that secrete half as much glue protein typically pupate on glass surfaces, and larvae that secrete very little glue protein typically pupate on media surfaces. In our experiment we did not measure the quantity of glue proteins released by the larvae so, we were unable to draw the conclusion that the difference in the pupation site preference was caused by the quantity of glue proteins released.

In our experiment light, temperature, humidity, pH, larval density was maintained constantly and even same species were used. But variation in PSP across different media was influenced by less competition, viscosity, dietary protein concentration and majorly by the nutrient (HMB) present in the Ensure® product. Hence observed variation in our experimental results was due to the effect of quantity and quality of the nutrients present in different media.

Conclusion

Our study revealed that PSP varies across different media, in control and mixed media greater percent of pupa found on the surface of media, whereas in test media greater percent of pupa found nearby/on the cotton plug. It can be concluded that PSP was an adaptable character that was used by the *Drosophila* to protect itself from the desiccation, disease, predators and mainly to increase the survivability of the emerging adult flies. Further in our study variation in PSP in different media was due to the variation in nutrient quantity and quality present in different media.

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