Effects of *ort*^{1} mutation and gender on the recovery time of *Drosophila melanogaster* after being anesthetized by CO_2

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Abstract

The purpose of this study was to determine the effect of gender and the *ort*^{1} mutation on the recovery time of *Drosophila melanogaster* after being exposed to CO_2. Previous studies suggested that the mutant *D. melanogaster* takes longer to recover from CO_2 anesthesia compared to the Oregon-R wild-type strain. Yet, there has been little research done on the effects of gender on the recovery time. To test the recovery time between the two genders and the two strains, we exposed two groups, a wild-type group and a mutant group of *D. melanogaster,* to CO_2. The *D. melanogaster* were in vials when they were exposed to CO_2 and after the exposure we monitored each replicate individually in Petri dishes. Recovery time and sex data were collected for each of the replicates. The difference in the recovery time between the wild type and the mutant was not significant and gender had no effect on the recovery time as the *p*-value was less than 0.5. However, the effects of gender on recovery time in *Drosophila melanogaster* are not the same for the wild-type and mutant strain.

Introduction

Drosophila melanogaster, commonly known as the fruit fly, is a rigorously studied organism in various research fields. It is one of the best understood eukaryotic organisms (Wixon & O'Kane 2000). Scientists who study active organisms like *D. melanogaster* need to administer an anesthetic to work on them (Colinet & Renault 2012). Anesthetic methods include chilling, CO_2 exposure and etherisation (Colinet & Renault 2012). The most popular anesthetic method in entomological research is CO_2 exposure (Colinet & Renault 2012). They found that there were significant metabolic effects due to CO_2 exposure; however, these effects were only observed during the short term recovery and nothing was observed for the long term recovery. Side effects of CO_2 exposure included, but were not limited to, an increase in hemolymph acidity which stops the beating of the heart (Colinet & Renault 2012).

The objective of our study was to determine the effects of gender and *ort*^{1} mutation on the recovery time of *D. melanogaster* after exposing them to CO_2. In addition, we wanted to

understand the role of *ort*^{1} gene in the nervous system of *D. melanogaster*. The *ort*^{1} mutation is a large deletion in the hclA gene on the third chromosome, which affects synaptic transmission in the visual system of *D. melanogaster*. This causes hypersensitivity to light compared to the Oregon-R wild-type strain (Iovchev *et al.* 2002). Therefore, we predicted that the *ort*^{1} mutant *D. melanogaster* will recover more slowly than the wild-type strain. Previous studies suggest that mutants take longer to recover than wild-type strains (Iovchev *et al.* 2002). Lheritier (1948) suggested that there is not a significant difference in the recovery time between wild-type male and wild-type female *D. melanogaster*. However based on other studies, we predicted that females will take longer to recover compared to males (Perron *et al*. 1972).

On the molecular level, *D. melanogaster* shares many similar features and physiological pathways with humans. In fact, 75% of known human disease genes can be found in *D. melanogaster* (Belen & Tong 2012). Therefore, by better understanding the differences in behavior between wild-type and mutant *D. melanogaster* that are caused by a genomic difference, we can investigate similar cases of genes directly influencing behaviors in humans. Moreover, our experiment is useful for other scientists who use CO_2 anesthesia to paralyze *D. melanogaster* to perform experiments, because it will help them know how long the flies will stay immobilized. CO_2 anesthesia is used for many purposes in *D. melanogaster*, such as identification of specimens, surgeries, dsRNA injection and sexing of insects (Colinet & Renault 2012).

H_{01}: Gender of the organism has no effect on recovery time in *D. melanogaster.*

H_{A1}: Gender of the organism has an effect on recovery time in *D. melanogaster.*

H_{02}: There is no difference in recovery time between wild-type and mutant *D. melanogaster*.

H_{A2}: There is a difference in recovery time between wild-type and mutant *D. melanogaster*.

H_{03}: The effect of gender on recovery time in *D. melanogaster* is the same for wild-type and mutant strain.

H_{A3}: The effect of gender on recovery time in *D. melanogaster* is not the same for wild-type and mutant strain.

Methods

After anesthetizing *D. melanogaster* with CO_2, we studied the difference in the recovery time between males and females of wild-type and mutant *ort*^{1} strain. To study the effect of the mutation and gender on the recovery time of *D. melanogaster*, we exposed 82 *D. melanogaster* (18 wild-type males, 23 wild-type females, 19 *ort*^{1} mutant males, and 22 *ort*^{1} females) to CO_2 for 150 seconds. According to previous studies, at least 15 seconds of CO_2 exposure is required to get full anesthetic effects in *D. melanogaster* (Lheritier 1948). Furthermore, exposure to CO_2 for longer than three minutes negatively effects growth and reproduction in insects (Brooks 1957). Therefore, we chose to expose *D. melanogaster* to CO_2 for 150 seconds as it was greater than the minimum exposure time required, and was not long enough to cause detrimental effects to the organisms.

We performed our experiment over two lab periods for which the environmental factors were kept constant. On both days the room temperature was 23°C. The light intensity was 638 lux on the first day and 675 lux on the second day. To ensure consistency, on both days, we set the needle of the CO_2 tank to 7.5 lb/inch^2. In the first lab period, we worked with wild-type *D. melanogaster,* while in the second lab period we worked with mutant *ort*^{1} *D. melanogaster.* Our six stock vials each contained approximately 15 flies and food.

First, as shown in Figure 1, we labeled Petri dishes and marked a spot in the center of the dish. Then, we exposed vials, one at a time, to CO_2 for 150 seconds. Immediately following the CO_2 exposure, we used forceps to transfer individual anesthetized replicates to separate Petri dishes as shown in Figure 1. We placed the *D. melanogaster* on the marked spot in the center of the dish. Each group member monitored a group of Petri dishes using a standard timer to record recovery time as shown in Figure 2. We defined recovery time as complete body movement away from the initial marked spot. Once recovery time was recorded, we observed each replicate through a dissecting microscope to distinguish between males and females. This procedure was repeated for all six vials of *D. melanogaster*.

In order to analyze our data, we needed an equal number of replicates for each treatment group. Hence, we used a random number generator to randomly remove replicates from our data to get n=18 for each group. We calculated the mean recovery times and 95% confidence intervals for the four treatment groups, and conducted a two-way ANOVA test to analyze our data.

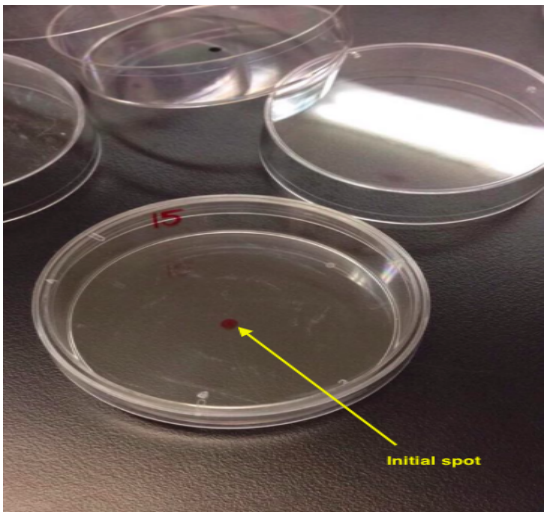

Figure 1. The initial spot where we placed anesthetized *D. melanogaster*.

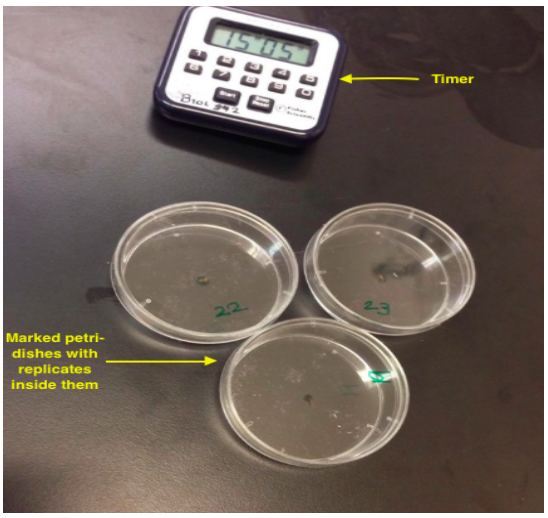

Figure 2. *D. melanogaster* were transferred to petri-dishes where we monitored movement and recorded recovery time.

Results

We found the mean recovery time for wild-type *D. melanogaster* males to be 359.2 seconds and wild-type *D. melanogaster* females to be 206.9 seconds. The 95% confidence intervals for wild-type *D. melanogaster* males and females were ±134.6 seconds and ±52.96 seconds, respectively. Furthermore, the mean recovery time for mutant ort^1 *D. melanogaster* males was 280.4 seconds and that of mutant ort^1 *D. melanogaster* females was 306.7 seconds. The 95% confidence intervals for mutant *D. melanogaster* males and females were ±44.49 seconds and ±68.64 seconds respectively. Using a two-way ANOVA test, we calculated the *p*-values for H_1, H_2, and H_3 to be 0.14, 0.81, and 0.04 respectively.

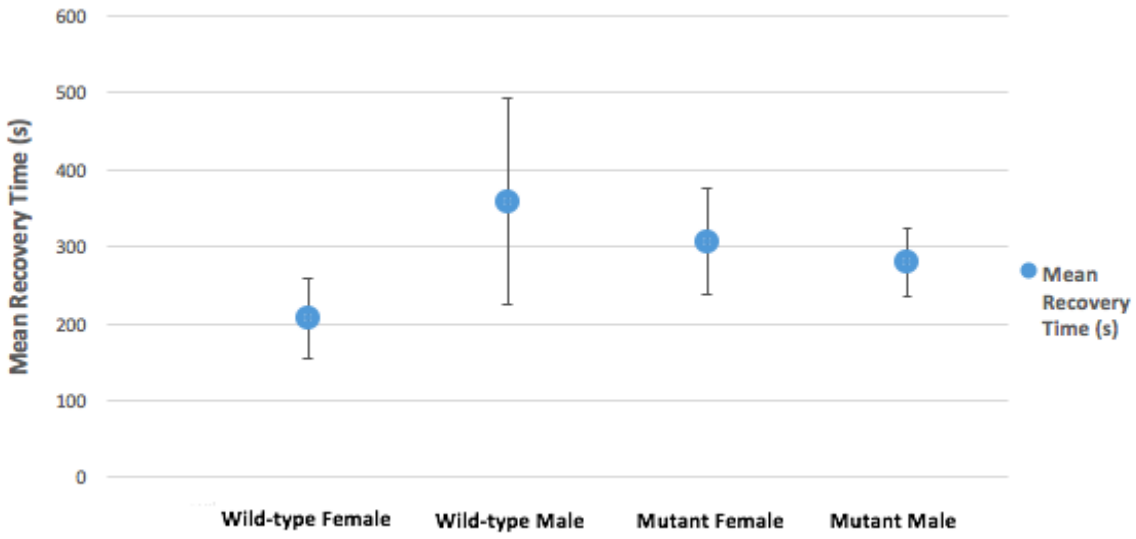

Figure 3. The mean recovery time (in seconds) after CO_2 exposure for each treatment group of *D. melanogaster*. For each treatment group, 18 replicates were used (n=18). The error bars represent 95% confidence intervals. The *p*-values calculated for H_1, H_2, and H_3 are 0.14, 0.81, and 0.04 respectively.

As seen in Figure 3, the mean recovery time for wild-type males was greater than that of wild-type females. Figure 3 also shows that mutant female *ort*^{1} *D. melanogaster* had a greater recovery time than mutant males. The largest variation was in male wild type and the smallest variation was in male *ort*^{1} *D. melanogaster.*

Discussion

First of all, with regards to H_{01}, our means were not significantly different, *p*-value (0.14 > 0.05). Therefore, we cannot reject the null hypothesis and cannot support the hypothesis that the gender of *D. melanogaster* has an effect on the recovery time of the fly. Oh *et al.* (2013) studied the sleep duration of different sexes of *D. melanogaster* and found that gender does not have an effect on the physiological pathway of waking up which is activated via histamine signaling. As previously stated, the *ort* mutation interferes with the histamine-gated channels and receptors (Gengs *et al.* 2002); therefore our result is consistent with Oh *et al.*'s (2013) findings.

Secondly, regarding H_{02}, the means calculated from the data did not show a significant difference (0.05 < *p*-value = 0.81). This means that we fail to reject the null hypothesis H_{02} that wild-type and *ort*^{1} *D. melanogaster* do not have significantly different recovery times. This finding contradicts our initial prediction. Oh *et al.* (2013) discuss the regulatory role of histamine receptors in wake-promoting signals in *D. melanogaster* and show that a deletion in the *ort* gene delays the recovery time of the organism. Furthermore, Iovchev *et al.* (2002) reported that *ort*^{1} flies have delayed recovery time after anesthetization because the *ort* gene is involved in the process of response to general anesthesia. Therefore, according to the literature, and due to the nature of the *ort*^{1} mutation and its negative effects on the histamine receptor physiological pathway of the organism (Gengs *et al.* 2002), we predicted that the

mutant strain would need more time to recover from the anesthesia. However, we did not find any significant difference between the recovery time of wild-type and the mutant strain after exposure to CO_2.

The reason for this inconsistency could be because a) we used a fixed concentration of CO_2 and Iovchev *et al.* used different doses of ether or b) the pathway for anesthetization with CO_2 and ether is different. In order to further investigate the possible effects of the *ort*^{1} mutation on the recovery rate compared to the wild type, different doses of CO_2 and/or different exposure durations to CO_2 could be used as treatments. Another explanation for not having found the predicted relationship between the recovery time and the *ort*^{1} mutation could be due to the biological variability of the organisms. In our observations, the mutant *D. melanogaster* were more brightly-colored and more transparent compared to the wild-types, which were darker. We also noticed that the mutants had comparatively narrower stripes on their bodies. It is possible that the mutant *D. melanogaster* were less mature (C. Pollock personal communication). Moreover, in the mutant vials, we did not find any larvae, whereas in the vials containing the wild-type *D. melanogaster*, there were a considerable number of larvae present which suggests the wild-type *D. melanogaster* had reached reproductive maturity. Thus the wild-type *D. melanogaster* may have been older than the mutants and this may be the reason for their faster recovery time.

Lastly, we rejected H_{03} because the calculated *p*-value (0.04) was less than 0.05; there is a significant difference in recovery time of females and males in wild-type and mutant *D. melanogaster* which means the effects of gender on recovery time in *D. melanogaster* is not the same for wild-type and the mutant strain. Exposure to CO_2 affects the central nervous system which immobilizes the *D. melanogaster* and causes a decrease in cardiac output

(Badre *et al.* 2005). Although these effects occur in both the wild-type as well as the mutant strain, according to our data there is a significant difference in the recovery between males and females of the wild-type and mutant *D. melanogaster*. Nassel (1999) emphasizes the role of histamine as a major neurotransmitter in synaptic transmission within the central nervous system of *D. melanogaster.* Furthermore, Iovchev *et al.* (2002) reported that the responses to the anesthetic were significantly different for both sexes of wild-type *D. melanogaster* whereas significantly different responses were not observed between mutant males and females, indicating that the response is different among the groups. Hence, our data agree with Iovchev *et al.* (2002). To elaborate, despite CO_2 exposure causing similar changes in the physiology of both genders and the mutant and wild-type when anesthetizing, it appears that the *ort* mutation and the gender still play a significant role in the amount of time the organism needs in the recovery portion of the experiment. Therefore, even though CO_2 exposure targets the same pathway in all *D. melanogaster* (mutant male, mutant female, wild-type male and wild-type female), the sex of the *D. melanogaster* and whether it is the *ort* mutation or wild-type play roles in the recovery time.

To determine the gender of the *D. melanogaster*, we put them under the light of a microscope which could have further influenced their recovery time as some organisms may be more sensitive to light and/or the warmth of the light. According to Hong *et al.* (2006), the temperature preference of *D. melanogaster* and its mechanisms to adapt to environmental temperature changes are directly carried out by histamine and histamine receptors. Hong *et al.* (2006) also found that a disruption in the histamine pathway, including an *ort* mutation, can cause an abnormal response to warmer or colder temperatures. For example, mutants with abnormalities affecting histamine (e.g., *ort* mutants) displayed lowered resistance to warmer

temperatures (Hong *et al.* 2006). Therefore, due to the warmer temperature caused by heat emitting from the light of the microscope, both wild-type and mutant *D. melanogaster* were likely to feel more stress (Hong *et al.* 2006), which could raise their cardiac output and awareness (Badre *et al.* 2005), which could ultimately result in a faster recovery. This effect could be more exaggerated in mutant flies as their tolerance to warmer temperatures is lower (Hong *et al.* 2006). Light signals can be perceived by *D. melanogaster* through its eyes and peripheral cells (Bulthoff 1982 & Plautz *et al.* 1997) and this may impact their recovery time. Moreover, Gao *et al.* (2008) noted that *D. melanogaster*, both wild-type and less so *ort* mutants, exhibit a positive phototaxic behavior towards light. This behavior can result in a shorter recovery time for the flies when under the bright light of the microscope.

To supply the replicates with a constant and equal pressure of CO_2 we made sure the gauge read the same number. To minimize human error with regards to judgment of what should be considered as recovery, we agreed on a definition before the experiment and marked the Petri-dishes accordingly. Furthermore, we each only observed a handful of *D. melanogaster* at a time in order to be able to carefully observe them and record the recovery time accurately. Despite our efforts to minimize different sources of uncertainty, there is still room for improvements to better the design and procedure, and eliminate errors. For instance, when transferring the individual flies from a Petri dish into another and onto the marked position, we used forceps to move the *D. melanogaster*. Different people transferring the *D. melanogaster* may not have applied the same amount of force. Lastly, we were not able to control the age and other biological variability of the *D. melanogaster* used, which could be a source of biological variation for our results.

Conclusion

In conclusion, we studied the effect of CO_2 anesthetization on different sexes of both *ort*^{1} mutant and wild type *D. melanogaster.* We found that there was no significant difference in recovery time between wild-type and mutant *D. melanogaster,* and gender of the organism had no effect on recovery time in *D. melanogaster.* However, the effects of gender on recovery time in *Drosophila melanogaster* are not the same for the wild-type and mutant strain.

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Literature Cited

Badre, NH, Martin, ME & Cooper, RL 2005, 'The physiological and behavioral effects of carbon dioxide on *Drosophila melanogaster* larvae', *Comparative Biochemistry and Physiology*, vol. 140 no. 3, pp. 363-376.

Belen, H & Tong, C 2012, '100 years of *Drosophila* research and its impact on vertebrate neuroscience: a history lesson for the future', *Nature Reviews Neuroscience,* vol. 11, no. 7, pp. 514-522.

Brooks, MA 1957, 'Growth-retarding effect of carbon-dioxide anaesthesia on the German cockroach', *Journal of Insect Physiology*, vol. 1 no. 1, pp. 76-84.

Bulthoff, H. 1982, '*Drosophila* mutants disturbed in visual orientation: I. Mutants affected in early visual processing', *Biological cybernetics,* vol. 45, no. 1, pp. 63-70.

Colinet, H & Renault, D 2012, 'Metabolic effects of CO_2 anaesthesia in *Drosophila melanogaster*', *Biology Letters*, vol. 8, no. 6, pp. 1050-1054.

Gao, S., Takemura, S., Ting, C., Huang, S., Lu, Z., Luan, H., Rister, J., Thum, A.S., Yang, M., Hong, S., Wang, J.W., Odenwald, W.F., White, B.H., Meinertzhagen, I.A. & Lee, C. 2008, 'The Neural Substrate of Spectral Preference in *Drosophila*', *Neuron,* vol. 60, no. 2, pp. 328-342.

Gengs, C, Leung, H, Skingsley, DR, Iovchev, MI, Yin, Z, Semenov, EP, Burg, MG, Hardie, RC & Pak, WL 2002, 'The target of *Drosophila* photoreceptor synaptic transmission is a histamine-gated chloride channel encoded by ort (hclA)', *The Journal of biological chemistry,* vol. 277, no. 44, pp. 42113-42120.

Iovchev, M, Kodrov, P, Wolstenholme, AJ, Pak, WL & Semenov, EP 2002, 'Altered drug resistance and recovery from paralysis in *Drosophila melanogaster* with a deficient histamine-gated chloride channel', *Journal of Neurogenetics*, vol. 16, no. 4, pp. 249-261.

Lheritier, P 1948, 'Sensitivity to CO2 in *Drosophila* - A review', *Heredity*, vol. 2, no. 3, pp. 325-348.

Nassel, DL 1999. 'Histamine in the brain of insects: A review', *Microscopy Research and Technique*, vol. 44, no. 2-3, pp. 121-136.

Oh, Y, Jang, D, Sonn, JY & Choe, J 2013, 'Histamine-HisCl1 receptor axis regulates wake-promoting signals in *Drosophila melanogaster*: e68269', *PLoS One,* vol. 8, no. 7.

Perron, J., Huot, L., Corrivault, G. and Chawla, S. (1972). Effects of carbon dioxide anaesthesia on *Drosophila melanogaster*. *Journal of Insect Physiology*, vol. 18 no 10, pp.1869-1874.

Plautz, J.D., Kaneko, M., Hall, J.C. & Kay, S.A. 1997, 'Independent photoreceptive circadian clocks throughout *Drosophila'*, *Science,* vol. 278, no. 5343, pp. 1632-1635.

Wixon, J & O'Kane, C 2000, 'Featured organism: *Drosophila melanogaster*', *Yeast*, vol. 17, no. 2, pp. 146-153.