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Cover Page Footnote

This research would not have been possible without funding from the Pence-Boyce Research Program and Catalyst at Olivet Nazarene University. The author would also like to thank the Honors Program, the Department of Chemistry and Geosciences, and Dr. Heyen for the constant support.



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ABSTRACT

Model organisms are widely used in research, especially in the context of complex situations. One model organism that has been widely used is the common fruit fly, *Drosophila melanogaster* (*D. mel*). *D. mel* are most commonly used in the context of genetics, but they have also been widely used in research focusing on general anesthetics. One value that has not been measured in *D. mel*, however, as it relates to general anesthetics, is the decrement times.

Flies were exposed to 40 μ L of the anesthetic isoflurane or sevoflurane in a centrifuge tube for 10 minutes, after which the flies were allowed to recover for various amounts of time. The anesthetic was then extracted using dichloromethane (DCM) and enflurane was added as an internal standard.

The decrement times of male flies with isoflurane were found to be approximately 30 seconds for the 50% decrement, two minutes for the 80%, and three minutes for the 90% decrement. For female flies the values found with isoflurane were found to be approximately 30 seconds for the 50% decrement and four minutes for the 80% decrement. The values found for the 50% decrement time of sevoflurane were about five minutes for the female flies and two minutes for the male flies, but the values found in female flies with sevoflurane were not consistent.

Though the data with sevoflurane were inconsistent, those collected with isoflurane give valuable insight into general anesthetics. This information can be used in future work involving drosophila flies and anesthetics in order to gain a deeper understanding of how inhalational anesthetics work

INTRODUCTION

The first publicized use of anesthetics was nearly two hundred years ago in 1846, and since then the use of anesthetics has become more common and safer for the patient.^{1,2} There are still many dangers of anesthetics, however, especially associated with prolonged and early exposure to anesthetic gases.^{3,4,5,6,7} Although many discoveries have been made, there are still many aspects of anesthesia that are not well understood. One way to further understand anesthesia is through the use of model organisms. A recent discovery that used model organisms found that stimulation or inhibition of certain neurons in the hypothalamus of mice had a correlation with quality and duration of sleep.⁸ To be used as a model organism, research must indicate that the organism responds to given stimuli in a way similar to how humans respond. This data can then be used to better understand the stimuli in humans. The fruit fly, *Drosophila melanogaster* (*D. mel*), is commonly used as a model organism, especially in the context of genetics, and has been said to be the model organism for genetics.⁹ Though genetics is the arena where *D. mel* has the most acclaim, the organism can be a model in many other applications.

One application for which *D. mel* has been used as a model is with anesthetics. Karunanithi et al. explored how *D. mel* are similar to humans in the way their minds

and bodies react to anesthetics.¹⁰ They present a basic overview of anesthesia and how it affects different levels of consciousness and discuss a few experiments with anesthetics and *D. mel*, such as using propofol-laced food in order to monitor activity of flies with different levels of anesthesia exposure. In 2017, MacMillan et al. explored the effects of isoflurane and sevoflurane anesthetics on many different aspects of health in *D. mel* including fecundity, starvation, and temperature tolerances. They found that *D. mel* generally recovers more quickly from sevoflurane than isoflurane after similar exposure times.¹¹ It was also found that with increased exposure time, the recovery time was more sex-dependent in isoflurane than in sevoflurane and that the fecundity of female flies was not significantly affected by either sevoflurane or isoflurane.

D. mel has been used in many different experiments to determine how anesthesia affects different organisms and how its effects can change based on genetics and sex.^{10,11,12,13,14,15}

Many properties of anesthetics have been measured in the organism. One property of anesthetics that is very important to those who work in the field of anesthesiology is the minimum alveolar concentration (MAC). The minimum alveolar concentration is a measure of how saturated the alveoli in the lungs must be with an anesthetic before approximately half of all humans will not react to a surgical incision.¹⁶ Zalucki et al. found the MAC value of isoflurane in *D. mel* to be 0.2-0.6% depending on the criteria used to determine if the fly is conscious.¹⁵ Consciousness was determined through a startle response, but the MAC was found to be higher if the test involved what the authors described to be more complex behavior, such as reacting to a light beam rather than reacting to a vibration.

One property of anesthetics that has not been measured in *D. mel* is the decrement times. The decrement time of an anesthetic is defined as the amount of time necessary for a given percentage of that anesthetic to have left the organism.¹⁶ In 1997, Bailey calculated the decrement times of isoflurane, sevoflurane, enflurane, and desflurane in humans.¹⁷ Bailey found that after six hours of exposure, enflurane has the longest 90% decrement time, at about 100 minutes, with isoflurane at 86 minutes, sevoflurane at 65 minutes, and desflurane being considerably lower than the rest at 14 minutes.

Knowing the decrement times of different anesthetic gases in *D. mel* will further establish the organism as a model for volatile anesthetic properties in humans. Additionally, due to their quick generation times, *D. mel* could be used to explore relationships between genetics and anesthetics, such as finding links between being more or less resistant to the effects of anesthetics. In this experiment, the decrement times of isoflurane and sevoflurane in male and female *D. mel* will be measured using gas chromatography-mass spectrometry (GC/MS).

MATERIALS AND METHODS

Wild-type drosophila flies (Carolina, catalog number 172100) were kept in culture vials and fed drosophila fly medium with yeast placed in the media and hydrated using sterile water. Flies were allowed to propagate and were transferred to new vials every month or so. The vials were stored in a Fisher Scientific 307C Isotemp Low

Temperature Incubator/Freezer set to 21.0 °C. No special attention was paid to using virgin female flies, thus flies were removed from vials and tested when necessary.

Because male and female drosophila flies have been found to have different recovery times, which would imply different decrement times, the flies were first sorted male and female.¹¹ This was done by removing flies from the culture vial to a new vial without medium. Flynap was then administered to the flies until they were all asleep, not exceeding two minutes of exposure. The flies were then placed on a notecard under a dissection microscope and sorted into two piles based on sex. Images of male and female flies have been included in **Figure 1**. It should also be noted that female flies are typically larger than male flies, but because this can vary based on genetics, all flies were sexed by flipping the fly over and checking the genitalia.

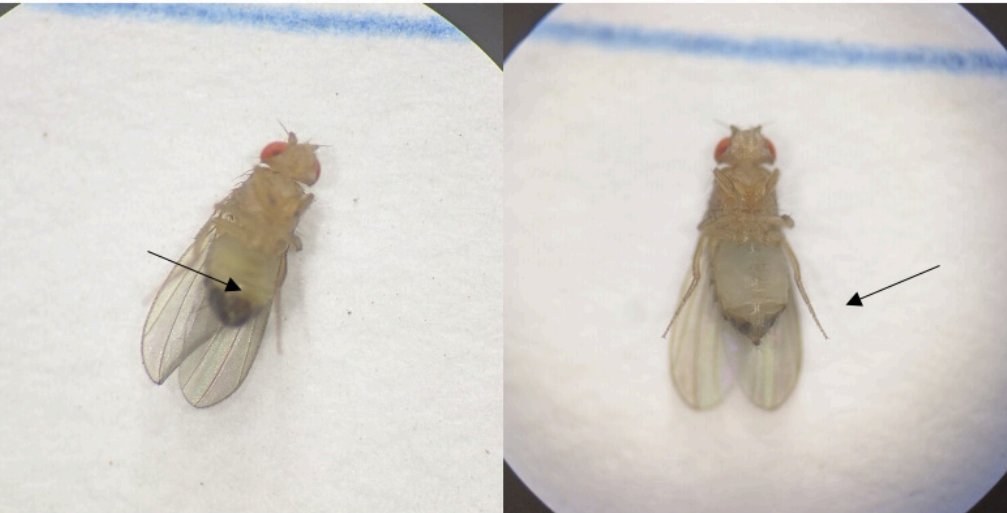


Figure 1: Male and female drosophila flies. Note the darker genitals and smaller size on the male (left), as opposed to the lighter, smooth genitals and larger size on the female (right).

After the flies were sorted, they were placed in empty culture vials overnight to allow them to recover and to ensure that the Flynap had no effect on responses to the anesthetics being tested. The following day, the flies were transferred from the culture vials into a conical centrifuge tube. A foam disc was then inserted into the top of the tube, to ensure that the flies would not be able to escape. Into this foam disc was injected 40 μ L of isoflurane or sevoflurane, depending on which anesthetic was being tested. This volume was chosen because it was used in a previous study and is enough to saturate the environment.¹¹ The cap was placed on the centrifuge tube to keep the anesthetic inside the tube, and the flies breathed the anesthetic for ten minutes. At ten minutes, the flies were transferred to a glass centrifuge tube and were either allowed to recover, or, in the case of the control group, immediately killed by pouring dichloromethane (DCM) into the vial. At this point, an enflurane internal standard was also added to the vial. Enflurane was chosen because it makes for a good internal standard when analyzing anesthetics due to its similar properties.¹⁸ The internal standard was added

as soon as possible to ensure any mistakes made during the procedure also affected the concentration of internal standard. The internal standard solution was made by diluting 40 μ L of enflurane in DCM to a total volume of 10 mL.

The flies were then homogenized using a glass stir rod, to extract all the anesthetic from their bodies by the DCM. This solution was then removed from the vial and diluted to 5 mL in order to be sure that every trial was the same volume no matter how much was removed into the GC/MS vial. Though the quantities could have been normalized by diluting the samples to a constant mass, it was more convenient to dilute to a constant volume and did not reduce the accuracy of the measurements any more than normalizing by other means.

Before data was collected, the response factor of the internal standard (IS) being used, enflurane, was calculated with both isoflurane and sevoflurane. These were run in equal concentrations and the following equation was used to calculate response factor (F):

$$F * \frac{Area_{IS}}{[IS]} = \frac{Area_A}{[A]}$$

Where *IS* represents the internal standard, and *A* represents the analyte, which was either isoflurane or sevoflurane depending on the trial. Two trials were run for both isoflurane and sevoflurane. These values can be found in Table 1.

TABLE 1: CALCULATION OF RESPONSE FACTORS

Response factors (F) calculated to be 6.945 and 0.626 for isoflurane and sevoflurane, respectively. Because equal volumes of internal standard (IS) and analyte (A) were used, the ratio of analyte to IS was equal to F. The average of the two runs was then calculated for use later.

Trial	Mean* Analyte Peak Area	Mean* Enflurane Peak Area	Mean* Response Factor (F)
Isoflurane	1284872	184986	6.945
Sevoflurane	117986	188549	0.626

*n=2

The GC/MS used was a Shimadzu GCMS-QP2010 SE, and the column used was a Rtx-5MS column of length 30.0 m, diameter 0.25 mm, and thickness 0.25 μ m. Since DCM elutes later than isoflurane, sevoflurane, and enflurane, the mass spectrometer sensor was turned off around the two-minute mark to avoid overstimulating the sensor. Typically the solvent elutes first, thus there is a solvent cut-off time, meaning the sensor waits to turn on until after the solvent has eluted. But because the solvent eluted later than the compounds being analyzed, it was decided to turn the sensor off after the analytes were measured.

The method used was adapted from MacMillan et al. and was as follows.¹¹ The column oven temperature was 30.0 °C. The injection temperature was 200.0 °C. A split injection mode was used with a split ratio of 1:50.0. The total flow was set to 54.7 mL/min with a column flow of 1.01 mL/min. The column oven temperature started at 30.0 °C and held at that temperature for 4.00 minutes. The temperature then increased at a rate of 40.00 °C/min to a temperature of 140.0 °C, which was held for 5.00 minutes.

RESULTS

Approximately thirty female *Drosophila melanogaster* flies were exposed to 40 µL of isoflurane in a conical centrifuge for ten minutes. The flies were then homogenized, and the anesthetic was extracted using dichloromethane (DCM). An enflurane internal standard was added and the sample was analyzed by gas chromatography-mass spectrometry (GC/MS) to determine the amount of anesthetic present. Different trials were run allowing the flies to recover for different periods of time, referred to as “recovery time” in Table 2. These data show the 50% decrement time of isoflurane in female *D. mel* flies to be one minute. A 75% decrement time for isoflurane was also found to be approximately four minutes.

TABLE 2: FEMALE ISOFLURANE DATA

Female flies have an approximate 50% decrement time of one minute using isoflurane. Percent decrement is calculated based on the control group.

Recovery Time	Number of flies	Isoflurane peak area	Enflurane peak area	Isoflurane concentration (ppm)	Isoflurane/fly (ppm)	Percent decrement
Control	30	15380	5983	1.48x10 ³	49.4	0
0.5 min	21	5106	5388	5.46x10 ²	26.0	47.34
1 min	30	7097	5447	7.50x10 ²	25.0	49.31
2 min	31	3751	5053	4.28x10 ²	13.8	72.05
3 min	32	4471	4496	5.73x10 ²	17.9	63.73
4 min	26	3350	6305	3.06x10 ²	11.8	76.15

The amount of isoflurane was calculated from the same equation used to calculate the response factor, *F*. Thus, the value calculated for the 0.5-minute recovery trial was calculated as follows:

$$F = \frac{(Area_{analyte}/Area_{IS})}{([analyte]/[IS])}$$

$$[analyte] = \left(\frac{Area_{analyte}/Area_{IS}}{F} \right) * ([IS])$$

The value used for the concentration of internal standard ([IS]) was 0.004, because the internal standard was prepared by diluting 40 µL to 10.00 mL with DCM. The value calculated for the concentration of anesthetic was then divided by the number of flies in that trial to standardize the amount of anesthetic present. Care was taken to remove

any dead flies from the centrifuge tube before exposure to anesthetic. Because these flies were not breathing the anesthetic, they would artificially decrease the amount of anesthetic detected per fly.

The same procedure as described above was used to determine percent decrements of isoflurane in male *D. mel* flies. These data were compiled in Table 3. As can be seen, the 50% decrement time of isoflurane in male flies was calculated to be approximately thirty seconds. The 80% decrement time was found to be two minutes, and the 90% decrement time was found to be three minutes, at which point the metabolism of the anesthetic seems to have slowed down, leading to a percent decrement of 90% after four minutes of recovery as well.

TABLE 3: MALE ISOFLURANE DATA

Male flies have an approximate 50% decrement time of 30 seconds using isoflurane. Percent decrement is calculated based on the control group.

Recovery Time	Number of flies	Isoflurane peak area	Enflurane peak area	Isoflurane concentration (ppm)	Isoflurane/fly (ppm)	Percent decrement
Control	29	21681	5739	2.18x10 ³	75.0	0
0.5 min	29	9010	4958	1.05x10 ³	36.1	51.90
1 min	21	2816	4505	3.60x10 ²	17.1	77.15
2 min	32	3978	4598	4.98x10 ²	15.6	79.24
3 min	25	1689	6202	1.57x10 ²	6.27	91.64
4 min	23	1414	5607	1.45x10 ²	6.31	91.58

The data collected for sevoflurane were less consistent than those collected for isoflurane in both male and female flies. The data collected for female flies using sevoflurane can be found in Table 4 and those collected with male flies in Table 5. Though there were some inconsistencies in the data collected using isoflurane (for example, the percent decrement after a three-minute recovery time being lower than that of a two-minute recovery in the female flies), they were not as bad as those noted during the collection of data using sevoflurane. One observation that can be made regarding the sevoflurane data, however, is how long the female flies were able to retain the anesthetic, showing only a 3% decrement after two minutes. This contradicts what was found by MacMillan et al. when measuring recovery times, which found that *D. mel* recover faster from sevoflurane.¹¹

TABLE 4: FEMALE SEVOFLURANE DATA

Recovery Time	Number of flies	Sevoflurane peak area	Enflurane peak area	Sevoflurane concentration (ppm)	Sevoflurane /fly (ppm)	Percent decrement
Control	28	4247	5407	5.02x10 ³	179	0
2 min	30	4905	6011	5.22x10 ³	174	3.04
5 min	31	1740	4714	2.36x10 ³	76.1	57.55
8 min	34	2969	4167	4.56x10 ³	134	25.30
10 min	25	0	4707	0	0	100

TABLE 5: MALE SEVOFLURANE DATA

Sevoflurane decrement times in male flies are inconsistent. Percent decrement is calculated based on the control group.

Recovery Time	Number of flies	Sevoflurane peak area	Enflurane peak area	Sevoflurane concentration (ppm)	Sevoflurane /fly (ppm)	Percent decrement
Control	31	7897	4598	1.10x10 ⁴	354	0
1 min	29	2081	4922	2.70x10 ³	93.2	73.68
2 min	27	4277	5918	4.62x10 ³	171	51.69
3 min	39	4560	9534	3.06x10 ³	78.4	77.86
5 min	32	4619	8626	3.42x10 ³	107	69.80
7 min	22	0	5304	0	0	100

DISCUSSION

The decrement times of isoflurane and sevoflurane in *D. mel* have not been measured and published to date. In order to measure this, male and female flies were separately exposed to 40 μ L of isoflurane or sevoflurane for ten minutes before being killed and extracting the anesthetic using dichloromethane. The relative amounts of anesthetic were measured using gas chromatography-mass spectrometry. It was found that the 90% decrement times for isoflurane for male flies was about three minutes. The 50% decrement times were also found to be around thirty seconds for female flies and slightly less for male flies, though an exact number for male flies was unknown due to the difficulty of extracting the anesthetic that quickly. The highest decrement times found for isoflurane in the female flies was the 80% decrement time at a little longer than four minutes.

It is difficult to measure specific decrement times accurately due to how they are measured. Because the flies must be killed in order to measure the decrement time, and because the time must be chosen before the percent decrement can be measured, it would be difficult to determine the exact time at which the anesthetic is at 50% the original concentration. Though it would be possible to use regression analysis to predict decrement times between those measured, the data were not consistent enough for this to be useful. Additionally, specific decrement times are not as useful clinically as knowing general decrement times, such as 50%, 80%, and 90%.

The decrement times of sevoflurane are harder to determine based on the data collected. For female flies, the 50% decrement time was found to be a little under five minutes, but with a recovery time of eight minutes the decrement was found to be only 25%. A trial with a recovery time of ten minutes using female flies found no sevoflurane peak, so it can be assumed that the 100% decrement time is somewhere between eight and ten minutes. For male flies using sevoflurane, the data was similarly inconsistent. As with the female flies, the only thing that can be assumed about the decrement times of sevoflurane in male flies is that the 100% decrement time is somewhere between five and seven minutes, as no sevoflurane peak was found after a recovery time of seven minutes.

Another inconsistency in the sevoflurane data is the longer decrement times of sevoflurane found as compared to those of isoflurane. This is inconsistent with previously published data that focused on recovery times rather than percent decrements. The data previously published found that *Drosophila* flies recover from isoflurane in approximately twelve minutes, whereas the recovery time found for sevoflurane was found to be approximately five minutes.¹¹ Based on this data, it would make sense to expect longer decrement times in isoflurane, but this is not what was observed. This also contrasts the data published by Bailey in 1997, which show that after 100 minutes of exposure, the 90% decrement times of isoflurane and sevoflurane in humans are 86 and 65 minutes, respectively.¹⁷ An explanation for this can be understood, however, by comparing the minimum alveolar concentrations (MACs) of isoflurane and sevoflurane.

As previously mentioned, the MAC is the percent of alveoli in the lungs that must be saturated for half of the population to not react to a surgical stimulus such as an incision. When measured in humans, these values are found to be approximately 0.85 and 1.71% for isoflurane and sevoflurane, respectively.^{19,20} Taking this into account, the longer decrement times in sevoflurane can be understood. That is, though the flies retain sevoflurane longer than they do isoflurane, they do not retain enough to remain unresponsive, and thus have a shorter recovery time with sevoflurane.

One possible reason for the inconsistency in the data for sevoflurane is that sevoflurane may be metabolized by *D. mel* in some way that is unknown and that can occur at different rates under different circumstances. Though the environment during collection of data was never greatly changed, there may be some unknown factor that caused this change in metabolism. Another possible reason is some genetic mutation that occurred in the flies during testing, causing a change in metabolism of sevoflurane.¹⁴

As mentioned before, the decrement times of anesthetics in *D. mel* have yet to be published, even though *D. mel* is a common model organism. Though the data collected using sevoflurane were inconsistent, the values found for isoflurane showed consistency and can be used in future work involving *drosophila* flies and anesthetics to gain a deeper understanding of how inhalational anesthetics work.

The mechanism of action of anesthetics is still largely debated. A study recently published found a correlation between stimulation or inhibition of certain neurons in the hypothalamus of mice and quality and duration of anesthetic-induced sleep, but

this experiment used injected anesthetics.⁸ The data collected in this experiment could be used in further research to determine how inhalational anesthetics affect the brain.

Additionally, the biochemical reactions behind the metabolism of anesthetics are still largely unknown. Knowing the decrement times of volatile anesthetics in *Drosophila* flies can help in the process of determining how anesthetics are metabolized by the flies, which can in turn help give evidence of how they are metabolized by humans. This could be done by determining the kinetics of the reactions occurring during the metabolism of anesthetics, one important factor of which is how long these reactions take. Though studies have been done exploring the kinetics of isoflurane in the body and in model organisms, the exact mechanism of metabolism is unknown.²¹

This experiment is limited by the fact that the data must be interpreted in the context of the procedure conducted. That is, the flies were exposed to anesthetic for ten minutes, whereas in some experiments, such as those exploring prolonged exposure to anesthetics, they may be exposed for much longer than that.^{4,5,6,7,22} The amount of time the flies were exposed to anesthetic in the present experiment was chosen because ten minutes is a relatively short amount of time, while still giving enough exposure to the anesthetic for the concentration to be detected. Additionally, this exposure time has been used in previous experiments using volatile anesthetics and *D. mel.*^{10,11} The amount of anesthetic the flies were exposed to saturates the centrifuge tube and makes adding any more anesthetic superfluous.¹¹ Though this does present a challenge when applying this data to other situations, the data is nonetheless novel and can still be helpful in future research.

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