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Drosophila melanogaster Pupae Orientation in Several Culture Conditions

Ahmad Fauzi[1*], *Diani* Fatmawati[1,2], *Siti* Zubaidah[3], and *Hendra* Susanto[3]

[1]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Malang, Jl. Raya Tlogomas 246 Malang 65144, Indonesia
[2]Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, 1732 Deogyeong-daero, Giheung-gu, Yongin-si, Gyeonggi-Do 17104, Republic of Korea
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jl. Semarang No. 5, Malang 65145, Indonesia

Abstract. Environmental conditions are indicated to affect pupal orientation in *Drosophila melanogaster*. The aim of this study was to analyze the differences in the number of upright, tilted, and flat pupae under 4 experimental conditions: uncontaminated conditions (condition 1), contaminated conditions for one generation (condition 2), contaminated conditions for two generations (condition 3), and the condition in which the contamination has been removed by five generations (condition 4). Each experiment involved 72 experimental units and each experimental unit consisting of one fruit fly culture. Each culture was derived from five pairs of wildtype fruit flies. The Kruskal-Wallis H Test was used as a hypothesis test while the Games-Howell was used for the post hoc test. The results of the analysis indicated that the pupal orientation was always significantly different in the four experimental conditions: condition 1 ($\chi^2(2)=51.769$, $p<0.001$), condition 2 ($\chi^2(2)=47.543$, $p<0.001$), condition 3 ($\chi^2(2))=48.835$, $p<0.001$), and condition 4 ($\chi^2(2)=49.972$, $p<0.001$). The number of pupae in the tilted orientation was always significantly higher than the other two orientations. On the other hand, the number of pupae in the upright orientation was not significantly different from the flat orientation in the four experimental conditions. The findings obtained in this study can be used as a basis for studying the behavior of fruit fly larvae under certain disease or environmental conditions, especially during the pupal formation stage.

1 Introduction

Drosophila melanogaster has been involved in various research in the field of biology [1–3]. This insect is used as a research subject in the fields of genetics [4], ecology [5], nanotoxicity [6], to microbiota studies [7]. The incentive for using fruit flies as subjects in various fields cannot be separated from their superior characteristics as model organisms, for example low maintenance costs, small size, and short generation time [8, 9]. In addition, fruit flies have various genes and metabolic pathways that are similar to humans, so Drosophila are able to

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[*] Corresponding author: ahmad_fauzi@umm.ac.id

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study various human health conditions in various controlled laboratory conditions [10]. With the development of various molecular techniques and complete genetic data on Drosophila, researchers can also easily model various genetic diseases and human degenerative diseases [11,12].

When involving Drosophila, researchers can also easily condition this organism's environment according to the external factors being studied. To ascertain the influence of these external factors, various assays were developed to analyze Drosophila's response to changes in environmental conditions [10,13,14]. Many assays can be performed on Drosophila, such as climbing assay [15–17], flight assay [18,19], feeding assay [20–22], flight assay [18,19], and sleep behavior assay [23,24]. In addition to these assays, pupae orientation is also a variable that is used as a research variable, although its use is still less massive when compared to other assays.

Pupa orientation assay can be found in various studies that examine the influence of strain and several environmental stress conditions. The location of pupation in Drosophila is regulated by various genes [25–27] and can be related to the behavior of this organism in the final stages of the larval phase [26]. The height and orientation of the pupa may also be related to the organism's muscle activity [28]. One of the main advantages of pupa orientation assay is its ability to measure morphological responses that are sensitive to various environmental changes and conditions. In connection with this statement, pupation can be influenced by the content of certain substances in the medium where it is cultured, such as the concentration of NaCl [27] as well as substances that are neurotoxic [29] and substances that affect the level of reactive oxygen species [30]. Pupational orientation data can also be linked to changes in proprioception [31] and is involved as an assay in research examining the impact of heavy metal [32]. In addition, measurement of pupa orientation also has the advantage of non-invasiveness due to this assay does not disturb the organism, so it allows for more accurate observation of the organism's response to the environment. Thus, the use of pupa orientation assay has the potential to become a reliable tool in identifying the environmental impact on organisms.

Although measurement of pupa orientation has great potential in providing insights into the organism's response to the environment, its potential still needs to be explored further. In addition, research on the influence of various conditions on pupa orientation in *D. melanogaster* is still relatively rare in Indonesia. This study involved a series of varied experimental conditions. Four experimental conditions were involved to cover a wide spectrum of exposure scenarios. This approach not only allows the examination of direct contamination effects, but also facilitates the evaluation of long-term consequences that may occur due to sustained exposure or persistent effects on future generations. By combining this innovative approach, this study can build a bridge between previous research and possible further development. The novelty of this study lies in the integration of the latest aspects of pupa orientation in a diverse set of experimental conditions. Through the campaign to use pupa orientation assay, this study aims to arouse the interest and awareness of Indonesian researchers to the great potential of this measurement, so that it can be better integrated into scientific research in this country.

2 Method

2.1 Research design and model organism

This study was conducted using a pretest-posttest experimental design involving the model organism *D. melanogaster* strain wildtype. The organisms were obtained from the Genetics Laboratory of Universitas Negeri Malang. The study was conducted in four different experimental conditions: uncontaminated (condition 1), contaminated for one generation (condition 2), contaminated for two generations (condition 3), and condition where the contamination has been removed for five generations (condition 4). Each experimental condition was repeated 72 times, and each experiment involved one culture of fruit flies that originated from five pairs of wildtype fruit flies.

2.2 Pupae orientation observation

The procedure for observing pupae orientation was based on the methodology that has been described by [13]. First, special culture tubes that are used for pupae behavior testing were prepared and labeled according to the experimental conditions that are being studied. Before starting the testing, each culture tube was filled with culture medium that has been adjusted to the predetermined concentration of contamination. After that, three pairs of *adult D. melanogaster* were placed in each test culture tube that has been filled with culture medium according to the different experimental conditions.

The *D. melanogaster* pairs in the culture tubes were given 10 days to mate. On Day 10, the observation of pupae orientation was conducted. Each pupa was carefully observed by counting the number of pupae that belong to a certain orientation category, namely "upright" (vertical position), "tilted" (oblique position), or "flat" (horizontal position) in each zone inside the culture tube.

In determining the pupae orientation category, the parameter that is used is the angle of inclination of the pupa against the gravity axis. Pupae is categorized as "upright" if the angle of inclination is between 0 or 180 degrees, as "tilted" at an angle around 45 or 225 degrees, and as "flat" at an angle around 90 or 270 degrees. This categorization allows us to obtain detailed information about the variation of pupae orientation in the face of gravity, which in turn can provide valuable insights into the organism's adaptive response to environmental changes.

2.3 Data analysis

After the data of pupa orientation from all experimental units were collected, two stages of analysis were performed. The first stage was aimed at processing the overall data from the four experimental conditions, while the second stage was aimed at analyzing the data in each experimental condition separately. In the first stage, a two-way Analysis of Variance (ANOVA) test was conducted. The number of pupae was positioned as the dependent variable, while the pupa orientation and experimental condition were the factor 1 and factor 2. Next, a post hoc test with Bonferroni correction was run to analyze the difference of means in each interaction. In the second stage, a hypothesis test using The Kruskal-Wallis H Test was used to evaluate whether there is a significant difference in pupa orientation between the experimental condition groups. If the test shows a significant difference, a post hoc Games-Howell test was used to identify the significantly different groups.

3 Results and Discussion

This study has collected data on pupa orientation from 288 experimental units that were spread across four experimental conditions. The average percentage of pupa orientation in each experimental condition is presented in Table 1. Based on Table 1, the tilted category dominates the pupa orientation in each experimental condition. Next, to ensure that there is a significant difference in pupa orientation in each experimental condition, a two-way ANOVA test was conducted. The summary of the ANOVA test results is presented in Table

2. Based on the results of the ANOVA test, there is a significant difference in the number of pupae based on their orientation (F=299.973, p<0.001) and based on the experimental condition (F=23.047, p<0.001). Furthermore, pupa orientation also interacts significantly with the experimental condition (F=2.803, p=0.012).

Table 1. Percentage of pupae in each orientation category.

Condition	**Mean ± Standard Error of the percentage of pupae in each orientation category**		
	Flat	**Tilted**	**Upright**
Uncontaminated	17.19 ± 1.61	72.04 ± 2.10	10.77 ± 1.41
Contaminated for a generation	11.78 ± 1.03	77.18 ± 1.79	11.04 ± 1.00
Contaminated for two generations	19.43 ± 1.14	64.47 ± 1.25	16.10 ± 1.11
Contamination has been removed	18.29 ± 1.57	68.79 ± 2.34	12.92 ± 1.64

Table 2. The results of the Two-Way ANOVA test to analyze the difference in the mean number of pupae in each orientation category and experimental conditions.

Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**
Orientation	201172.528	2	100586.264	299.973	< .001
Condition	23183.844	3	7727.948	23.047	< .001
Orientation ✻ Condition	5639.667	6	939.944	2.803	0.012
Residuals	92547.625	276	335.317		

Next, the difference in mean for each interaction group is visualized in the graph in Figure 1. In relation to the graph, the post hoc test results for the interaction group are presented in Table 3. Based on Figure 1 and Table 3, the number of tilted pupae in all experimental conditions occupied the top position. On the other hand, the upright pupae occupied the bottom position.

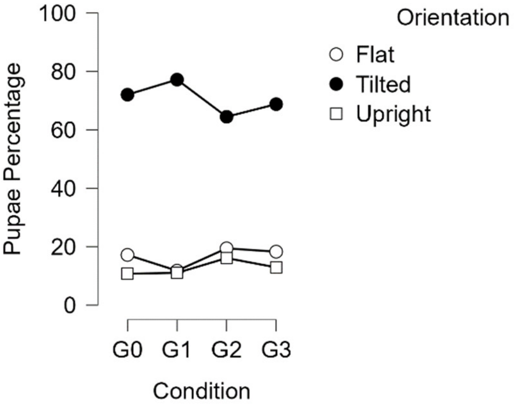

Fig. 1. Descriptive plot that visualizes the percentage fluctuation of each pupal orientation category.

Table 3. Post Hoc Comparisons with Bonferroni correction for the interaction of pupal orientation categories with experimental conditions (G0=uncontaminated, G1=contaminated for one generation, G2=contaminated for two generations, and G3=contamination removed).

Interaction		Mean Difference	SE	t	p_{bonf}
Flat G0	Tilted G0	-53.667	5.286	-10.152	< .001
	Upright G0	6.958	5.286	1.316	1
	Flat G1	8.833	5.286	1.671	1
	Tilted G1	-32.667	5.286	-6.18	< .001
	Upright G1	9.75	5.286	1.844	1
	Flat G2	-9.167	5.286	-1.734	1
	Tilted G2	-74.958	5.286	-14.18	< .001
	Upright G2	-5.167	5.286	-0.977	1
	Flat G3	-2.958	5.286	-0.56	1
	Tilted G3	-57.417	5.286	-10.862	< .001
	Upright G3	1.917	5.286	0.363	1
Tilted G0	Upright G0	60.625	5.286	11.469	< .001
	Flat G1	62.5	5.286	11.823	< .001
	Tilted G1	21	5.286	3.973	0.006
	Upright G1	63.417	5.286	11.997	< .001
	Flat G2	44.5	5.286	8.418	< .001
	Tilted G2	-21.292	5.286	-4.028	0.005
	Upright G2	48.5	5.286	9.175	< .001
	Flat G3	50.708	5.286	9.593	< .001
	Tilted G3	-3.75	5.286	-0.709	1
	Upright G3	55.583	5.286	10.515	< .001
Upright G0	Flat G1	1.875	5.286	0.355	1
	Tilted G1	-39.625	5.286	-7.496	< .001
	Upright G1	2.792	5.286	0.528	1
	Flat G2	-16.125	5.286	-3.05	0.165
	Tilted G2	-81.917	5.286	-15.497	< .001
	Upright G2	-12.125	5.286	-2.294	1
	Flat G3	-9.917	5.286	-1.876	1
	Tilted G3	-64.375	5.286	-12.178	< .001
	Upright G3	-5.042	5.286	-0.954	1
Flat G1	Tilted G1	-41.5	5.286	-7.851	< .001
	Upright G1	0.917	5.286	0.173	1
	Flat G2	-18	5.286	-3.405	0.05
	Tilted G2	-83.792	5.286	-15.851	< .001

Interaction		**Mean Difference**	**SE**	**t**	**p_{bonf}**
	Upright G2	-14	5.286	-2.648	0.564
	Flat G3	-11.792	5.286	-2.231	1
	Tilted G3	-66.25	5.286	-12.533	< .001
	Upright G3	-6.917	5.286	-1.308	1
Tilted G1	Upright G1	42.417	5.286	8.024	< .001
	Flat G2	23.5	5.286	4.446	< .001
	Tilted G2	-42.292	5.286	-8.001	< .001
	Upright G2	27.5	5.286	5.202	< .001
	Flat G3	29.708	5.286	5.62	< .001
	Tilted G3	-24.75	5.286	-4.682	< .001
	Upright G3	34.583	5.286	6.542	< .001
Upright G1	Flat G2	-18.917	5.286	-3.579	0.027
	Tilted G2	-84.708	5.286	-16.025	< .001
	Upright G2	-14.917	5.286	-2.822	0.338
	Flat G3	-12.708	5.286	-2.404	1
	Tilted G3	-67.167	5.286	-12.706	< .001
	Upright G3	-7.833	5.286	-1.482	1
Flat G2	Tilted G2	-65.792	5.286	-12.446	< .001
	Upright G2	4	5.286	0.757	1
	Flat G3	6.208	5.286	1.174	1
	Tilted G3	-48.25	5.286	-9.128	< .001
	Upright G3	11.083	5.286	2.097	1
Tilted G2	Upright G2	69.792	5.286	13.203	< .001
	Flat G3	72	5.286	13.621	< .001
	Tilted G3	17.542	5.286	3.318	0.068
	Upright G3	76.875	5.286	14.543	< .001
Upright G2	Flat G3	2.208	5.286	0.418	1
	Tilted G3	-52.25	5.286	-9.884	< .001
	Upright G3	7.083	5.286	1.34	1
Flat G3	Tilted G3	-54.458	5.286	-10.302	< .001
	Upright G3	4.875	5.286	0.922	1
Tilted G3	Upright G3	59.333	5.286	11.224	< .001

Furthermore, the Kruskal-Wallis H Test was employed to assess potential variations in pupal orientation across the four unique experimental conditions. The summarized results of this analysis are presented in Table 4. The outcomes of this analysis unveiled significant differences in pupal orientation among all the experimental conditions : conditions without contamination ($\chi^2(2)=51.769$, $p<0.001$), contamination for one generation ($\chi^2(2)=47.540$,

$p<0.001$), contamination for two generations ($\chi^2(2)=48.835$, $p<0.001$), and conditions with eliminated contamination over several generations ($\chi^2(2)=49.972$, $p<0.001$). These findings emphasize that the orientation of pupae remained consistently influenced by the specific experimental conditions, indicating that the manipulated variables played a role in shaping pupal behavior. To further investigate the significance of these differences, post hoc analysis was conducted using the Games-Howell test, which provides a deeper understanding of the specific variations between each condition (Table 5).

Table 4. The Kruskal-Wallis H Test results for each experimental condition.

Condition	Statistics	df	p
Uncontaminated	51.769	2	< 0.001
Contaminated for a generation	47.540	2	< 0.001
Contaminated for two generations	48.835	2	< 0.001
Contamination has been removed	49.972	2	< 0.001

Table 5. Games-Howell Post Hoc Comparisons for each experimental condition.

Condition	Comparison	Mean Difference	SE	t	df	p_{tukey}
Uncontaminated	Upright - Titled	-61.268	2.534	-24.179	40.245	< .001
	Upright - Flat	-6.417	2.142	-2.995	45.235	0.012
	Titled - Flat	54.851	2.649	20.706	43.067	< .001
Contaminated for a generation	Upright - Titled	-66.142	2.052	-32.238	36.219	< .001
	Upright - Flat	-0.733	1.443	-0.508	45.961	0.868
	Titled - Flat	65.408	2.066	31.652	36.861	< .001
Contaminated for two generations	Upright - Titled	-48.371	1.674	-28.891	45.351	< .001
	Upright - Flat	-3.324	1.593	-2.087	45.965	0.104
	Titled - Flat	45.047	1.695	26.576	45.611	< .001
Contamination has been removed	Upright - Titled	-55.867	2.86	-19.532	41.152	< .001
	Upright - Flat	-5.373	2.271	-2.366	45.919	0.057
	Titled - Flat	50.494	2.822	17.891	40.205	< .001

The complex relationship between larval behavior during prepupation [26] and various environmental factors [27] can be the reason for assuming there is a significant difference in the number of pupae position. The microenvironmental conditions around the pupa are an important factor in pupa development [33,34]. Light, temperature and humidity are common abiotic factors that may influence the position of the larvae attached to the vertical surface before becoming pupae [35,36].

Furthermore, in nature, pupal orientation can also be influenced by the insect's access to nutritional sources. Even though organism does not consume food during the pupal stage [37], it needs nutrition during the stages before and after becoming a pupa [38]. Correspondingly, different nutritional conditions will also influence pupa development, for example how long it will take for the pupa to eclosion into an adult fly [39,40]. Several previous studies also reported that several substances contained in the fly media will influence the position of Drosophila pupae, such as the content of toxic compounds [29] and the concentration of NaCl in the media [27].

The positioning of *D. melanogaster* pupae in relation to the gravitational axis is influenced by a complex interplay of factors. In one study, it was observed that active *D. melanogaster* larvae often construct extensive tunnels within agar substrates and encase their pupae within these tunnels [26]. These embedded larvae exhibit a more extended period of development from egg to pupariation compared to larvae that pupate on the surface [26]. This suggests that the construction behaviors of larvae, which likely involve their alignment with the gravitational axis, may be influenced by developmental timing. In contrast, another study found no significant correlation between larval development duration and pupation elevation [41], indicating the involvement of additional factors. The reasons underlying this wandering behavior remain unclear but could be attributed to fluctuations in temperature, variations in food quality, or environmental factors such as interactions with conspecifics or exposure to potential threats like parasitoids [42]. Consequently, it appears that the orientation of *D. melanogaster* pupae in relation to the gravitational axis is subject to regulation by a combination of physiological and environmental factors.

Regarding the Games-Howell analysis, the findings consistently highlighted that the number of pupae in the tilted significantly exceeded those in the upright and flat orientations across all experimental conditions. Conversely, the number of pupae in the upright orientation did not exhibit statistically significant differences when compared to those in the flat orientation within the framework of the four experimental conditions. These outcomes underscore the robust influence of the experimental conditions on the prevalence of pupal orientations, particularly the tilted orientation, which consistently stands out as the most prevalent.

The consistency of the tilted orientation as the pupal orientation with the highest percentage in all experimental conditions indicates the existence of an important adaptation mechanism or result experienced by Drosophila. One speculation that can be put forward is that the tilted orientation provides various benefits for the pupa, such as enhanced survival, developmental efficiency, or response to environmental cues.

One potential reason for the high percentage of tilted is related to the anatomical alignment of the pupa with the axis of gravity. In line with this explanation, previous studies reported that adult flies are very sensitive to the direction of gravity's pull [43]. Although that study does not directly relate to pupa orientation, the research findings suggest that gravity has an important influence on Drosophila orientation. The tilted orientation allows the pupa to stick firmly to the wall and has optimal flexibility for fly metamorphosis. This orientation may also optimize energy savings during the pupal stage as well as accommodate maximum eclosion of adult flies. In line with laboratory conditions, Drosophila larvae in nature also often live in a variety of complex habitats, in terms of surface, texture or slope angle of the substrate. Tilted orientation may have the potential to increase the pupa's adaptability to various micro-environmental conditions and maximize the pupa's interaction with the surface, different, more flexible. However, although these speculations are logical, further study regarding the adaptive significance of tilted orientation is necessary.

The findings of this study also revealed consistent differences in the number of pupae based on their orientation. These findings, in line with recommendation from the other studies, indicate pupal orientation as a distinctive consistent feature and a potential biomarker in various investigations [44,45]. By not collecting or processing the pupae, researchers can directly collect pupa orientation data through direct observation without disturbing the flies being studied. This non-invasive data collection method is beneficial in research involving insects [46,47]. Given its ease in collecting data, this assay is recommended to be applied as an alternative variable when researchers examine the influence of various internal and external factors on several aspects of biology. In line with several previous studies that have examined genetic conditions [25–27], other studies that attempt to examine the role of other genes involved in pupal pupation also need to be continued.

To date, pupal orientation has been less frequently explored and exploited than pupal height [48–50]. This research has explored pupa height when influenced by dietary protein restriction [49], radiation exposure [51], to certain metabolic conditions [52]. On the other hand, by monitoring changes in the percentage of pupal orientation, the researchers were able to explore Drosophila's response to diverse conditions. Just like several previous studies, several researchers have studied various stress triggers on pupal orientation, such as exposure to heavy metals [32] and other toxic substance [29]. Investigating the influence of various external factors such as temperature, humidity and gravity will provide a unique perspective on the adaptability and plasticity of species. This knowledge can have implications for understanding broader ecological patterns and responses to environmental change.

4 Conclusion

This study sheds light on the pivotal role of pupal orientation as a dynamic and informative parameter in the context of *D. melanogaster* research. The findings presented herein underscore the significance of this variable in assessing behavioral responses, potential stressors, and environmental influences. The analyses conducted, utilizing both ANOVA and Kruskal-Wallis tests, consistently revealed the substantial impact of experimental conditions on pupal orientation. This was reflected not only in the individual effects of orientation and condition but also in their compelling interaction. The robust patterns observed across various experimental scenarios highlight the reliability and sensitivity of pupal orientation as a measure of developmental dynamics. Moreover, the Games-Howell post hoc test accentuated the distinctiveness of the tilted orientation, reaffirming its prominence as a significant feature across different conditions.

These results collectively underscore the potential of pupal orientation as an invaluable tool for researchers investigating a range of topics, including toxicity assessments, ecological studies, and beyond. By harnessing the power of this unassuming yet insightful parameter, future studies can unlock a deeper understanding of how Drosophila navigate the challenges of their environment, respond to stressors, and adapt to changing conditions. As the scientific community continues to explore innovative avenues to decode the intricacies of biological systems, embracing the multifaceted significance of pupal orientation holds the promise of illuminating new dimensions in our comprehension of species-environment interactions.

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