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Development of a system to measure cardiac function in the drosophila melanogaster animal model

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ABSTRACT

DEVELOPMENT OF A SYSTEM TO MEASURE CARDIAC FUNCTION IN THE *DROSOPHILA MELANOGASTER* ANIMAL MODEL

by
Heather Anne Reinhardt

Previous studies have shown that several genes are evolutionarily conserved from lower organisms to man, where a number of genes have been directly linked to cardiac function and disease. The *Drosophila melanogaster* (Fruit Fly) has a genome similar to humans and therefore has proven to be an important animal model to study the role of genetics in cardiac function and disease development. Fruit flies are an important basic science model because of the ease with which genes can be manipulated and quickly expressed due to the short lifespan of the fly. Although standard techniques exist to affect the genome of the fly, measurement of fly cardiac function is not well established due to technical difficulties stemming from the fly size and fragility. This work describes a system developed to measure in vivo cardiac function of an adult Fruit Fly. This involved design of a novel anesthesia chamber to accurately anesthetize fruit flies, construction of a microscope-image analysis system to visualize the fruit fly heart beating in real time, implementation of an image analysis method to analyze fruit fly heart rate, and design and implementation of an electrical pacing system to stress the fruit fly heart. This system was tested with standard yellow white (*yw*) fruit flies. Data obtained confirmed previous studies showing that aging affects fruit fly heart rate (9 day: 285.2 ± 5.8 bpm, 30 day: 221.7 ± 8.1 bpm, 53 day: 195.1 ± 9 bpm) and ability to handle pacing stress. This study determined that a previously believed cardio-protective anesthesia (Triethylamine, FlyNap®) is in fact a cardiac depressant, where incremental increases in FlyNap® dose

decreases fruit fly heart rate linearly, and also affects the fly heart's response to pacing stress. In summary, a system was developed to measure cardiac function in fruit flies, which allows the future study of cardiac function in genetically manipulated fruit flies under normal and diseased conditions.

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by
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DEVELOPMENT OF A SYSTEM TO MEASURE CARDIAC FUNCTION IN THE *DROSOPHILA MELANOGASTER* ANIMAL MODEL

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CHAPTER 1

INTRODUCTION

1.1 Objective

The objective of this work is to develop and implement a system that will measure and analyze the heart rate of *Drosophila melanogaster* (Fruit Fly). The average heart rate will be determined by anesthetizing the flies with a carefully measured amount of anesthesia and then recording a video of their beating hearts under normal and stressed conditions. A normal condition is when the flies' basal heart rate is recorded at room temperature. A stressed condition will be induced by electrical pacing and by varying the frequency applied. A novel anesthesia chamber will be developed to determine the effect of anesthesia by changing the amount of anesthesia administered. An image analysis program will be used to determine the average heart rate. The above methods will be used to compare the values obtained from 9 day-old, 30 day-old, and 53 day-old male flies.

1.2 Relationship between Fruit Flies and Man

Drosophila melanogaster (Fruit Fly) was first studied Thomas H. Morgan in 1910, and used to show that chromosomes carry genetic information [1]. Only within the last decade have links between Fly and man been made, and the genetic factors regulating cardiac function even more recently [5].

The fly also has a simple heart, which is why it is a valuable animal model for the study of cardiac disease, which is the leading cause of death among patients in the United States with an estimated 950,000 deaths per year [6]. In studying cardiovascular disease,

heart rate is monitored in humans and is used as a criterion of comparing normal and diseased conditions.

In humans, the likelihood of experiencing heart failure is 70 times greater in people who are over the age of 65 than in people between 20 and 34 years old. Also, cardiac function declines with age in human beings [2]. Moreover, man undergoes cardiac problems as a result of various types of stress, and is more prone to experience these problems with age.

Man has evolutionarily conserved cardiovascular disease-causing genes that are found in the Fruit Fly. Cardiomyopathy, conduction, hypertension, atherosclerosis, and vascular defects have all been found to have disease-related genes in both the human and in the fly (as detailed in Appendix B) and may be acting in common physiological processes or pathways [6]. Furthermore, genes that control the early stages of heart development are also conserved in vertebrates and invertebrates [6]. Therefore, an accurate method to quantify Fruit Fly cardiac function, as well as changes due to disease, aging and gene manipulation conditions.

1.3 Literature Survey

The following literature review discusses the importance of *Drosophila melanogaster* in the measurement of cardiac function and response to stimuli in previous research.

1.3.1 Significance of *Drosophila melanogaster*

Drosophila melanogaster has been a valuable animal model for almost a century in genetic research for several reasons. Flies commonly have a lifespan of between 45 and 60 days [2] and can easily and quickly reproduce. Female flies can lay up to 500 eggs in ten days. Flies are born two weeks after fertilization [2]. Flies are also easy and cheap to maintain in any environment. Other laboratory animals do not have such a short lifespan. For example, mice live an average of two to six years [3]. Mice also take 21 days to be born after fertilization [3] and are much more costly to maintain. In addition, the entire genetic sequence of *Drosophila melanogaster* has been mapped out since 1998. This makes mutant genes readily accessible to investigators through various vendor companies specializing in fly genetics.

Another important distinction between flies and other animal models is their number of chromosomes (the structures which contain genetic information). Fly larvae have giant chromosomes in their salivary glands that can be studied. Fruit flies only have 4 pairs of chromosomes, as opposed to humans, which have 23 pairs and mice, which have 20 pairs. Three of these pairs are autosomes and the other is a pair of sex chromosomes. Males do not have genetic recombination. Thomas Hunt Morgan, with his colleagues, studied x-linked inheritance and showed genes located on the same chromosome do not undergo independent assortment [4]. Studying x-linked traits helped to confirm that genes are in fact on chromosomes while studying linked traits helped to begin mapping out the genetic sequence of chromosomes [4]. The entire code contains about 132 million bases and nearly 13,767 genes [4].

Recent developments have led to ~19,000 P-element insertions and deletions throughout the flies' entire genome. This allows for rapid screening [5] of the genome and the mapping of candidate genes. This is not available for other species, only *Drosophila melanogaster* [5].

Lastly, flies are being studied because of their relevance to humans. 61% of known human genetically linked diseases have a genetic match in *Drosophila's* genome. Fifty percent of fly proteins have mammalian analogues [4]. This evolutionary conservation and other key aspects in the Fruit Fly animal model have led it to be studied in Parkinson disease, Huntington's disease, and Alzheimer's disease as well as in the study of cancer, drug abuse, diabetes and immunity [4]. In their review, Bodmer and Bier [6] report that there are currently 1682 known human disease genes, 74% of which have *Drosophila* homologs. A third of these known genes are as highly conserved as genes known to be functionally equivalent between flies and humans [6].

Much research has been conducted in using *Drosophila melanogaster* as a model for heart development. Little research has been done on the fly, however, in its connection to cardiovascular disease. The reason for this is that it's difficult to observe the adult fly's heart *in-vivo* [5].

Therefore, the Fruit Fly proves to be a valuable animal model in basic science research. A combination of biochemical and cardiac physiology measurement-techniques would prove powerful in studying cardiac disease.

1.3.2 *Drosophila melanogaster* Anatomy

Drosophila melanogaster is a small insect whose body consists of a head, a thorax, and an abdomen (Figure 1.1). A fly's body is between 2.5 and 3 mm in length by 2 mm wide and is normally yellow brown in color with black stripes. Female flies are larger than males. A pair of compound red eyes is found on the head in addition to three pairs of smaller, simple eyes [1].

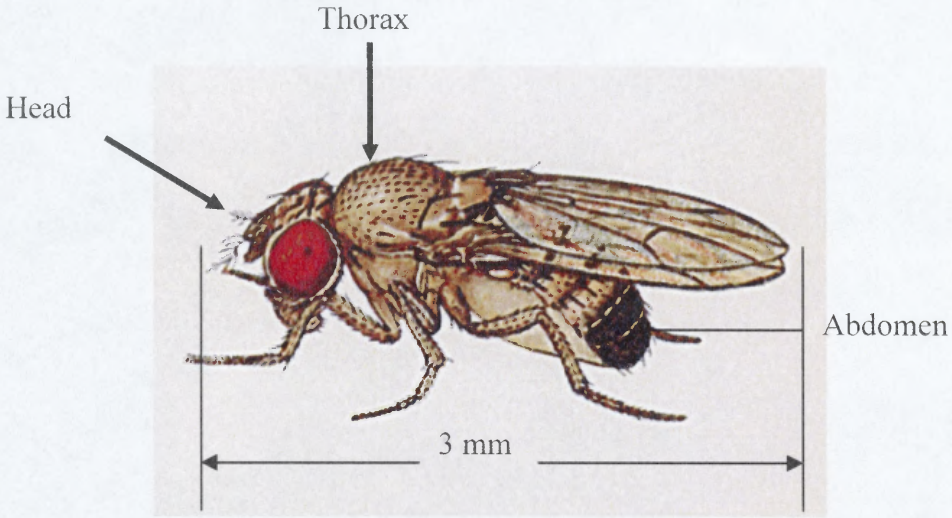


Figure 1.1 Wild Type *Drosophila melanogaster*, taken from Patterson [7]

The heart of a fly is a tubular-shaped organ that runs parallel to the body of the fly. It can be located under a 5x objective on a microscope by first finding two tracheal tubes. Between these two tubes the fly's heart can be seen beating, (Figure 1.2). It is located between and normal to the fly's tracheal tubes, which are parallel to the black stripes that are shown.

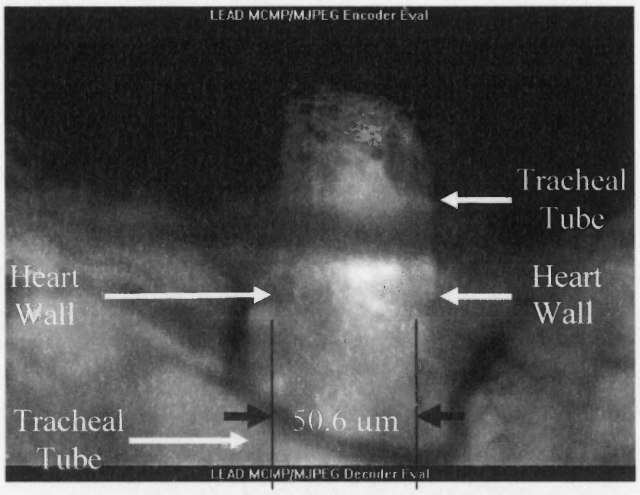


Figure 1.2 The heart of *Drosophila melanogaster*

Drosophila melanogaster's heart is a simple organ with a simple function. The heart is a tubular shaped muscle that pumps endolymph or hemolymph containing energy substrates [2] from the abdomen to the thorax and ultimately to the head. The pattern that the heart beats in is called a tri-phasic pattern, consisting of a period of systole (contraction), diastole (relaxation), and diastasis (a pause period). During diastole, when the heart walls are farthest apart, we were able to measure the distance between these wall and obtained a value of 50.6 μm. This compares 76 ± 3 μm [5] to values obtained by other investigators.

Drosophila melanogaster's heart is referred to as a dorsal vessel which is made up of an anterior aorta and a posterior heart [8]. The hemolymph is drawn into three pairs of slit-like openings, called ostia, that are found in the posterior half of the dorsal vessel and pumped through the heart into the aorta where it flows out of the open-ended tubular structure [8].

Lastly, the heart of *Drosophila melanogaster* is simple compared to higher organisms. However, this simple organism is intricate in terms of its structure, cell types and patterns of gene expression [9]. The heart of *Drosophila* is made of a few cell types.

Two inner rows of contractile cardiac cells form the lumen of the heart and several types of pericardial cells lay beyond these which may serve in filtration of the hemolymph [9]. This vessel is attached to the epidermis by alary muscles which are attached at the segmental borders [9]. In addition, the early stages of development of both the vertebrate heart and the fly heart are very similar. Both hearts are formed by bilaterally symmetric precursor cells that migrate to and assemble at the embryonic midline [9].

1.3.3 Current Methods for Measuring the Heart Rate of *Drosophila melanogaster*

Paternostro et. al [2] used a Nikon Diaphot-TMD inverted microscope with a Nomarski optics and a X10 (numerical aperture 0.25) objective. A light beam was focused solely on the first ventricle of the flies' hearts. Flies were placed on their backs, perpendicular to the light path, and their wings were stuck into double-sided tape, exposing their heart [2]. A Sony DXC-101 video camera captured the images of the beating heart on VHS tape. Slow-motion replay allowed the heart rate measurements to take place. The end-diastole and end-systole heart wall positions were measured on still images at the midpoint between the two major tracheal tubes [2]. It was reported that lower voltages or durations were unable to consistently pace the heart [2].

Wessells et. al [10, 11] visualized flies under a 10X objective using interference contrast optics. Because the flies were darkly pigmented, making it difficult for edge-tracing software to be used, manual counting was performed to determine heart rate. Placing flies on a temperature-controlled stage did this. A stereomicroscope was used to observe the flies for fifteen seconds. Each fly was counted five times with a 10 to 20

second pause between counts. The average of these five measurements was taken to determine the actual heart rate [10].

Sláma et. al [12] employed four methods to measure heart rate. The first was an optocardiographic method based on recording of the reflected pulse-light that was modulated by subintegumental movements of the heart and other tissues [12]. 1-5 KHz pulse-light was emitted by a 640 nm LED. The beam was applied externally to the pericardial region of the abdominal tergites through an optic fibre. The reflected light was collected by a second optic fibre, and transferred to a phototransistor of the amplifier and recorded [12]. A second method was contact thermography which used miniature thermo resistor sensors placed on the outer integumental surface overlying the pericardial region [12]. The third was a visual-electric method which involved visual observation of the systolic contractions under a stereomicroscope with simultaneous recording of the heart's movements by manually using a fast-response electrical switch [12]. A fourth method used was another optocardiographic method where the pulse light was applied at a ventral location [12].

Johnson et. al [13] studied *Drosophila* pupae because they are nearly transparent, making the heart more visible. An Olympus BH2 microscope that was fitted with an optical data acquisition system was used. Pupae were placed in this microscope. A Sensortek TS-4 allowed the stage to be temperature-controlled. Heart rate was measured using an electrocardiogram at temperatures of 20°C, 25°C, 30°C, 35°C, and 37°C. Pupae were allowed a minute and a half to adjust to the temperature and then their heart rates were recorded. Each heart produced a signal which was amplified and then digitized

with a Metrabyte DAS-8 AD converter at 100 Hz. The signals were then stored on a microcomputer [14].

Johnson et. al [14] tested flies that were in the transition between larva and pupa [13, 14]. Pupa were placed under an Olympus BH2 microscope that had a DC illuminator power supply. This minimized any possible trauma to the heart. A drop of water was put on the animal helped to stabilize the temperature between the animal and the slide and to increase light intensity. An eyepiece of the microscope contained a phototransistor that monitored changes in light intensity as heartbeat [14]. Voltage changes were amplified and recorded digitally for later analysis [14].

Zornik et. al [8, 12, 16] used a microscope-based technique [13, 15] to optically detect heart rate. Each subject was injected with 40 nL of solution with a glass micropipette. Each animal received only one injection, and no less than seven subjects were used for each concentration of a compound [8]. The substances were injected into the anterior of the central nervous system dorsal vessel to make sure that the chemicals diffused properly without causing damage to either the cardiac or neural tissues [8]. The baseline heart rate was recorded for two minutes before each injection. Following injection, the animal's heart rate was recorded for ten minutes. The heart rates were calculated in thirty- second intervals and then averaged.

Johnson et. al [16] placed fly pupa on a glass slide that was maintained at 25°C by a Sensortek TS-4 unit. An Olympus binocular compound microscope was used that was fitted with a phototransistor in an eyepiece that detected movements of the heart. The signal was pre-amplified by a 741C operational amplifier circuit, and then put through a

low pass filter and amplified by a Grass 79D polygraph. A 486i computer recorded the data through a Metrabyte DAS9 analog to digital converter at 100 Hz [16].

1.3.4 Previous *Drosophila* Cardiac Function Studies

Dowse et. al also considered certain mutations in larval *Drosophila melanogaster* that might have an affect on the heart's ability to beat. There are certain mutant flies whose hearts' ability to contract and produce action potentials are severely limited or inhibited at or above certain temperatures. These are the nap^{ts} flies, or *no action potential*$^{temperature\ sensitive}$ flies. Heart rhythms of these flies were increasingly more erratic with increasing temperature compared to those of the wild type [17].

Johnson et. al [13] sought to explore the roles of receptors, cAMP, cGMP, G-proteins, and calcium in affecting the heart rate of larval *Drosophila melanogaster*. In their research, Johnson et. al determined that both the α_1 adrenergic receptor and the 5-hydroxytryptamine$_2$ (5-HT_2) are important in regulating heart rate. cGMP may be integral in a signaling pathway for heart rate regulation as well. Pertussis toxin and both agonists and antagonists of the α_1 adrenergic receptor and 5-HT_2 affect the rhythm of the heart, which means that two G proteins are involved in regulating heart rate. An increase in cAMP was shown to significantly alter heart rate, but not as much as neurotransmitters[13]. Moreover, Dowse et. al [17] shows that the mutant *rutabaga*, which results in a reduction of adenylate cyclase activity, does not affect heart rate [17].

Gu and Singh [18] explored the role of ion channels in the ability of the heart to beat. This research was done in an effort to determine if the heartbeat of *Drosophila melanogaster* is myogenic (the beats are intrinsic to the muscle itself) or neurogenic (the

beats are a result of string or train of neural impulses) by nature [18]. Using tetrodotoxin (TTX), these researchers discovered that sodium channels do not play a significant role in the ability of the flies' hearts to beat. These results also suggested that the heart beat pattern of the fly is myogenic by nature. Both calcium and potassium channels were indicated to play a role in the heart's ability to beat.

Most recently, a study has been conducted by Wolf et. al [5] in the heart rate measurement of *conscious*, restrained, adult flies. This method is called optical coherence tomography and is used to show that a conscious, mutant adult fly can be accurately measured for heart rate. The mutant fly was developed that contained a trans-gene that has been associated with cardiomyopathy in humans. It is not an edge tracing technique that has been used previously [2, 10], but is a method that measures the changes in the cardiac chambers during systole and diastole. The chamber dimensions were measured and recorded. The end-diastolic dimension (EDD) was found to be 76 ± 3 μm and the end-systolic dimension (ESD) was found to be 8 ± 2 μm. The fractional shortening (FS) was determined to be 90 ± 2 %. Heart rate, but not EDD, ESD or FS was found to be temperature dependent, with a mean heart rate of 271 ± 13 bpm at 22°C vs. 511 ± 26 bpm at 38°C.

The above-mentioned studies all investigated the heart rate of *Drosophila melanogaster* using different techniques. These studies have shown that both aging and stressing the heart result in a decrease in the heart rate of flies.

1.3.5 Previous Studies on the Effect of Aging on Cardiac Function

Paternostro et. al [2] investigated the effect of stress on the aging process in wild type and transgenic flies. At room temperature (22°) as well as at an elevated temperature (28°), the resting heart rate was found to decline as the flies aged (Figure 1.3). The resting heart rate in humans also declines with age [2].

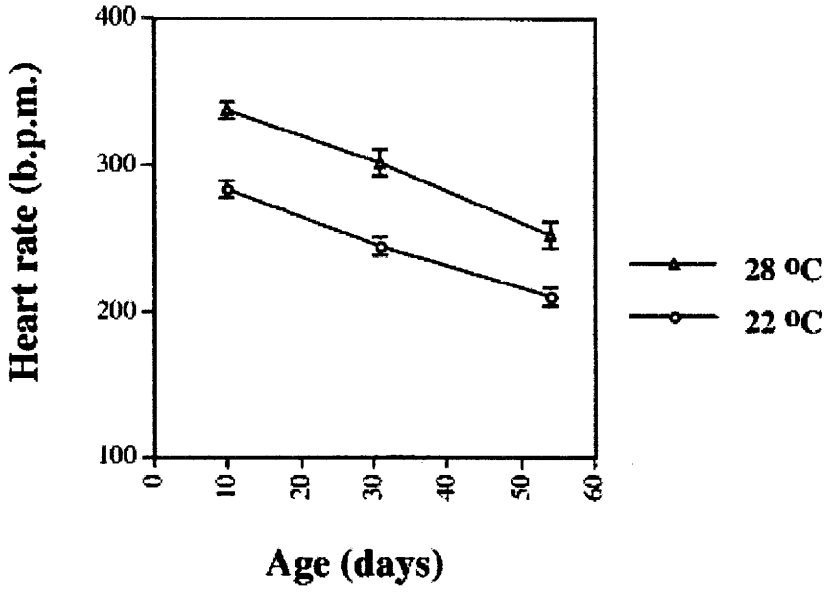
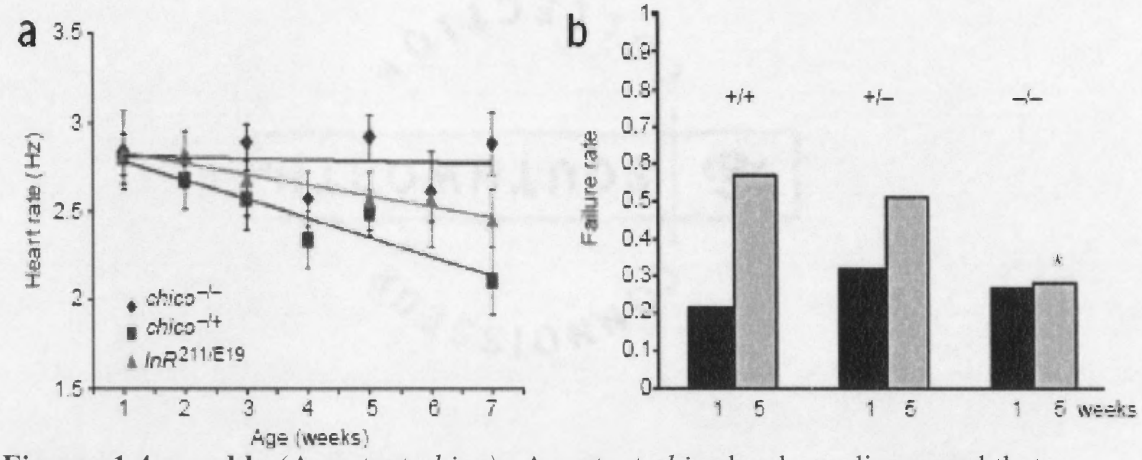


Figure 1.3 Heart rate declines with age [2]

Paternostro et. al [2] also found that temperature-induced stress resulted in an increase in heart rate. The higher the temperature, the higher the heart rate for all ages involved. Electrical pacing was used to find the highest achievable heart rate for both young and old flies. Paternostro et al. [2] reported that the maximal frequency for 10 day old flies at 22°C is 411 ± 13 beats per minute. Fibrillation, indicated by a tremor-like response in the fly's heart, occurred in only 20 % of the young flies but in 70% of the older flies. This indicates a relationship between aging and decline in heart function. Although all of the young flies that underwent this fibrillation recovered within two minutes, 40% of the older flies never recovered. At 28°, the pacing experiment was performed. The maximal

heart rate achievable here for 6 day-old flies was 498 ± 9.6 beats per minute. It was lower for older flies. The automated heart rate detection program determined a coefficient of variation, which was much larger in older flies.

Wessells et.al [11] demonstrated that age-related changes in heart function are reduced or absent in long-lived flies when the amount of insulin is reduced in the fly. The insulin-IGF receptor is known to regulate lifespan. This paper describes a decreasing change in resting heart rate over a period of seven weeks, as well as an increase in the rate of stress-induced heart failure. It is shown that in mutations of this insulin receptor or where insulin-like peptides are reduced, flies either do not experience or minimally experience this declining heart rate. This paper also shows that over-expression of a phosphatase dPTENor a transcription factor dFOXO also inhibit a decline in heart rate [11]. Figures 1.4 a and b demonstrate that the mutant *chico* phenotypically exhibits both low failure rates with age as well as a maintained heart rate with age.



Figures 1.4 a and b (A mutant *chico)*: A mutant *chico* has been discovered that maintains a higher heart rate throughout the aging process and also experiences a lower failure rate throughout the aging process [11]

1.3.6 Detrimental Effect of Anesthesia

The use of anesthetics is a common medical procedure. Use of a general anesthesia provides a temporary loss of consciousness. Local anesthesia, such as lidocaine, results in a temporary loss of sensation at a specific site in the body through the application of drugs. Regional anesthesia results in a reversible loss of sensation and possible loss of movement by blocking sections of the nerve and spinal cord. Anesthesia is either injected or inhaled. Age, weight, and medical history significantly influence the dosage of anesthesia that is applied.

In animals and humans, the application of too much anesthesia (overdosing) can result in reduced blood pressure, weakened heart rate, cardiac dysrhythmias, and a change in respiration. Therefore, when conducting studies with the use of anesthesia, the physiological conditions of the patient must be considered and carefully monitored. This can be executed by carefully administering an appropriate, specific amount of anesthesia.

Previous studies have been performed involving the use of anesthesia on *Drosophila melanogaster.* These have included understanding the effect of anesthesia on the mating behavior of flies [19] as well as understanding the effect of anesthesia on an escape response [20]. These studies have indicated that anesthesia has an effect on different behaviors of the fly. To what extent *does* anesthesia affect *Drosophila melanogaster*? The effects of anesthesia need to be carefully understood when using it to conduct a physiological study.

Barron [19] explored the roles of both chilling and CO_2 anesthesia on the mating behavior of *Drosophila melanogaster*. Results indicated that both methods increased copulation latencies even if they were applied 20 hours before the behavioral study. The

CO_2 slowed mating more than chilling did. This suggests that the recovery period may not be long enough, and that when conducting behavioral studies, one should avoid using anesthesia [19].

Lin and Nash [20] sought to study the neural pathway involved in the visual escape response of *Drosophila melanogaster*. This paper determined necessary concentrations of halothane gas that must be given to flies to obtain certain responses.

Moreover, Walcourt and Nash [21] have also shown that a mutation, known as *mud*, which stands for *mushroom body defect*, has caused an increased sensitivity to halothane.

Krishnan and Nash [22] also studied *har (halothane resistant)* mutants. They discovered that these mutant flies were more resistant to the effects of halothane than wild-type flies. These mutants' behaviors were less affected than those of the wild type flies. The study was conducted by allowing halothane to flow into the chamber. At the end of the study, flies' resistances were determined by visualizing their location in the chamber. Flies lying on their backs on the bottom of the chamber were considered anesthetized. Flies that were higher up in the chamber were considered to be more resistant [22].

Another well-known anesthetic used for research involving flies is known as FlyNap® (an anesthetic by Carolina Biological Supply Company containing triethylamine) [23]. This product contains triethylamine. Its material safety data sheet reports that it is a "severe irritant to eyes and mucous membranes. Prolonged exposure may cause damage to heart, liver and/or kidneys [24]." This chemical is used in physiological research in addition to being used globally in genetics classrooms.

Current methods for FlyNap® application include chambers that are filled with 1 ml of FlyNap®, or using absorbent wands that are dipped into the anesthetic and then placed into a vial containing the flies [23].

Paternostro et. al [2] used an absorbent wand dipped in FlyNap®. This wand was placed into the vial that contained the flies. Flies were taken from the vial as soon as they were immobile [2]. This study did not expose the flies to a precisely measured amount of anesthesia, nor did it provide a precise exposure time. Investigators reported that there was not an immediate depression in heart rate after the removal of the FlyNap®. The heart rate was very similar immediately after anesthesia removal and 30 to 40 minutes after anesthesia removal. Mean heart rate was found to be 283 ± 6 bpm (beats per minute) versus 282 ± 4 bpm for 10 day-old flies, and in 48 day-old flies 213 ± 6 bpm versus 211 ± 11 bpm. Therefore, Paternostro et. al [2] concluded that FlyNap® is a cardio-protective agent.

Wessells and Bodmer [10] report that FlyNap® will immobilize *Drosophila* without acutely affecting heart rate [10]. They also report that flies become more sensitive to FlyNap® exposure with advancing age. Their research found that a 10-minute exposure of FlyNap® could be given to flies up to 5 weeks of age in a slow-release *Drosophila* anesthetizing chamber [10]. The resulting heart rate and function was not different from flies that had been immobilized using glue or adhesive tape [10].

In summary, previous studies have indicated that anesthesia does affect cardiac physiology and function [2,13,16,18-23]. However, previous studies have not specified exact exposure time or dosage of anesthesia to apply. There is therefore a need to address these issues.

1.3.7 Current Methods for Stressing the Heart

A stress is an organism's total response to environmental demands or pressures [25]. Stress may be physical or emotional. In dealing with *Drosophila melanogaster*, the stresses are physical. Researchers have used various methods to cause stress on the heart.

Temperature

Paternostro et. al [2] applied a temperature stress by anesthetizing their flies and placing them into an incubator containing a Diaphot microscope. Two minutes after insertion, a video camera began recording the flies' hearts. In protocol 1, the incubator started at 22°C and over the course of an hour was gradually increased to 28°C [2]. The second protocol called for the flies to be anesthetized and then placed into the incubator. After two minutes, videos were recorded. This was done for temperatures of 28°C, 32°C, 35°C, and 38°C [2].

Dowse et. al [11] studied fly larvae. Larvae were stressed by using temperatures. Placed on a slide exposing their hearts, larvae were exposed to temperatures of 20°C, 25°C, 30°C, 35°C, and 37°C. The temperatures tested went as low as 18°C, 16°C, and 14°C. Between each temperature, there was rest period of 1.5-minutes. At each temperature, a thirty-second video was recorded. A Sensortek unit helped maintain the temperature [17].

Vermeulen and Bijlsma [26]used temperatures to determine the temperature dependence of lifespan. Flies were housed in incubators at temperatures of 21°C, 25°C, and 29°C. Flies were transferred to fresh vials twice a week and the number of dead flies were counted [26].

Electrical Pacing

Researchers [2,13] also used external pacing to stress the heart by way of a model 611 square wave stimulator. Micromanipulators were used to place electrodes (with Sigma gel electrode gel) on the surface of the abdomen. Pulse trains of 20-second duration with a recovery period of one minute were applied. This was done in increasing steps from 5 Hz to 8 Hz at 22°C. At 28°C, the pacing was performed from 6 Hz to 9 Hz. The duration of the electrical stimuli was 30 ms, and the voltage that was applied was 40 V [2].

Wessells et. al [13] prepared glass slides that had electrodes soldered onto them and covered with a layer of tin foil, leaving loops exposed (figure 1.5 a and b). A gap between the tin foil 2 mm in width was created. Conductive jelly was spread along each side of the slide. A maximum of five flies were placed onto the slide at a time, perpendicular to the foil [13]. A square wave stimulator was attached by way of leads to the loops attached to the electrodes. 40 V was applied at 6 Hz for 30 seconds, following which the flies' responses were recorded. Three responses were observed: recovery of normal heart beat, fibrillation or cardiac arrest [10].

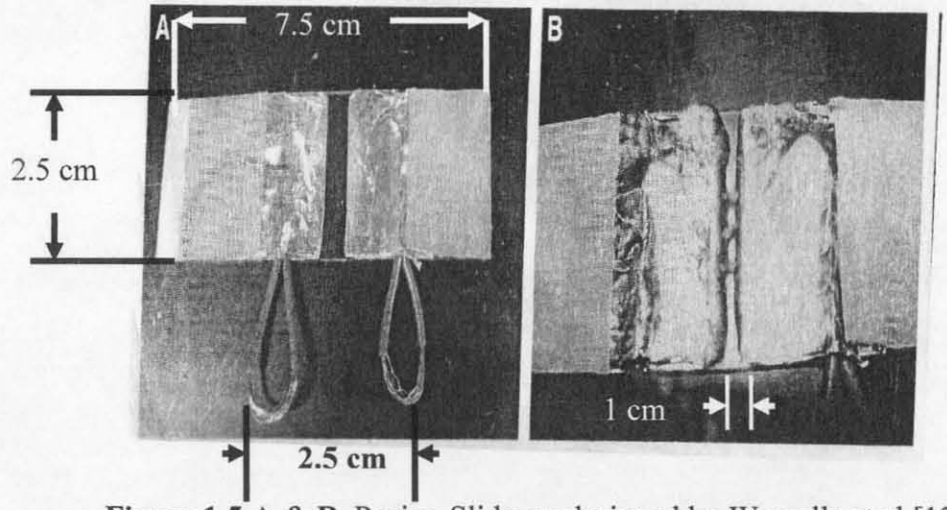


Figure 1.5 A & B Pacing Slide, as designed by Wessells et al [13]

Drug Effect

To investigate the role of dynamin in cardiac function, Johnson et. al [14] used neurotransmitters to stress the heart. Norepinephrine, dopamine, serotonin were used to examine the effects of these drugs on the heart's ability to beat [14].

Johnson et. al [13] investigated the role of neurotransmitters on the heart to see their effects on the heart's ability to beat. Mutant strains were also tested to see if there were any differences in drug responses. Following treatment, heartbeat was recorded for two minutes at 20°C, 25°C, 30°C, 35°C, and 37°C. The last thirty seconds were used for analysis [13].

Zornik et. al [8] also used neurotransmitters to gauge their role in affecting heart rate. Serotonin, octopamine, dopamine, acetylcholine, nicotine, caffeine, and muscarine were injected into larva, pupa, and adult flies in an attempt to alter heart rate. Amino acids were also injected into these animals to study their effects. GABA, glutamate and proctolin were the proteins used [8].

Johnson et. al [16] tested nine neuropeptides, which had been isolated from Drosophila melanogaster, as well as five neuropeptides that had been isolated from the central nervous system of *Limulus* to see their effects on the heart rate of the fly. These substances were injected into the dorsal, caudal end of the fly near the heart. Following injection, the flies were placed at 25°C and their heart rates were recorded [16].

1.3.8 Image Analysis

Custom software programs can be used to analyze recorded videos of the flies beating hearts to determine heart rate. Depending on the computer, this could be a faster or slower process. In addition, programs could contain errors, leading to flawed data. Therefore, visually counting the number of heartbeats within a given time frame [2,10,13] can lead to an accurate measurement of total heart rate. This is the gold standard used by researchers. In addition, positioning of the fly needs to be exactly the same for many programs to work.

Paternostro et. al [2], although reporting that manually counting the heart rate is more accurate, used a custom-designed digital image-processing method to measure heart rate and its variation directly from a fly's video signals. A Pentium II-based microcomputer was used to store the videos and a high-resolution frame grabber (sampling frequency of 30 frames/sec) was used. Ten two-second videos were recorded (60 frames each) and custom software was used to develop a time-space image signal to represent the time course of image intensity along a line segment of pixels that crossed the ventricular lumen [2]. A low pass filter was then used, reducing the noise, and heart rate was determined by counting the peaks in the signal. This procedure was repeated

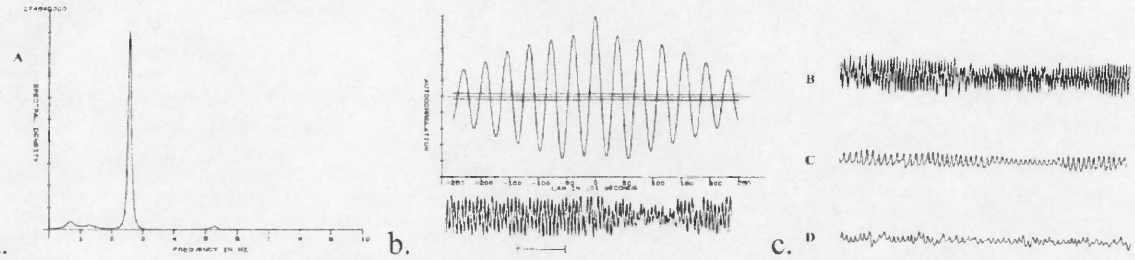
with unfiltered data to obtain an autocorrelation and the spectral density by Fast Fourier Transform [2].

Wessells et. al [13] used manual counting to determine the number of heart beats within a given time frame due to the dark pigment of the flies making it difficult for the edge-tracing software to be used [10].

Dowse et. al [11] used a phototransistor fitted to an eyepiece. When movements of the heart occurred, the signals that were received by this phototransistor were amplified using a 741C operational amplifier for pre-amplification and a Grass 79D Polygraph. The output was recorded digitally by way of a Sony Betamax recorder, sampling frequency of 44KHz, through a Nakamichi DHP-100 digital audio processor. A Metrabyte DAS8 analog to digital converter was used to download the data, which was then stored in a 486i computer for digital analysis (using custom-designed software) and re-digitized to a frequency of 100Hz. A plot of thirty seconds of data was made for each temperature. The following two digital-signal techniques were used: Autocorrelation analysis (which gives a good estimate of the strength and significance of any periodicity in the data) and Maximum Entropy Spectral Analysis (a high-resolution, highly sensitive spectral analysis system used to estimate the heartbeat frequency) [11]. Autocorrelation was used to quantify the strength of the heartbeats [17]. From the signal, the first peak was counted as number one and the third peak was measured as a fraction of the first peak, i.e. a correlation coefficient. [11]. An objective measure of the strength of the rhythmicity of the signal can be made because if the time series is more irregular, then the decline in the autocorrelation envelope will be more steep [11].

Maximum Entropy Spectral Analysis (MESA) is a technique that consists of choosing the most random time series with its correlation function coinciding with a set sequence of estimated values. It was developed to because classical Fast Fourier Transformation techniques were developed to define a stationary state of the processes they were calculated for. MESA should predict the correlation function of observed time series by way of maximizing the entropy of the process in theoretical and informative meaning [27, 28].

Johnson et. al [15] used Maximum Entropy Spectral Analysis to estimate heart rate. Autocorrelation was used to determine the regularity of heartbeat [14]. Rhythmicity of the signal was indicated by positive and negative peaks of correlation (Figures 1.6 a, b and c) and the decay envelope indicated the regularity of the signal [14].



Figures 1.6 a, b and c a: Spectral analysis shows a peak frequency of heartbeat. b & c: Autocorrelation techniques show how regular the signals is and how rhythmic [14]

Johnson et. al [12] used Maximum Entropy Spectral Analysis to analyze the data using autocorrelation techniques. Raw data plots as well as spectral analysis helped to estimate the heart rate. The strength of the heartbeat was determined using the autocorrelation function [13]. The faster the decline in strength, the less regular the heart rate was [13].

Johnson et. al [14, 17] sed custom software to analyze the data. Maximum Entropy Spectral Analysis (MESA) software estimated the heart rate. Autocorrelation techniques determined the significance of the MESA frequency peaks. The decaying envelope of the autocorrelation determined the strength and regularity of the heart rate [14, 17].

Such automated techniques have been developed in order to speed up heart rate analysis in large groups of flies. However, the gold standard to successfully measure heart rates is hand counting, which continues to be used to verify the accuracy of automated methods.

1.3.9 Conclusions Drawn From Literature Review

Researchers have used many methods to determine an average heart rate under normal and stressed conditions. Temperature, electrical stimuli, and injected drugs have proven to be useful tools to achieve elevated heart rates, thereby modeling a stressed cardiac situation. The tools to determine average heart rate may be different, but they all successfully measure heart rate. Table 1.1 summarizes the heart rates as reported by previous studies [2, 5, 13,15].

Much research has been spent studying cardiac function in *Drosophila melanogaster*. The fly has proven itself a great tool for investigative purposes for several reasons: the fly has a short lifespan of around sixty days, it is easy and cheap to maintain, the fly's genome has been mapped out and mutations can easily be made within it, and human disease genes have been conserved in the fly. With today's society having as many health problems related to genetics, the fly is a likely candidate to pursue these

studies. Therefore, *Drosophila melanogaster* is an important animal model in studying diseases or illnesses of the heart.

Studies have been performed to investigate the role of anesthesia in the fly. Investigators have shown that certain forms of anesthesia, such as CO2 or chilling affect behaviors in *Drosophila melanogaster*. Others have shown that FlyNap® does not alter fly heart rate. These papers differ in their suggested amounts of anesthesia as well as application methods.

As demonstrated, the fly is a great candidate for a cardiac disease model. A system must be implemented that can acutely study the flies' hearts. Furthermore, this system needs to monitor the flies as closely to conscious conditions as possible to obtain an accurate heart rate value.

A characterized method for measuring cardiac function with well-described dosage of anesthesia should be established.

Table 1.1 Summary of Adult Fly Heart Rates Under Normal (Room Temperature-25°C) Conditions

Researcher	Adult Fly Heart Rate (BPM)
Paternostro et al [2]	6 day-old flies: 310 ± 6 10 day-old flies: 286 ± 3 30 day-old flies: 249 ± 5 47 day-old flies: 245 ± 9 54 day-old flies: 220 ± 3
Wessells et. al [10]	1 week-old flies: 174 BPM 5 week-old flies: 144 BPM
Johnson et. al [15]	2.41 ± 0.04 Hz

Table 1.2 Summary of Adult Fly Heart Rates Under Temperature Stress

Researcher	Adult Fly Heart Rate (BPM)
Paternostro et al [2]	35°C: 10 Day Old Flies: 440 ± 7 30 day-old Flies: 372 ± 8 54 Day Old Flies: 288 ± 10
Johnson et. al [15]	20°C: 2.28 ± 0.05 Hz 30°C: 2.72 ± 0.04 Hz 35°C: 3.08 ± 0.05 Hz 37°C: 3.33 ± 0.05 Hz
Wolf et. al [5]	22°C: 271 ± 13 BPM 38°C: 511 ± 26 BPM

Table 1.3 Summary of Adult Fly Heart Rates Under Paced Conditions

Researcher	Maximum Achievable Paced Heart Rate (BPM)
Paternostro et al [2]	22°C: 10 Day Old Flies: 411 ± 13 59 day-old Flies: 303 ± 8 28°C: 6 Day Old Flies: 498 ± 9.6 59 day-old Flies: 414 ± 18

CHAPTER 2

MATERIALS AND METHODS

2.1 Design of a Fly Cardiac Function Measurement System

Our goal was to develop an accurate way in which to measure *Drosophila melanogaster* cardiac function. Previous methods successfully measured cardiac function. We wanted to develop an improved system that would be user-friendly and not alter the physiology of the fly in the process. Finding an improved method to appropriately stress the Fruit Fly heart and measure cardiac response was also an important goal.

Design of an Anesthesia Chamber

Carbon dioxide, as an anesthesia method was found to be useful in sorting flies, but not in studies longer than three minutes in duration. A CO2 gun usually provides this anesthetic. A tank of carbon dioxide also provides a plate with a continuous flow of carbon dioxide. After the gun anesthetizes the flies, the flies are transferred to the carbon dioxide plate, where a continuous gas flow keeps the flies asleep. Because the CO_2 plate is not optically clear, this is not a practical procedure to study the flies under a microscope.

The Carolina Biological Supply Company supplied an anesthetic agent called FlyNap® (Figure 2.1), which would knock the flies out for genetics studies that required more time to manipulate the flies.

Figure 2.1 FlyNap®, supplied by Carolina Biological [23]

The Carolina Drosophila Manual states "the length of time the flies remain anesthetized depends on the amount of FlyNap® on the wand, and on the number and age of the flies in the culture vial [29]." This statement begs a very important question: How much anesthesia is enough? On another page, the manual informs the reader that FlyNap® safely anesthetizes flies for fifty minutes to several hours. The manual does not provide an exact measurement of anesthetic that the wand can hold. The company stated that the wand holds between 35 and 40 µl.

Another method that the Carolina Biological Supply Company offers to apply FlyNap® is the Carolina Anesthetizer (Figure 2.2). This is designed to slowly release ether. The chamber can be adapted so that ether can be more quickly applied to the flies. Flies remain anesthized by ether for 5-10-minutes.

Figure 2.2 The Carolina anesthetizer from Carolina Biological Supply Company [23]

The company also does not provide any information on the interaction of the anesthetic with the flies' physiology, including cardiovascular and neural effects.

Therefore, before doing any physiological studies, we needed to determine the effects of FlyNap® on our flies' cardiovascular systems and their heart rates. Although reported by other investigators that there is not, in fact, an adverse effect of FlyNap® on heart rate, we needed to verify these claims. To do so, we tested flies that were exposed to FlyNap®.

In these tests, we noticed that flies were falling asleep at different, inconsistent times from one trial to another. We also noticed that flies that brushed up against the actual liquid anesthetic got stuck and then died. This led us to believe that the necessity for an exactly measured amount of anesthesia was great, as well as a specified delivery time. In addition, it seemed essential separate the flies from the actual anesthetic. We also noticed that after two minutes, fifteen seconds all of the flies seemed to be asleep. The flies lying on their sides or on their backs indicated this. There wasn't any visible leg or body movement.

At earlier times, although the flies could be seen lying on their sides or backs, their legs or heads could be seen moving. Quickly placing them onto a microscope slide and observing body movements under a 5x objective lens better observed this. This helped us to reach the conclusion that 2.5-minutes of exposure was a safe delivery time.

This led to the design of a new anesthesia chamber that would allow an exact amount of anesthesia be delivered and would separate the flies from falling into the liquid anesthetic. We used a polypropylene conical tube to make the chamber. It is a BD Falcon conical tube (item number 352098). We cut off the bottom at the 20-ml mark,

located near the conical end of the tube. One and a fifth of an inch was cut off of the tube. The edges were filed down so that they were smooth. This is the end where a cotton ball would be inserted, ensuring that the flies could not escape. On the other end, where the cap screws onto the tube, we used silicone glue to secure an upright, 100 µm-pore filter. We had also filed the edges of this to round it so that filter would fit into the tube. The cap could easily be screwed on over this (Figures 2.3 a & b)

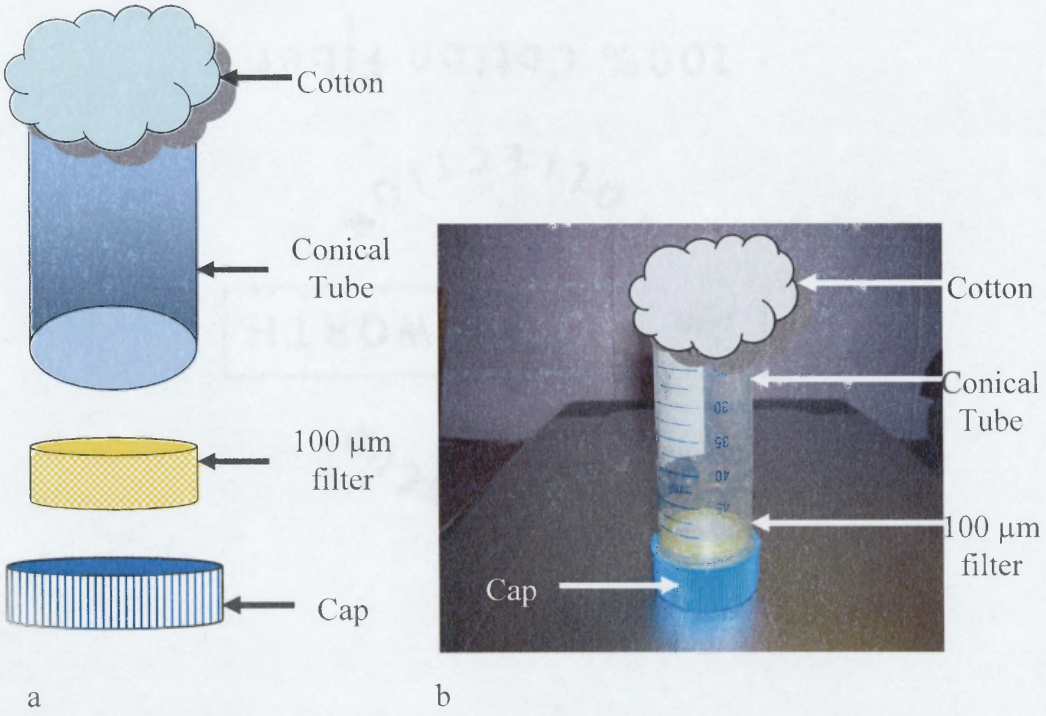


Figure 2.3 a & b The Anesthesia Chamber.

The idea behind this design is that the flies would be quickly knocked out by using the CO_2 gun and then brushed into the anesthesia chamber. A cotton ball is then inserted. Next, a calibrated Eppendorf pipette man is used to deliver 40 µl of FlyNap® into the cap, which would then be screwed onto the other end of the tube. A timer is used

to make sure that anesthesia was delivered for exactly 2.5-minutes. As flies fell asleep they fell onto the filter, which would prevent them from falling into the liquid anesthetic.

Next, with the new anesthesia chamber, it was necessary to test the anesthesia's effect on the heart rate of *Drosophila melanogaster*. 2.5, 5, 7.5, and 10-minute exposure times were decided upon. Groups of ten flies were placed into chambers, exposed to anesthesia for these amounts of time, and then monitored for their heart rate.

Drosophila Handling and Positioning

After receiving anesthesia, flies were placed out onto a white box in contrast with their dark bodies. This allowed the investigator to work closely with them.

To place flies onto the heart rate slide, forceps were used to lay the flies flat on their bellies. Using a paintbrush and the forceps (Figures 2.4 a and b), the wings were spread out and then stuck into the tape. The wings had to be spread because when they are folded in, they cover the heart and made it very difficult to see. In addition, it was very important to make sure the flies were placed completely horizontal to the slide. Positioning on any other angle resulted in inaccurate, unclear images of the heart.

Figure 2.4 a & b Forceps (a) and paintbrush (b) used to manipulate flies

To ensure that we were not twisting the bodies of the flies, causing them harm, we used magnifying glasses (Figure 2.5) to see the fly and position the fly. This allowed us to use the paintbrush and the forceps carefully.

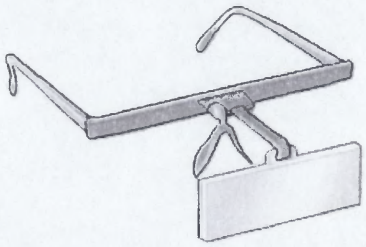


Figure 2.5 Magnifying glasses used to better visualize the flies

Drosophila Heart Imaging

Previous studies have discussed using double-sided tape to place the flies on. We discovered this to be optically unclear and therefore unfavorable. The development of a better method on which to place the flies to visualize their hearts became crucial. This heart rate slide contained a piece of double-sided tape running the lengths of each of its short sides. Using forceps, a piece of optically clear Scotch tape was laid perpendicular and across these, stuck into and connecting the two pieces of double-sided tape. A thermocouple was taped onto the top of the slide, making it possible to measure the slide's temperature. A similar thermocouple was taped onto the microscope to measure air temperature. The temperatures were interpreted and monitored using a Digisense Dual JT-E-K Thermocouple Thermometer by Cole Parmer.

Dual temperature monitoring allowed the investigator to make sure that all studies were conducted under similar temperature conditions. This ensured that temperature did not rise too high or fall too low. Slide temperatures were generally in the range of 24°C

to 26°C. Keeping a semi-constant temperature with continuous monitoring was crucial for our studies because temperature is a known stress to the cardiovascular system.

For heart rate measurements, any number of flies can be placed onto the tape. For our purposes however, there were only between ten and fifteen flies in the chamber at a given time.

An upright Nikon Ecclipse E200 microscope was used for several reasons. First, the microscope was reasonably priced as opposed to inverted microscopes (microscopes that have lights that shine the object from above and provide a large working area for the investigator because objectives do not get in the way), which are more costly. In addition, the stage was adjustable in three planes. For our purposes, we needed a longer working distance (the distance between the stage and the bottom of the objective). This was so that we could maneuver forceps or paintbrushes underneath the objective in case there were any last minute fly-adjustments that needed to be made. The longer working distance also allowed the flies to be visualized but not crushed by the objective lenses.

Next, the microscope also contained an infrared reducing filter. This ensured that the light source did not emit too much heat, which was important for two reasons. First, as previously mentioned, higher temperatures are known to increase heart rate. We did not want any outside factors to influence our studies. Also, in addition to increasing heart rate, too much heat can physically harm the flies.

The microscope also contained an adjustable light source so that the amount of light being shined onto the fly could be changed. This allowed us to avoid illuminating areas of the flies' bodies we were not interested in. In addition, this feature allowed us to keep the light at a minimum amount so the very small organs were not washed out.

Flies were first visualized under a 5x objective. The heart was placed in the center field of the objective. Using the course and fine objectives, the heart was brought into focus. The shutter was then opened, allowing the beating heart to be displayed on the computer (Dell Optixplex GX280) screen.

Using a video camera (MTI RC300) that was placed on top of the microscope, up to a 1-second video was recorded of each fly under the 5x. This was to be used for identification and verification purposes. The camera was attached to a power source that was adjusted by a gain knob. The value that was necessary to set this gain knob was determined through trial and error- how much light and magnification was needed. Figures 2.6 a, b and c show the microscope used to visualize the flies as well as a basic diagram of the heart rate slide and temperature monitors.

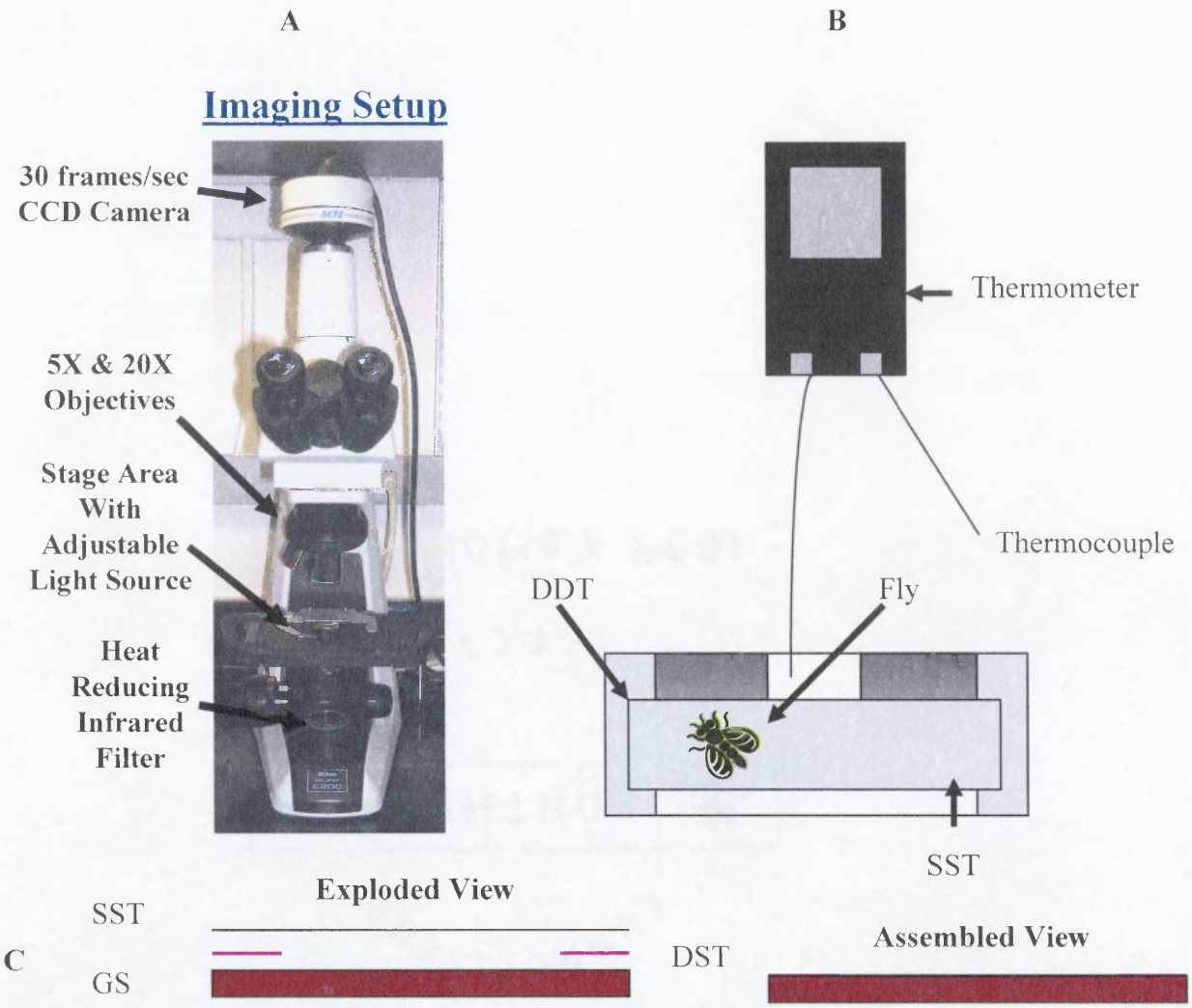


Figure 2.6 a, b, and c Imaging Set-up: (a) microscope set-up (b) top view of the imaging slide (c) side view of the imaging slide (where the following abbreviations are used: SST- single-sided tape; DST- double-sided tape; GS- glass slide)

Next, the 20x objective was moved into place. At this point, only the course adjustment was used to further focus the fly heart. A twenty-second video was then recorded under the 20x condition. These videos were to be used later to calculate an average heart rate for each fly. A picture of the flies' orientation and the corresponding heart pictures under both a 5x and a 20x object is shown in Figure 2.7.

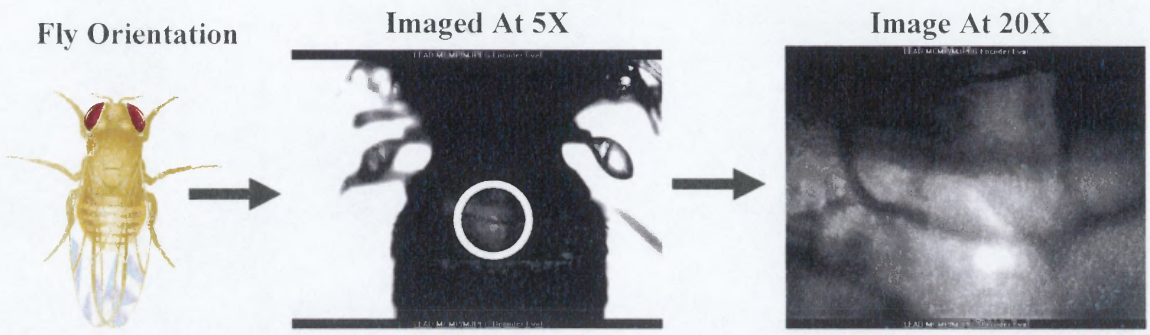


Figure 2.7 *Drosophila melanogaster's* Orientation For Room Temperature Heart Rate Measurements

2.2 Development of a Method to Stress the Flies' Hearts

Following the procedures used by Wessells et. al [10], a protocol was developed that would stress the heart of the fly. Wessells et. al used a slide that contained wire soldered 2.5 cm apart. Each of these wires was covered with conductive aluminum foil, leaving loops of wire exposed on which to attach leads to the electric stimulator. A gap of 1 cm was left between the pieces of aluminum foil. Conductive jelly was spread along each of the edges of the wire, leaving a gap between the jelly layers on which to lay the fly. With this set-up, nothing less than 40 V would effectively pace the heart [10].

Initially, two pacing slides, with inner gaps of 2.5 mm and 3 mm were made. According to Wessells et al. [10], electrode gel filled the extraneous space and flies were set into it. Upon testing these procedures, we found it very difficult to pace and test the flies. Flies were oriented in the following manner, visualized in the microscope and on the computer screen.

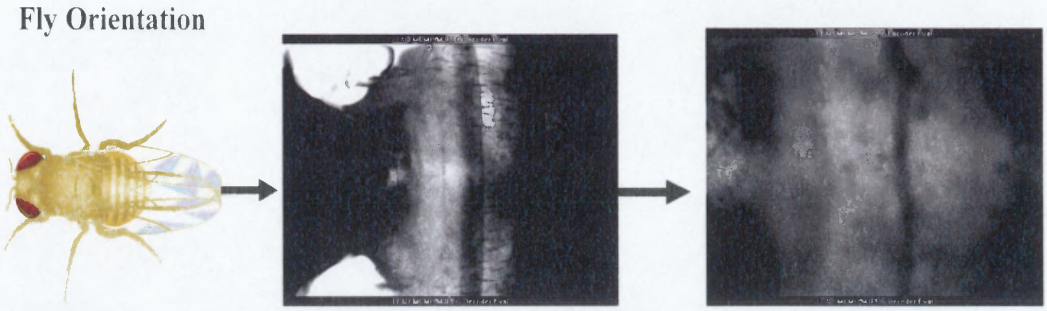


Figure 2.8 Fly Pacing Orientation and Corresponding 5x and 20x Heart Pictures and Orientations

Although the flies' heads and abdomens were placed in the jelly, the flies were not anchored. The gap used was 1 cm wide and the flies' bodies were between 2.5 mm and 3 mm. Therefore, when the electrical stimulator was turned on, the flies would begin to shake violently or move. Consequently, further analysis of heart rate was nearly impossible because the fly was moving so rapidly that the video taken could have been showing a paced heart *or* a heart beating regularly but in a violently shaking organism. It made calculating heart rate very difficult.

We also found that placing the jelly inside the gap caused optical problems as well. The light shining through the jelly caused very bad glares and caused regions to be so bright that the region of interest was not visible. If even the slightest bit of gel from the gap got onto the body of the flies, the heart could not be visualized.

It became apparent that a method that would better anchor the flies as well as pace them and keep them from moving was needed.

The design that was used to pace the flies involved making a spacer. The spacer was comprised of four cover slips -100 μm thick - which were used to help create a very small gap between our pacing metal, on which the fly could lay across, leaving only a very small abdominal region exposed. The cover slips were bonded together using UV

glue. A piece of lead (Atlantic Nuclear, thickness of 0.015 in.) was cut and glued down with clear silicone glue to a glass microscope slide. This lead is composed of 95.75% lead, 2.5% antimony, and 1.75% tin. It had a measured resistivity of 2 ohms. Silicone glue was spread along one of the outer edges of another piece of cut lead. The spacer was held in place, and the other piece of lead metal was slid up against the spacer. Removal of the spacer left a very small gap of 400μm.

Next, wires were soldered onto the ends of these pieces of lead (Figure 2.9). These wires were attached to banana clips, which would be used to plug into the ground and the positive voltage plugs on the stimulator. On the other ends of each of the pieces of lead, single wires were soldered. These wires were to be connected to an oscilloscope probe. The purpose of this was to be sure that the input voltage was the same as the output voltage. For example, we found 4 V necessary for electrical pacing. We wanted to be sure that not only was our stimulator supplying 4 V, but our flies were also receiving 4 V. This was necessary to determine any voltage loss that may have occurred due to resistance of the fly, jelly, or lead.

It was also important to consider the interaction between the electrical pacing gel and the lead when the voltage source is turned on. In ECG, in order for the signal to pass from the body to the electrode, an electrically-conductive path must be determined [28]. Oxidative and reductive chemical reactions take place at the source of contact between the metal and the gel [30]. Commonly, silver - silver-chloride (chosen because it causes less noise in the resulting signal) is used as the metal in electrodes for electromyograms [30]. The silver-chloride layer allows current from the muscle to pass more freely across the junction between the electrolyte and the electrode [30]. This material does not cause

significant offset potential (a voltage stored by the electrode that will add to the ECG signal and interfere with it) [28]. Stainless steel materials, however, have poor offset characteristics [28].

Researchers, when electrically pacing fly hearts, have used aluminum to stress the fly. We chose to use lead, however, because of its availability, low voltage requirements to adequately pace the fly, and the flexibility of the material to be cut and manipulated on a 2.5 cm x 7.5 cm microscope slide.

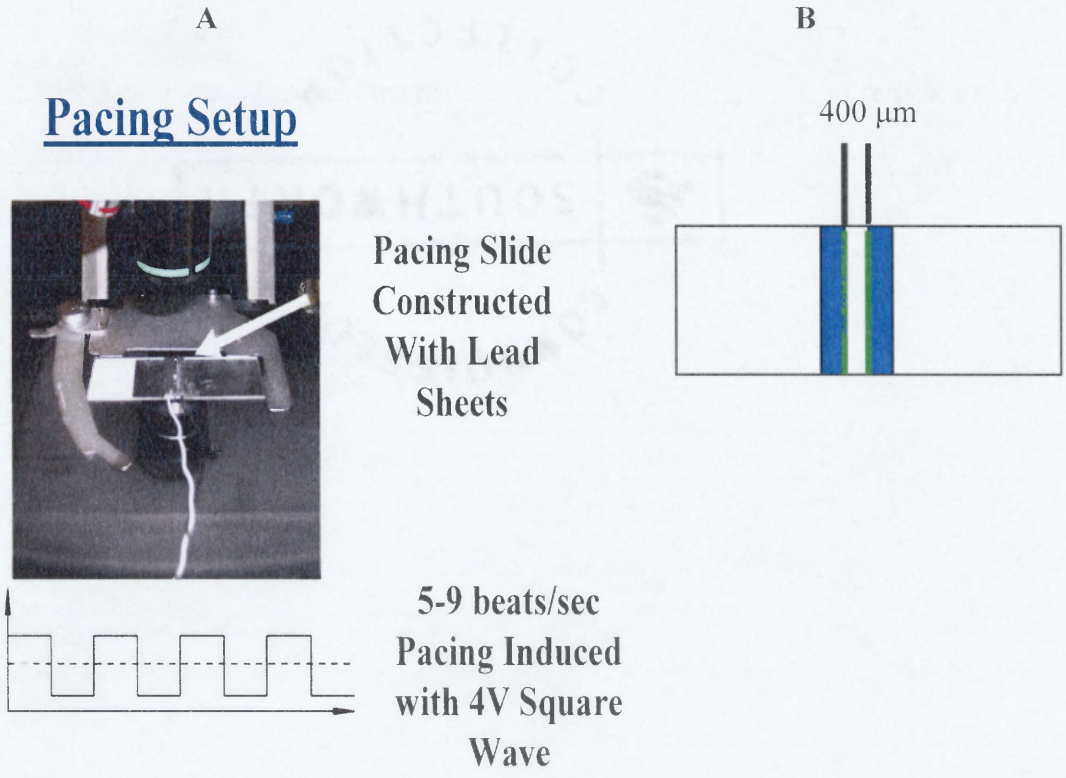


Figure 2.9 Pacing Slide

Having completed the pacing slide, it was important to determine if there was any voltage loss due to the fly, jelly, or lead. To do this, the pacing slide was first tested by itself by connecting it to the voltage source (a square wave stimulator) and applying 4

volts. The oscilloscope screen showed that 3.7 V was actually being applied, so there was a voltage loss of 0.3 V. Applying the jelly to the pacing slide also resulted in a received voltage of 3.7 V. Therefore, the jelly did not cause any further loss. Lastly, flies were placed into the jelly, with their head touching one piece of lead/jelly and their abdomens touching the other. 4 V was applied, and the oscilloscope showed that between 3.6 and 3.7 V was achieved. This was confirmed on numerous occasions.

Signa electrical pacing jelly comes in two varieties. One is more conductive than the other. We used the more conductive of the two- Signa gel electrode gel, highly conductive multi-purpose electrolyte. This was tested and allowed the flies to be more easily paced. It also did not cause any voltage loss. Lastly, the gel provided a means to anchor the flies' bodies. We found that lining the inner edges of the pacing metal provided the most stable form of electrical pacing, resulting in no body movement. Very little gel was used for this. A very thin line of gel was used (~ 2 mm thick) on each side. We also found that squeezing the gel out of its original vessel caused unnecessary globbing. Therefore, we used a needle to apply our thin line.

It was also necessary to determine whether or not there was a delay between the time that the voltage source was actually turned on and the time that the fly received the voltage. This was accomplished through watching the computer screen and witnessing the flies' physical state (body movements and heart rate) and also watching the oscilloscope as it increased from reading 0 V to 3.7 V. For example, one could literally turn on the electrical stimulator and begin to watch the computer screen. At a given point in time, the electrical stimuli would literally "turn on" and the flies heart beat pattern could be seen changing into a more rhythmic pattern. We found there to be almost a

seven second delay from the time our stimulator was physically turned on and the time the fly actually began to be paced.

Upon testing 40 V, we discovered that this was too much voltage to be applying. This made the flies either severely jump on the slide, die, or both jump and then die. By continuously lowering the voltage, we found that at 4 V, the flies' hearts were adequately paced. To discover the maximum frequency that could be achieved, we first set the voltage to 4 V, and then turned the frequency to 5 Hz. This was the lowest frequency tested in any of the literature that was reviewed [2]. We subsequently raised the voltage when the flies' hearts would not reach a given frequency. We kept increasing the voltage until it began to hurt the fly, which was indicated by body movement. The maximum frequency that was achieved was 8 Hz. 4 V was sufficient to achieve this frequency. Figure 2.10 outlines the procedure that we took to determine a maximum voltage required and the maximum frequency that could be achieved.

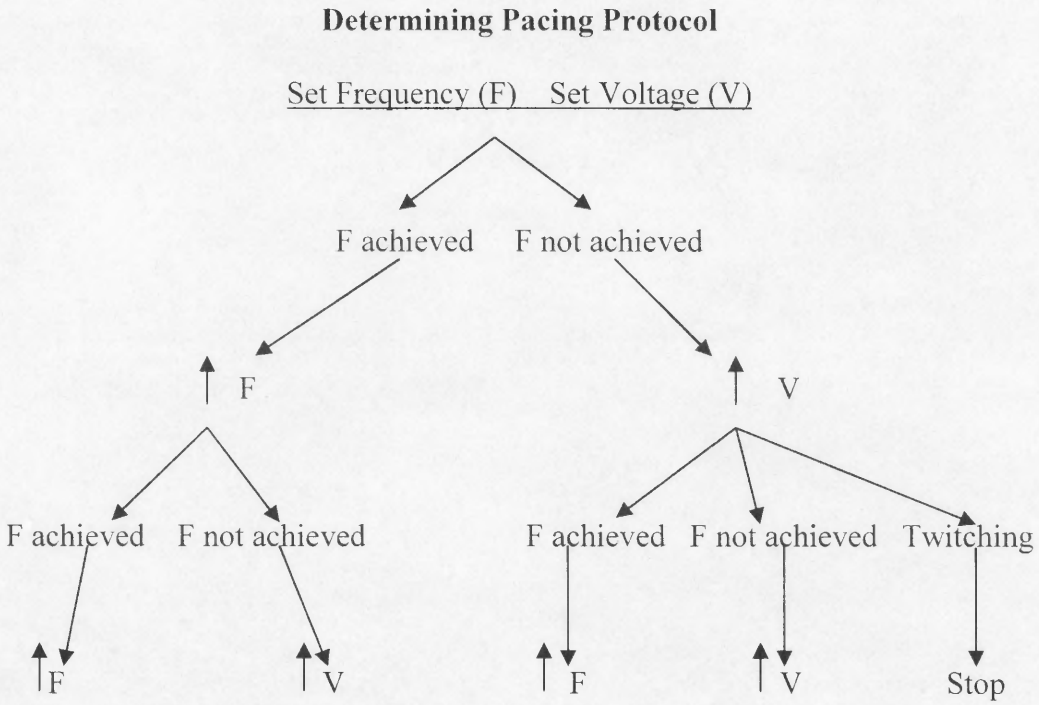


Figure 2.10 Determining the pacing protocol

This diagram allowed us to determine an optimum frequency and voltage required to electrically stimulate and pace the flies' hearts. When a twitching motion was observed, we knew that the fly was in some sort of pain and the voltage had to be decreased.

A variable duty cycle stimulator was used to deliver the voltage. The stimulator used was a Grass Instruments model SD5A-F231XG. Figure 2.11 shows a Grass Telefactor square wave stimulator, model SD9.

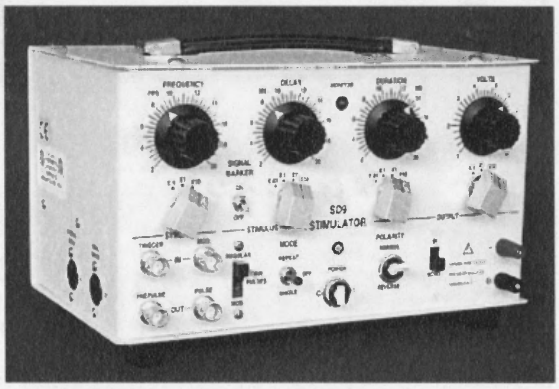


Figure 2.11 Square Wave Stimulator SD9 by Grass Telefactor [31]

The duration of the pulses from the electric stimulator was set to be 30 ms [2]. A stimulator is a device that allows a voltage to be repeatedly delivered under controlled conditions. The following is a definition of pulse duration: In a pulse waveform, the interval between (a) the time, during the first transition, that the pulse amplitude reaches a specified fraction (level) of its final amplitude, and (b) the time the pulse amplitude drops, on the last transition, to the same level. The interval between the 50% points of the final amplitude is usually used to determine or define pulse duration, and this is understood to be the case unless otherwise specified [32].

In deciding upon stimuli duration, we investigated the role of the duty cycle. The definition of duty cycle is the total specified period of operation. A train of pulses with a 50% duty-cycle is called a square wave [33]. Pulse-width modulation (PWM) is used in some music synthesizers to vary the duty-cycle of an oscillator during the performance, which has a subtle effect on the tone colors obtained [33]. The duty cycle can be calculated by dividing the time that the signal is in the high state by the period (time required to complete one cycle) of the cycle and multiplying by 100%. The period can be calculated by dividing 1 by the frequency (the number of complete cycles per second that the signal contains).

Using the above calculation, for elecrical pacing at 5 Hz, the period is 0.2 s. Electrically pacing at 6 Hz, the period is 0.17 s. Electrically pacing 7 Hz, the period is 0.14 s. Electrically pacing at 8 Hz, the period is 0.125 s. Electrically pacing at 9 Hz, the period is 0.11 s. For a pacing duration of 30 ms, the duty cycle would be calculated by by dividing this duration by the period of the cycle (which is changing with each pacing frequency) and multiplying by 100%. Therefore, by changing the pulse-width, or

duration of the stimuli, the duty cycle can be changed. We attempted a duty cycle of 50%, but it appeared that the flies' physiologies were being disturbed (indicated by tremors and severe shaking). Figure 2.12 shows a wafeform with a duty cycle that is 15%- corresponding to a pulse duration of 30 ms and a frequency of 5 Hz.

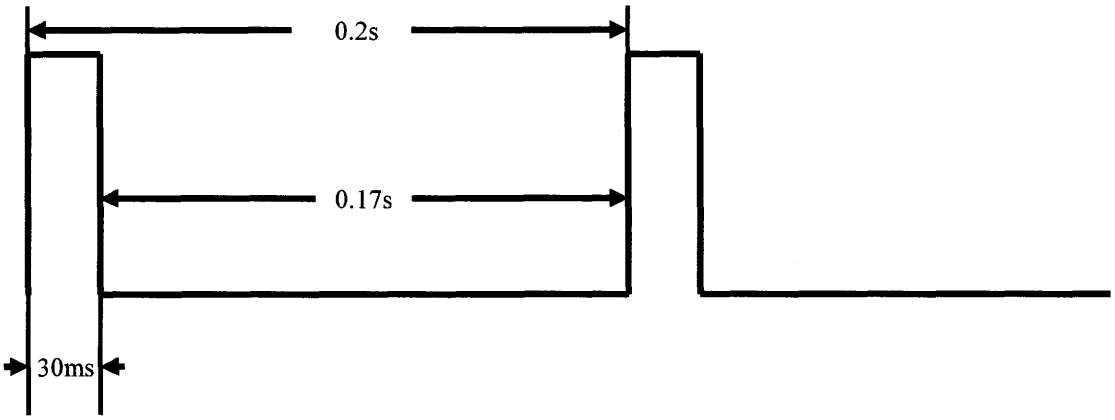


Figure 2.12 A wafeform with a frequency of 5 Hz and a duty cycle of 15%

Finally, each fly's heart was located in the above-mentioned method and a one second video under the 5x objective was recorded for it. A twenty-second video was recorded without any electric stimuli for each fly under the 20x objective. The stimulator was then set at duration = 30 ms, voltage = 4 V, and frequency = 5 Hz. As soon as the fly was visibly being paced- detected by both visually seeing the heart being paced on the computer screen as well as seeing the voltage reach its threshold on the oscilloscope- the camera was turned on and a twenty second video was recorded. Following twenty seconds, both the stimulator and the camera were turned off for a one minute rest period. This was then repeated for frequencies up to and including 9 Hz. The next fly was then placed onto the pacing slide and the procedure was then repeated.

2.3 Cardiac Function Analysis Method

The videos were recorded on a camera that had a frame rate of 29.97 frames/sec. Other investigators [2, 10, 13] seemed to break down long videos into shorter intervals and then determine an average heart rate.

On Microsoft Excel, a workbook for each day that videos were recorded was set up. For example, on 8/12/05, four tubes containing ten flies each were anesthetized and had their heart rates measured after being exposed to 2.5, 5, 7.5 and 10-minutes of anesthesia. The Excel workbook was set up so that it recorded the time of day the heart rate was recorded, the slide temperature, the air temperature, and the anesthesia exposure time for each fly.

Each twenty-second video was then opened and saved as a batch on an avi-to bitmap program. Another program, Image J, (available through the NIH website http://www.nih.com/ [34]) then imported all of the individual video frames. Image J allowed us to darken the background of the videos so that the heart walls were more visible if necessary. It was decided that the twenty-second videos were to be broken down into 10, 2-second intervals, each with its own heart rate, from which an average would be found.

We chose to record twenty-second videos for several reasons. First, we needed a length of time that would provide an adequate picture of what was really happening to each fly heart. As previously mentioned, the fly's heartbeat pattern is triphasic, containing pause periods. From our observations, under anesthesia, some of these pauses can last up to two seconds. If we chose a small time interval, such as two seconds, we could get a value of "0" as the heart rate of the fly, which is not accurate. Therefore, we

chose a video of twenty-second duration. Longer videos would require an automated video analysis method. Next, after choosing a video length of twenty-seconds, we chose to break down each video into ten two-second intervals. We did this so that we could obtain an accurate average heart rate. For example, if we counted the number of beats over the full twenty seconds, we would not get a clear picture of what was happening at various points over the course of time. We needed to have average heart rates at various points to get a clear picture. We then averaged these ten averages to get the average heart rate. Averaging over twenty seconds suggests that there is a constant average heart rate. This is not the case with the Fruit Fly.

Each fly had its own Excel spreadsheet that needed a starting frame. The spreadsheet was then programmed to find an expected end frame- sixty frames after the starting frame.

The frame right before the first contraction took place was then found. This was the starting frame and was recorded as such on the spreadsheet. Next, the number of complete heartbeats was counted within sixty frames. If the 60^{th} frame was in the middle of a beat, we went back to the frame where that particular contraction began and counted that as the end frame. The idea was to get an average heart rate contained within or close to 60 frames. For example, if the starting frame was 12, the end frame should be 72. However, if the 10^{th} beat began on frame 67, but was an incomplete beat at frame 72, the end frame was taken as 67, containing 9 beats. We were accurately able to

This was done for ten intervals. At the end of these intervals, Excel was programmed to calculate an average heart rate, a standard error about the mean, a

standard deviation, a total count (the number of intervals or samples used), and an error percent.

For the different age flies, separate workbooks were designed that would total all of the nine day-old flies under 2.5-minutes of anesthesia for example, and compute an average as well as an standard error about the mean, and plot these numbers against those results for 5-minutes, 7.5-minutes, and 10-minutes. An unpaired significance test, or student's t-test was then used to determine if there was a significant difference between average heart rates under the different anesthesia levels.

Another spreadsheet was set up so that the average heart rate for each age group was compared under each anesthesia level. For example, one graph shows the average heart rate under 2.5-minutes of anesthesia for 9, 30 and 53 day-old flies. This was then done for 5, 7.5 and 10-minute anesthesia dosages.

Lastly, spreadsheets were set up so that the average achieved heart rate under each set frequency was calculated. All the individual flies were totaled to give a large n-number, and an average achieved heart rate under that set frequency was calculated. Standard error bars were given to show the deviation within samples. Each fly video was scored on a scale of 1-4, with 1 having the worst clarity and 4 having the best clarity.

There are several positive and negative issues related to our image analysis program that should be mentioned. First, there is the issue of sampling. The main sampling problem that could occur is not satisfying the Nyquist frequency requirement. The Nyquist frequency is two times the maximum sampling frequency. Our camera, which captures images at about 30 frames per second (30 Hz), satisfies the Nyquist frequency requirement. The Nyquist frequency is 30 Hz and half of the Nyquist

frequency is 15 Hz. The maximum frequency at which our flies are paced is 9 Hz, well below the Nyquist frequency.

A faster camera, however, would be able to capture more details about the heart beat patter. Furthermore, although a faster frame rate camera would be more useful to minimize or eliminate sampling problems that could possibly occur, it is fairly expensive.

In addition, a faster camera would definitely necessitate designing or purchasing an automated image-analysis program. If we were to purchase and use a camera that captures images at 60 frames/sec, a 20-second video would consist of 1200 frames. This would make counting by hand a very long process and would cut our productivity in half.

Next, as stated earlier, it is not always possible to align the fly completely vertically. The camera that was used for our purposes could easily rotate a full 360°. Therefore, even though the fly was not actually completely vertical with respect to the slide, we could always manipulate the camera so that the image that was being recorded was being recorded as a vertical image. This setup would allow future image analysis programs to analyze the videos because the fly would always be in the same orientation.

Also, our Excel workbook was really useful in that once we had a template set up for both the average heart rate under anesthesia and for electrical pacing, as soon as we entered in data the necessary calculations and graphing was performed.

2.4 Development, Maintenance, and Growth of Flies

The flies that were used in this study were known as wild type *Drosophila melanogaster*. Wild type flies typically have normal characteristics for fruit flies- yellow body with black stripes and red eyes. The wild type flies used for our purposes were noted as *yw*:

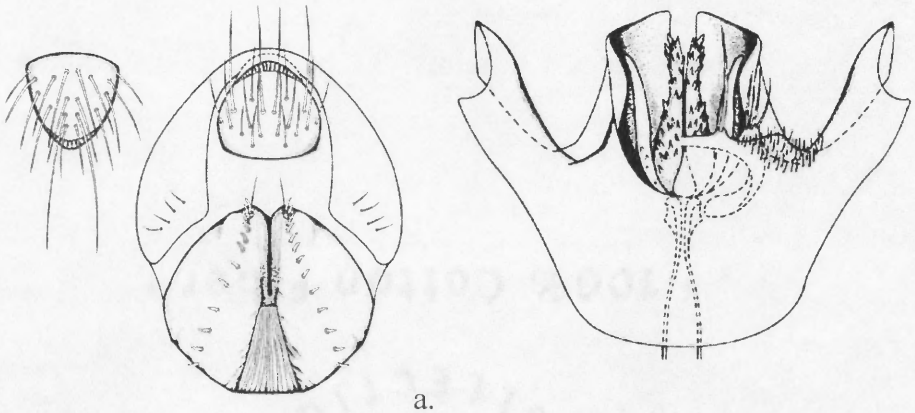
these flies were yellow body with white eyes. These are mutant phenotypes for the fly. In flies, any further mutation will cause the eye color to be closer to red than white. So, if the body color remains the same and a mutation is being studied, investigators are able to visibly see which flies express the mutation and which ones don't.

Flies were put into vials with no more than ten flies per plastic vial. They were transferred to fresh vials every four days to ensure that unhealthy, dirty conditions did not have any adverse effect on age longevity or heart rate conditions. Vials were plugged with cotton to ensure that air could get into the vials while preventing the flies' escape from their housing environment.

Flies were fed an agar/molasses-based food during the length of the studies. For a complete protocol on the preparation of fly food, please refer to Appendix B. Flies were mated at room temperature in clear, plastic bottles that were filled with one inch of fly food. Yeast was sprinkled on top of the food. The male to female ratio for the mating was 1:3.

A microscope (Nikon Type 104) was used to separate young and old flies as well as gender. Newborn virgin female flies were selected for mating. Having a nearly white appearance identified virgins. Their bodies had not yet turned yellow and they had not yet developed their characteristic black stripes. For the mutant, virgins also appeared white and their eyes were a slight white/pink color- they had not yet turned dark orange.

Turning them on their backs and looking at their sex organs identified gender of the flies. Figures 2.13 a & b demonstrate the difference between a female and male fly. Under a microscope, it is easy to identify the male because his sex organs are a red color, with a red patch on the tip of the abdomen. The female's sex organs are white.



a. b.

Figure 2.13 a & b The female (a) and male (b) fly [34,[35]

After mating these flies, the parent flies were transferred to fresh bottles to begin laying more offspring three or four days later. This was done so that the bottle did not become overcrowded with fly larvae and adult flies.

At room temperature, flies are born about two weeks after the larvae are laid. This time can be shorter if the larvae are housed in a higher temperature or can be longer if housed in colder temperatures. After the flies necessary for our studies were born and separated out into housing vials, the vials were placed into an incubator set at 25°C. Flies were only removed from this incubator to be used in heart rate or stress analyses.

For sorting of flies as well as transferal from one vial to another, CO_2 was used. A CO_2 gun used in tandem with a tank attached to a CO_2 plate enabled the user to temporarily knock out flies in vials or bottles so that the flies could be easily transferred from one bottle to another. The gun allowed the user to dump the flies onto the CO_2 plate, where a constant, steady stream of CO_2 was applied for sorting purposes.

2.5 Polymerase Chain Reaction Procedure

Polymerase chain reaction, or PCR, is a procedure that researchers use to amplify a single strand of DNA into billions of copies of the same strand. This allows further testing to be performed on that DNA sample [36].

Polymerases are enzymes that take one copy of DNA and make a second copy of it. A double strand of DNA- a double helix- must first be detached (denatured by breaking chemical bonds that exist between nucleotide bases) into separate strands, creating two templates. DNA bases bind in a lock and key fashion. A binds to T and C binds to G. The polymerase enzyme comes along and attaches nucleotide bases- A,C,T, or G- to these two strands, creating two identical double-strands of DNA.

Although DNA amplification occurs naturally in the body, laboratory simulation requires reagents to be added into the PCR "mix." Other than the DNA, the two other components are necessary for copying DNA. These are the a supply of free nucleotide bases (A, C, G and T) as well as a primer. The free nucleotides are necessary because every time that a DNA double-helix is denatured and used as two template strands, the enzyme needs free bases to attach to the template to make a complete double helix. A primer is a small sequence of nucleotides that binds to the region of interest. It is typically 15 to 30 nucleotides long. These sequences initiate the copying process. There are also typically forward and reverse primers. One primer binds to one of the DNA template strands, the other primer binds to the other half of the DNA double helix, or the complimentary strand. The polymerase enzyme is called a Taq.

The PCR reaction occurs in three steps. The first of step is the denaturation process that happens at high temperatures between 75°C and 95°C. The time of this

process can vary depending on the DNA sample itself. Typically, 30 seconds is necessary. The second step occurs at a much lower temperature of about 55°C for 20 seconds [36]. Again, this time can vary depending on the actual DNA samples. The annealing of the primer occurs during this step. The final step is the amplification process, where the Taq enzyme adds free bases to the template strands. Every newly synthesized strand can provide two template strands *for the next strands to be synthesized.* This step is repeated for at least 30 cycles [36]. After these cycles, 1 strand of DNA can produce over a billion copies. Figure 2.14 outlines the process of a PCR DNA amplification.

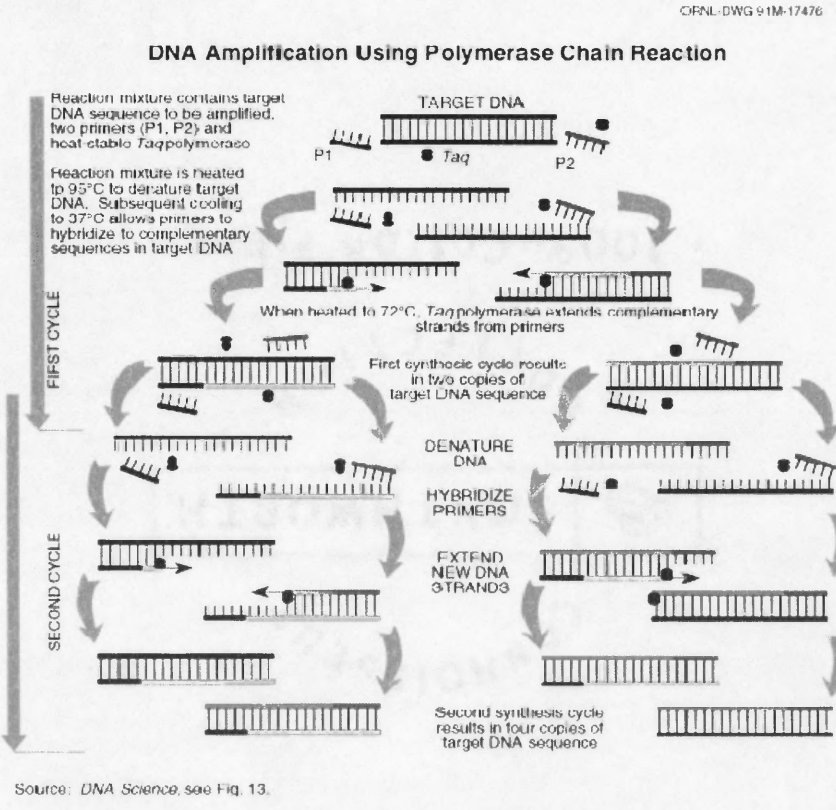


Figure 2.14 PCR Amplification Process [37]

After the above process, a sample of the PCR product, mixed with a dye, is loaded into a well on a gel made from agarose. The gel is placed into an electrophoresis

machine that pulls the negatively charged DNA through the gel towards the positive side of the machine. This results in bands forming on the gel that can be seen under a camera that picks up these images. Every DNA segment that is being amplified by PCR has a specific size. Larger sizes do not move as far along the gel under a given voltage and time. Smaller sized fragments move farther along the gel (Figure 2.15). Figure 2.15 is a picture of a PCR product taken by Dr. Yongkyu Park. This shows a ladder on the left, which has bands that appear at different base pair weights. This is used as a comparative factor to see if the researcher gets the appropriate size of DNA that they are looking for. In this picture, it can be seen that in one fly, the mutant, the region of interest has been knocked out under the M, for mutant. The W stands for wild type. PKA, or Protein Kinase A, is being used a control to ensure that the DNA is good and not a contaminated sample. This is because Protein Kinase A is present in all the flies, not just the wild type flies. This is a great PCR product.

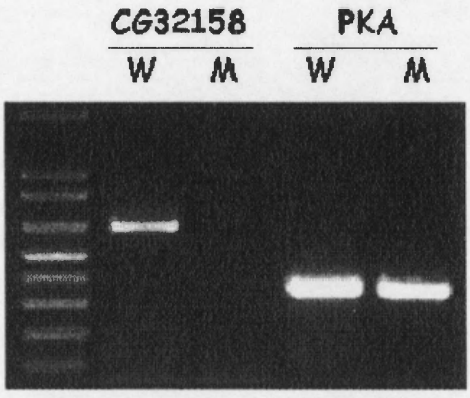


Figure 2.15 Demonstrating that a mutant knock out fly is in fact missing a segment of its original DNA that the wild type fly (on left) has in tact [38].

CHAPTER 3

RESULTS

3.1 Comparing Methods of Anesthesia

Initial experiments were run to determine which anesthesia method was the preferred method- carbon dioxide, ether, or FlyNap®. First, ether was tested on YW and YW[C06449] flies (a mutant fly). This was done in a hood environment as ether fumes should not be inhaled. Ether resulted in the flies becoming immobile between forty and fifty seconds. For the first trial, the flies had begun to move after six minutes of ether removal, and only one video was able to be recorded. The second trial resulted in five flies being recorded, but with movement after four minutes of ether removal. The third trial resulted in movement after five minutes of ether removal. Lastly, the fourth trial resulted in movement after four minutes of ether removal. Incidentally, movement was characterized by leg movements or by flies attempting to flip over onto their bellies.

Additional flies that were used to test ether exposure time showed that over-exposure to ether resulted in the flies spreading their wings or beginning to struggle.

We then compared the results of the ether test to a FlyNap® trial. On average, the results of repetitive studies of testing on FlyNap® indicated that for a vial containing between 5 and 15 flies, flies seemed to fall asleep at two minutes, plus or minus ten seconds. To measure ten flies, it took about five minutes to place them all on the heart rate slide. It took about eight additional minutes to measure all the heart rates. Flies

were still asleep by the end of this time period. Comparison of these two methods indicated that FlyNap® was indeed the better choice when performing physiological tests.

Next, after determining that FlyNap® was the best anesthetic to use, it was necessary to determine an appropriate application method. The methods tested included the use of a Q-tip, the Carolina Biological Supply Company application wand, and our newly designed anesthesia chamber. Both the Q-tip and the anesthesia chamber were used so that an exact amount of 40µl of FlyNap® could be applied to the flies.

The following results were obtained to determine if there was a significant difference ($p<0.05$) using the student's t-test between the heart rates achieved using the wand method, supplied by Carolina Biological Supply Company, and a Q-tip.

On July 5th, 2005, July 6th, 2005 and July 13th, 2005 seven day-old YW (wild type) flies were tested in an experiment to measure the above-mentioned significant difference. The samples were pooled together to give a larger n-number so that there would be less variability between samples. It was found that there was a significant difference between the heart rates achieved using the wand vs. those achieved using the Q-tip for this group. The average heart achieved for the Q-tip method was 305.2 ± 1.3 bpm. This group was comprised of 33 samples. The wand method achieved an average heart rate of 257.2 ± 1.9 bpm, with an n-number of 25. The Q-tip method resulted in a higher resting heart rate. Regardless of this outcome, the Q-tip method still was unable to prevent flies from flying or walking into the cotton end and getting stuck in the FlyNap®. If the flies walked into the cotton end which contained the FlyNap®, the flies got stuck

and then died almost immediately or they got wet and then died. The necessity for a better anesthesia method was evident.

A new anesthesia chamber was designed and developed. Next, the wand method was compared with the novel anesthesia chamber method. The following experiments and results were obtained. Tests of the wand method vs. the anesthesia chamber method were performed on four separate occasions. Results from these four occasions were pooled together into one larger group. This increased the n-numbers that were being compared.

Pooled tests on July 17th and July 18th resulted in a t-test value of 0.7919. The wand group had an n-number of 15 and an average heart rate of 321.3 ± 21.3 bpm . The n-number for the chamber group was thirteen and had an average heart rate of 328.1 ± 13.6. The t-test value of 0.7919 ($p<0.05$) indicated that the difference between these two achieved heart rates was not significant. Figure 3.1 demonstrates this relationship.

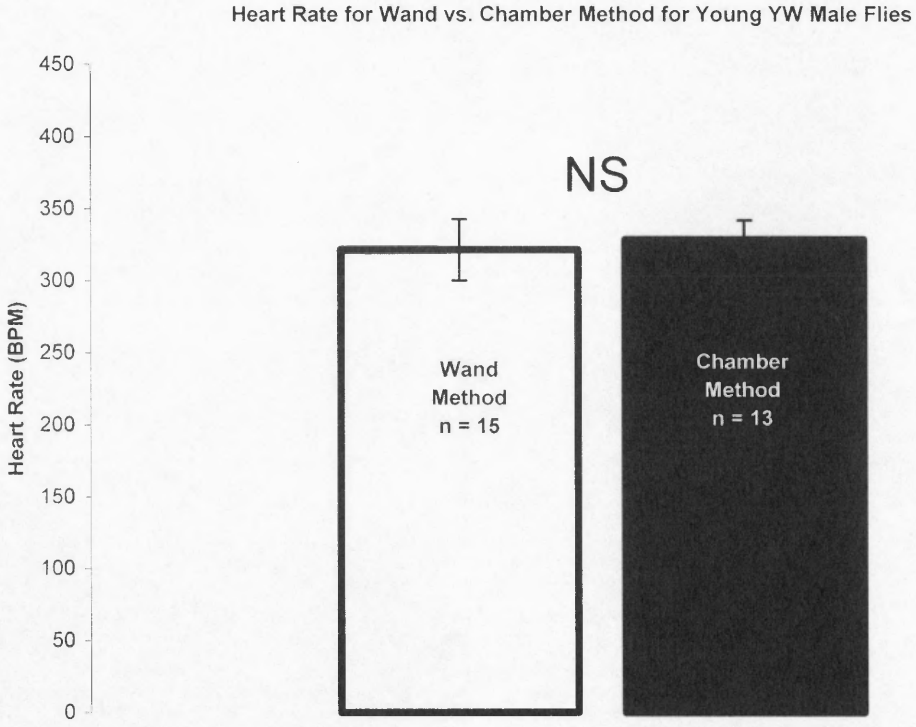


Figure 3.1 Comparison of Wand and Anesthesia Chamber
NS indicates that the student's t-test resulted in an insignificant difference between the two methods

3.2 The Effect of Aging on Cardiac Function

In three separate cases, male YW flies achieved senescence at 47, 35 and 34 days (Figure 3.2). This data was pooled and an average percent survival was calculated per week. The best fit for this data was a second order, polynomial fit. After the eighth week, almost all of the flies were dead. Equations such as the logistic equation, which is a non-linear equation that can be used to model biological functions, were not chosen because of this population's rapid death rate towards the end of its lifespan.

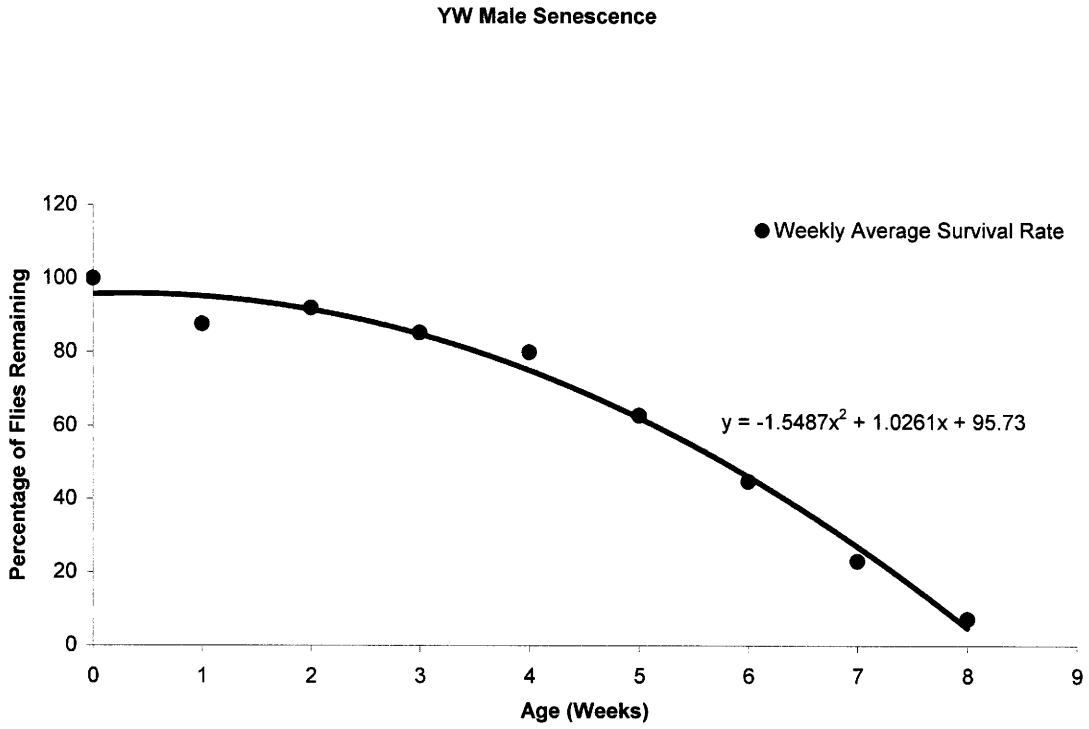


Figure 3.2 Senescence of the wild type flies

3.3 Effect of Aging on Heart Rate

A heart rate study would need a larger n-number for each age group so that a more accurate, clear picture could be achieved as to what was exactly happening to the heart rate with age. This would also reduce the variability within the samples.

Flies of the ages 9, 30 and 53 days of age were tested for their heart rate. These ages seem to represent young, middle-aged, and old flies quite accurately and were modeled after Paternostro et. al. [2]. The results of this study, under 2.5-minutes of anesthesia for the YW male fly are demonstrated by Figure 3.3.

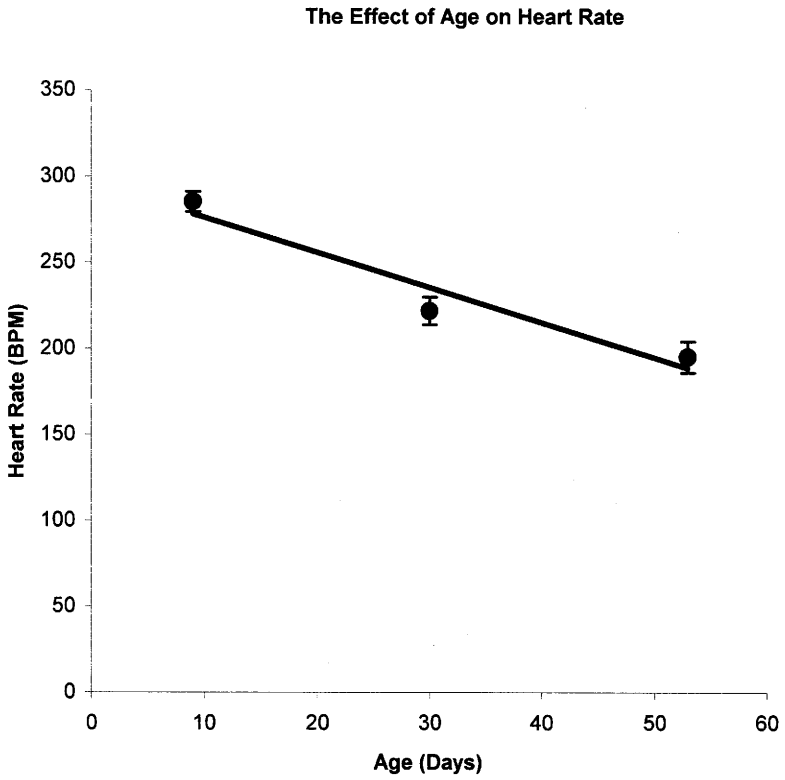


Figure 3.3 The Aging Effect under 2.5-minutes of Anesthesia

Table 3.1 shows the average heart rate values obtained for both male and female wild type flies at the 2.5-minute anesthesia level. Table 3.2 displays the results of the student's t-test for the age comparisons, indicating that there is a significant difference in the average heart rates for the 9, 30 and 53 day old wild type fly.

Table 3.1 Summary of the heart rates obtained at the 2.5-minute anesthesia level

Fly	**Gender**	**Average Heart Rate (BPM)**		
		9 Days Old	**30 Days Old**	**53 Days Old**
Wild Type	Male	285.2 ± 5.8	221.7 ± 8.1	195.1 ± 9

Table 3.2 Results of the Student's t-test For 9, 30 and 53 day-old flies under 2.5 minutes of anesthesia

Comparison	T Test Value	Significance ($p<0.05$)
9 & 30 Day-Old Flies	$2.49 * 10^{-7}$	Significant Difference
9 & 53 Day-Old Flies	$4.65 * 10^{-9}$	Significant Difference
30 & 53 Day-Old Flies	0.0350	Significant Difference

3.4 The Effect of Anesthesia on Heart Rate

Upon observing differences in heart rates that were obtained when the FlyNap® wand was kept in the fly vial and withdrawn at different times- depending when the flies fell asleep- it was necessary to establish what exactly was a good amount of anesthesia to use. While it was crucial that the flies remained knocked out for the time during which they were to be studied, it was also important that the anesthesia have as little to no effect on heart rate. Therefore, it was pertinent to verify the appropriate anesthesia level.

Again, literature has reported conflicting views on appropriate levels and FlyNap® exposure time [2, 10, 11]. Reports of removal of the wand once the flies are asleep (which observations have shown that this time can vary from one fly to another), up to five minutes is appropriate, and no damage is done when exposure time is left as long as ten minutes for flies up to five weeks of age. To determine an exact time, we chose 2.5-minutes (an exposure time around 30 seconds past the time flies were observed to typically fall asleep), 5-minutes (exposure of 3 minutes past time flies typically fall asleep), 7.5-minutes (5.5-minutes past when flies typically fall asleep), and 10-minutes (8

minutes past when flies typically fall asleep). The results of these anesthesia studies are discussed below.

It was found that the effect of anesthesia is constant throughout the aging process. It has the same decreasing trend for all the ages that were studied.

A graph was compiled to show these three ages on one same graph for the wild type flies. Using Excel, a trend-line was plotted for each age. A trend-line is a line that best fits a set of data points. A formula providing the both the y-intercept and the slope of the line can be calculated. One can compare the slopes to see if there is a significant change between one trend line and another. The values of the slopes obtained for the 9, 30 and 53 day-old flies indicate that the effect of anesthesia on the fly is conserved throughout the aging process (Figure 3.4).

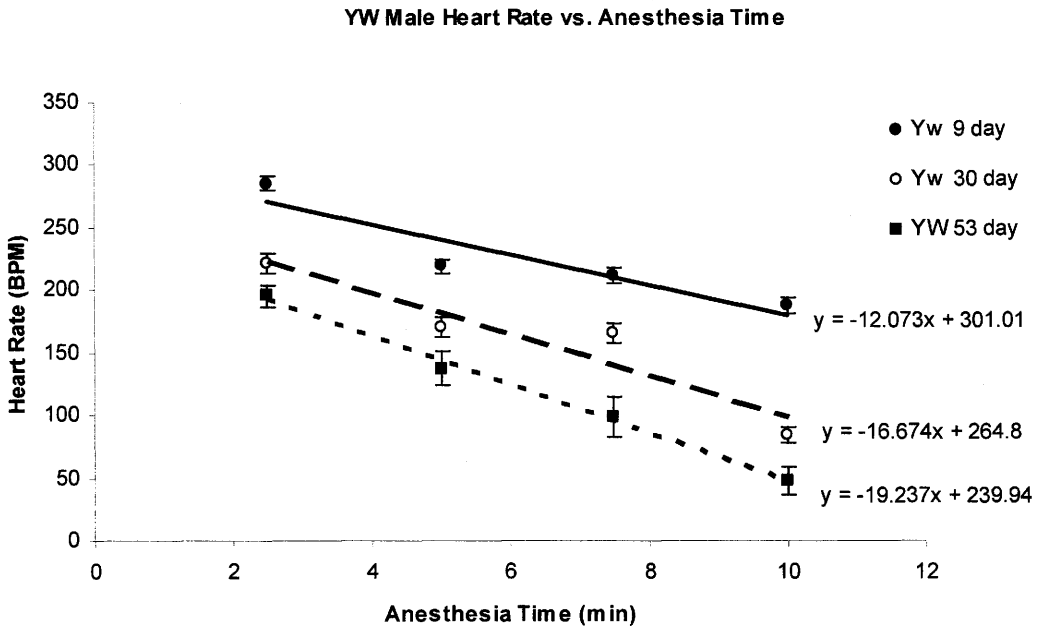


Figure 3.4 Total of effect of anesthesia

For 9 day-old flies, it was found that there is indeed a decrease in heart rate with FlyNap® exposure time (Figure 3.5). This study indicated that there was a significant decrease ($p<0.5$) (Table 3.3) in heart rate from the 2.5-minute exposure level to the 5-minute level. There seemed to be a plateau between the 5 and the 7.5-minute exposure level, showing an insignificant decrease ($p<0.5$) in heart rate. From the 7.5-minute exposure level to that of the 10-minute exposure level, there was also a significant decrease ($p<0.5$) in heart rate.

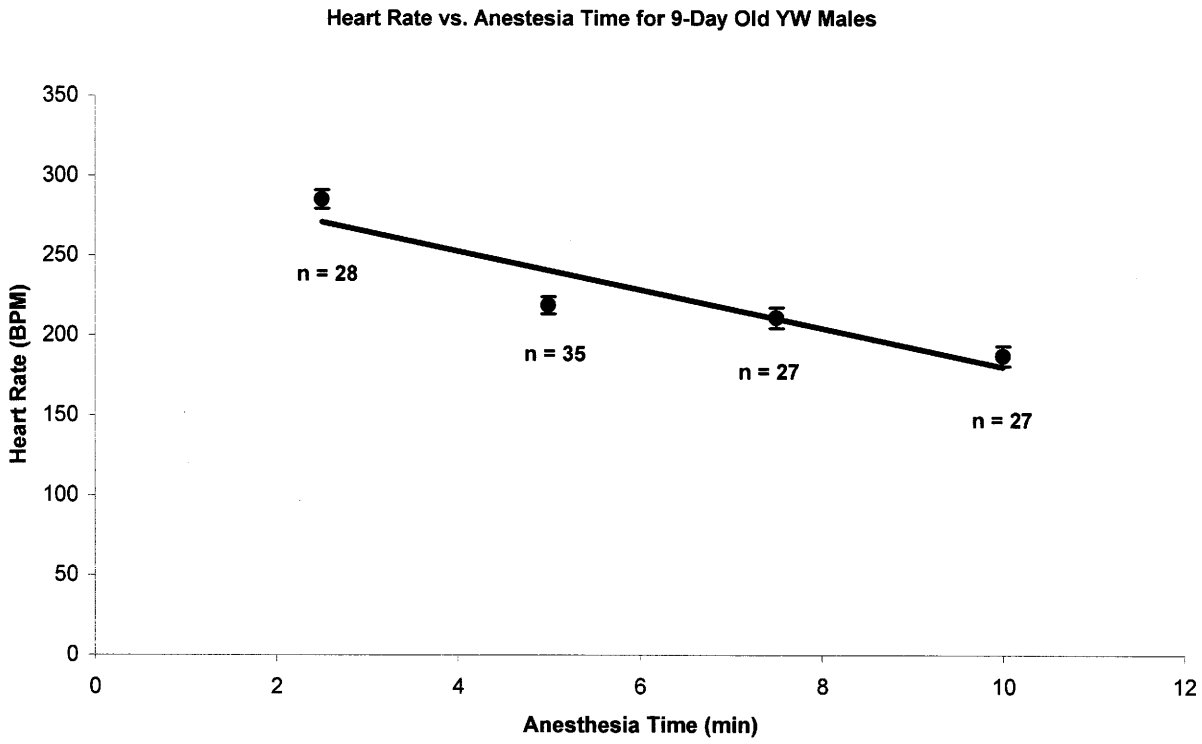


Figure 3.5 Effect of anesthesia on heart rate of wild type flies

Table 3.3 Results of the Student's t-test For 9 day-old flies and the anesthesia effect

Comparison	T Test Value	Significance ($p<0.05$)
2.5 & 5	$1.8588 * 10^{-11}$	Significant Difference
2.5 & 7.5	$2.2556 * 10^{-11}$	Significant Difference
2.5 & 10	$7.7885 * 10^{-16}$	Significant Difference
5 & 7.5	0.3604	No Significant Difference
5 & 10	0.0004	Significant Difference
7.5 & 10	0.0111	Significant Difference

As we were not only interested in young flies, but the effect of aging on heart rate, we checked the effect of anesthesia on middle-aged flies (30 days old). YW male flies were studied.

30 day-old wild type, or YW flies, appeared to have the same trend (Figure 3.6) due to the effect of anesthesia that the 9 day-old YW flies had. Comparisons between the 2.5 and 5-minute levels as well as the 7.5 and 10-minute levels resulted in significant differences ($p<0.05$) between decreasing heart rate values (Table 3.4). A comparison between the 5 and the 7.5- minute levels resulted in an insignificant decline in heart rate.

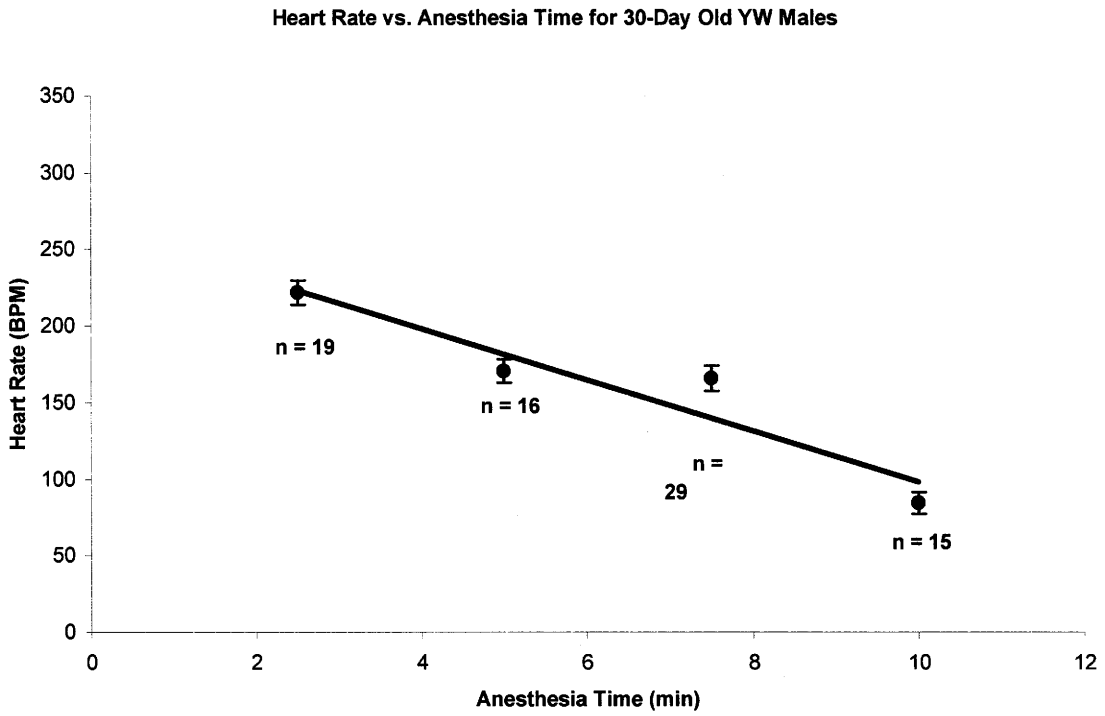


Figure 3.6 Effect of anesthesia on 30 day-old wild type flies

Table 3.4 Results of the Student's t-test For 30 day-old flies and the anesthesia effect

Comparison	T Test Value	Significance ($p<0.05$)
2.5 & 5	$6.0719 * 10^{-5}$	Significant Difference
2.5 & 7.5	0.0044	Significant Difference
2.5 & 10	$3.6738 * 10^{-14}$	Significant Difference
5 & 7.5	0.3288	No Significant Difference
5 & 10	$4.3958 * 10^{-9}$	Significant Difference
7.5 & 10	$3.8943 * 10^{-9}$	Significant Difference

Lastly, an anesthesia study (Figure 3.7) was also conducted for 53 day-old flies. Wild type flies were tested at this age. For all anesthesia level comparisons (2.5 & 5, 5 &

7.5, and 7.5 & 10) there was a significant difference (Table 3.5) between the heart rates that were achieved.

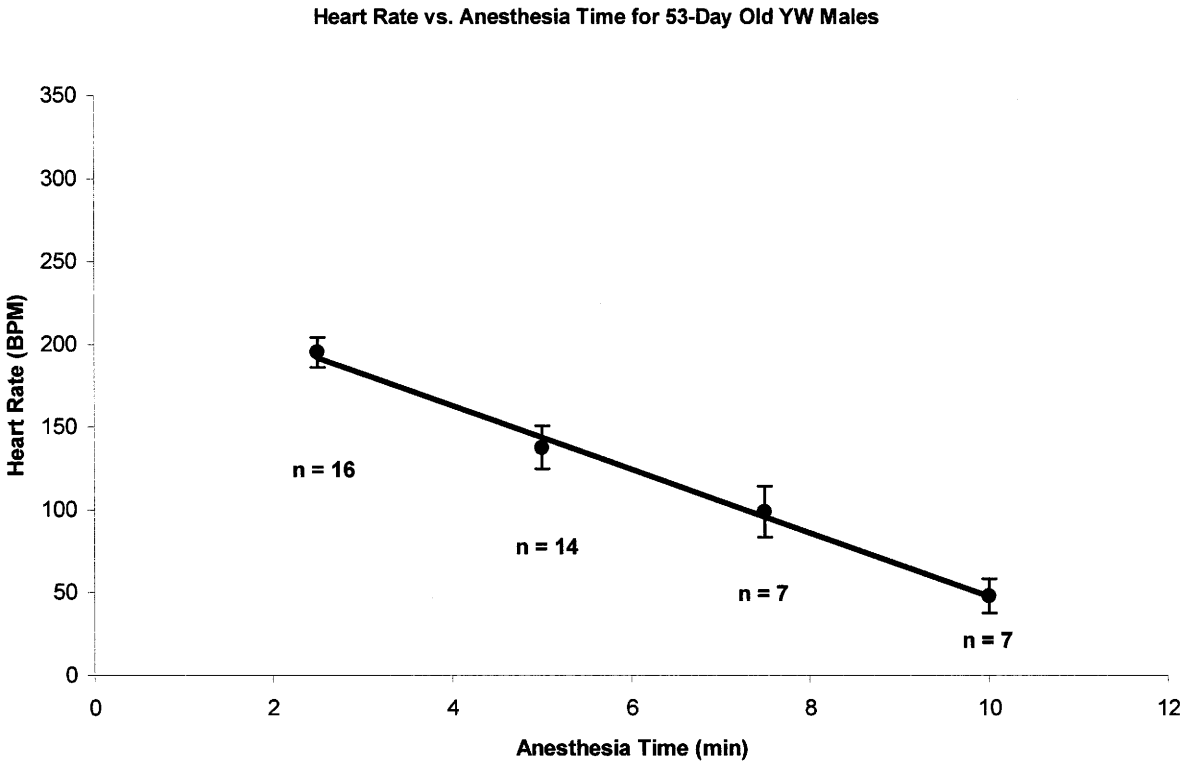


Figure 3.7 Effect of anesthesia on 53 day-old wild type flies

Table 3.5 Results of the Student's t-test For 53 day-old flies and the anesthesia effect

Comparison	T Test Value	Significance ($p<0.05$)
2.5 & 5	0.0013	Significant Difference
2.5 & 7.5	0.0003	Significant Difference
2.5 & 10	$2.1561 * 10^{-8}$	Significant Difference
5 & 7.5	0.0749	No Significant Difference
5 & 10	$3.6731 * 10^{-9}$	Significant Difference
7.5 & 10	0.0203	Significant Difference

Lastly, a table was constructed to summarize the effect of anesthesia on heart rate. This was done for the three studied ages (Table 3.6).

For the wild type fly, using a student's unpaired t-test, there was a significant difference (Table 3.7) in the heart rates that were achieved for each age at the corresponding anesthesia level. For example, at the 2.5-minute anesthesia level, there was a significant decrease in heart rate between the 9 and the 30 day-old YW flies. There was also a significant difference in heart rate between the 9 and the 53 day-old YW flies. In addition, there was a significant difference in heart rate between the 30 and the 53 day-old YW flies. This trait was conserved for the three additional anesthesia levels of 5, 7.5 and 10-minutes.

Table 3.6 Total anesthesia effect

Fly	**Anesthesia Level**	**Average Heart Rate (BPM)**		
		9 Days Old	**30 Days Old**	**53 Days Old**
Wild Type	2.5	285.2 ± 5.8	221.7 ± 8.1	195.1 ± 9
Wild Type	5	218.9 ± 5.5	170.4 ± 7.7	137.4 ± 13
Wild Type	7.5	211 ± 6.5	165.8 ± 8.2	98.6 ± 15.5
Wild Type	10	187.2 ± 6.3	84.3 ± 7	47.7 ± 10.4

Table 3.7 Results of the student's t-test for all age and anesthesia comparisons

Age Groups	Comparison	T Test Value	Significance ($p<0.05$)
9 & 30	2.5	$2.4862 * 10^{-7}$	Significant Difference
9 & 30	5	$1.5935 * 10^{-5}$	Significant Difference
9 & 30	7.5	$7.3164 * 10^{-5}$	Significant Difference
9 & 30	10	$1.3578 * 10^{-12}$	Significant Difference
9 & 53	2.5	$4.6535 * 10^{-9}$	Significant Difference
9 & 53	5	$1.8000 * 10^{-5}$	Significant Difference
9 & 53	7.5	0.0077	Significant Difference
9 & 53	10	$2.2253 * 10^{-7}$	Significant Difference
30 & 53	2.5	0.0350	Significant Difference
30 & 53	5	0.0398	Significant Difference
30 & 53	7.5	0.0035	Significant Difference
30 & 53	10	0.0135	Significant Difference

3.5 The Effect of Pacing-Induced Stress on Cardiac Function

As mentioned earlier, it was necessary to establish a protocol for pacing these flies. We wanted to stress the heart and it was easiest to accomplish this through electrical stimuli. Electrical pacing was performed on 9, 30 and 53 day-old YW flies only.

Electrical stimuli resulted in the flies' hearts being able to beat at a much faster pace than its resting heart rate. Observations showed that 40 V made the flies jump and

violently shake while on the pacing slide. Some of these flies died after pacing had ceased.

In addition, electrical stimuli at lower frequencies seemed to increase the heart rate. As frequency was further increased, the heart rate began to decline. This is evident in the younger flies. As the flies got older, however, the ability of the heart to beat at a faster pace, although stimulated, declined. Figure 3.8 displays the effect of age on the heart's ability to be paced.

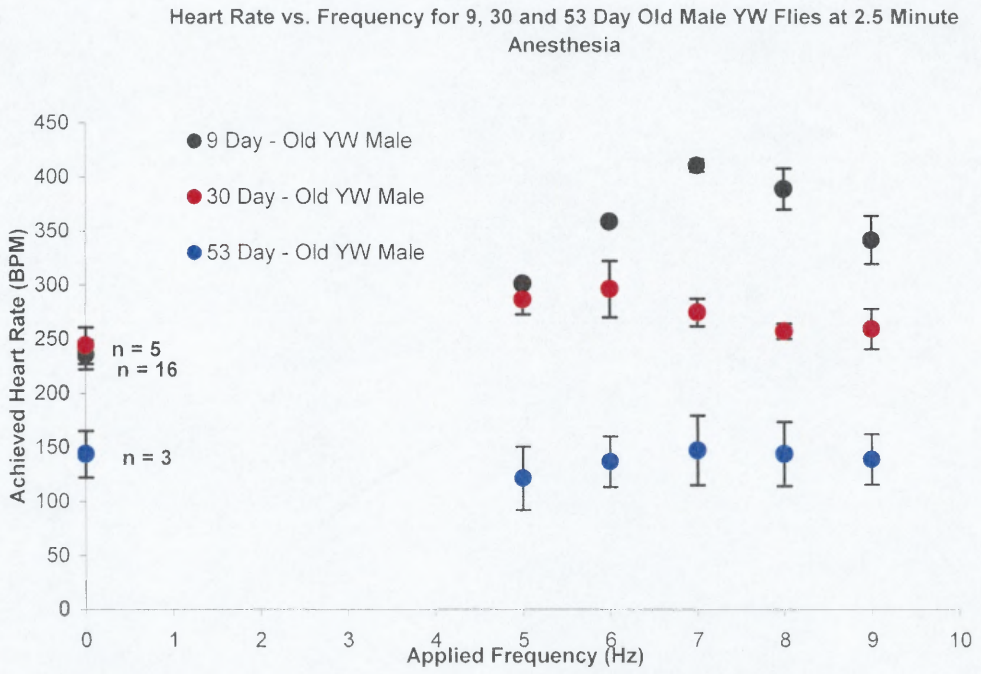


Figure 3.8 Achieved heart rates for 9, 30, and 53 day-old flies under 2.5-minutes of anesthesia

For 9 day-old YW flies, the maximum heart rate achieved was found when the frequency was set at 7 Hz. This is equal to 420 beats per minute. This was found to be the case for both the 2.5-minute and 5-minute anesthesia levels. Both 7.5-minute and 10-minute anesthesia levels resulted in the maximum frequency being achieved under a set frequency of 6 Hz. Figure 3.9 demonstrates this.

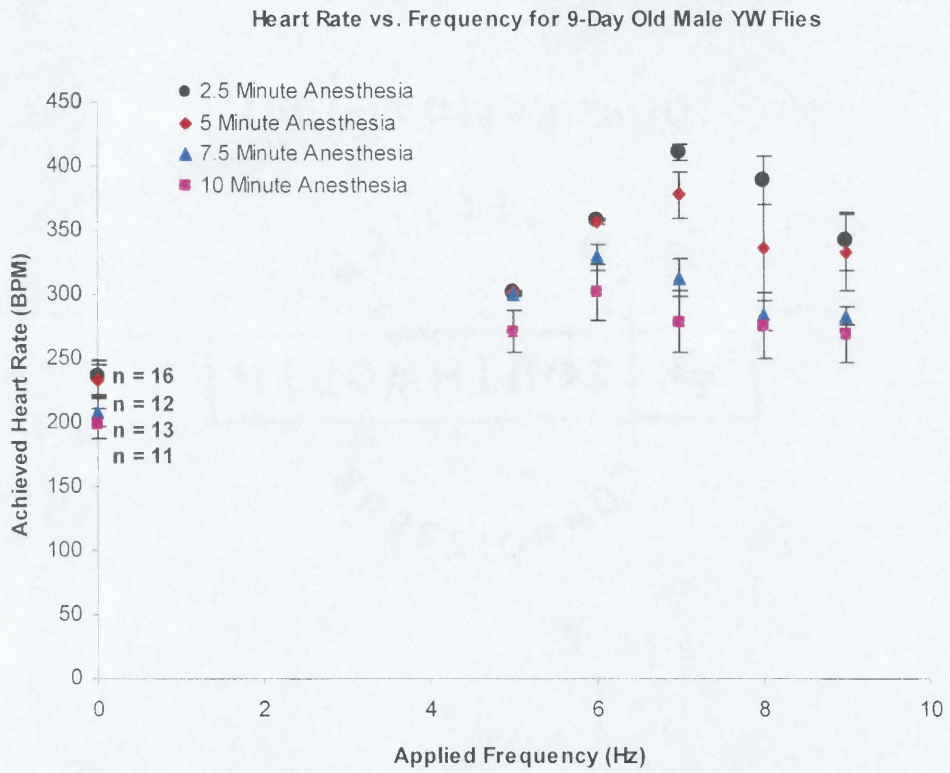


Figure 3.9 Total effect of electrical pacing on 9 day-old wild type flies

As demonstrated above, anesthesia seems to affect the heart's ability to be electrically paced. Without any electrical pacing, there was no significant difference (Table 3.8) between heart rates achieved under any of the anesthesia levels. Under 5 Hz, there was a significant difference of achieved heart rates between the 5 and the 7.5-minute anesthesia levels. At both 6 Hz and 7 Hz, the combinations of 2.5-minute and 5-minute levels resulted in no significant difference between achieved heart rates, as did the 7.5-minute and 10-minute combination. All other combinations for the 6 and 7 Hz frequencies resulted in significant differences between achieved heart rates. Both 8 Hz and 9 Hz electrical pacing resulted in significant differences in achieved heart rates between the 2.5 and 7.5-minute combinations as well as the 2.5-minute and 10-minute

combinations. All other 8 and 9 Hz frequency combinations resulted in no significant differences in achieved heart rates.

Table 3.8 Results of the student's t-test ($p<0.05$) for 9 day-old flies under the 2.5, 5, 7.5 and 10- minute anesthesia levels

Comparisons (Measured By Frequency)	**2.5 & 5**	**2.5 & 7.5**	**2.5 & 10**	**5 & 7.5**	**5 & 10**	**7.5 & 10**
0	T = 0.8663	T = 0.1251	T = 0.0506	T = 0.1585	T = 0.0648	T = 0.5790
Conclusion	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant
5	T = 0.4494	T = 0.1942	T = 0.0962	T = 0.0463	T = 0.0884	T = 0.1123
Conclusion	Not Significant	Not Significant	Not Significant	Significant	Not Significant	Not Significant
6	T = 0.1705	T = 0.0161	T = 0.0253	T = 0.0258	T = 0.0310	T = 0.2616
Conclusion	Not Significant	Significant	Significant	Significant	Significant	Not Significant
7	T = 0.1111	T = 1.5421 * 10^{-5}	T = 0.0002	T = 0.0043	T = 0.0043	T = 0.2442
Conclusion	Not Significant	Significant	Significant	Significant	Significant	Not Significant
8	T = 0.1952	T = 9.0180 * 10^{-5}	T = 0.0020	T = 0.1775	T = 0.1759	T = 0.7709
Conclusion	Not Significant	Significant	Significant	Not Significant	Not Significant	Not Significant
9	T = 0.8146	T = 0.0246	T = 0.0310	T = 0.1340	T = 0.1026	T = 0.5528
Conclusion	Not Significant	Significant	Significant	Not Significant	Not Significant	Not Significant

Table 3.9 A, B, C, and D displays the achieved heart rates under all anesthesia levels while being electrically paced for the 9 day-old YW fly.

Tables 3.9 A, B, C & D Total effect of anesthesia on the heart rate of wild type flies under electrical pacing.

A

Applied Frequency	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
Anesthesia Dose (Min)	2.5	2.5	2.5	2.5	2.5	2.5
Achieved Heart Rate (BPM)	235.5 ± 13.4	300.8 ± 0.8	358.3 ± 0.6	410.6 ± 5.8	388.8 ± 19.0	341.6 ± 22.3

B

Applied Frequency	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
Anesthesia Dose (Min)	5	5	5	5	5	5
Achieved Heart Rate (BPM)	232.4 ± 12.5	301.7 ± 0.8	355.7 ± 1.6	377.4 ± 18.6	335.7 ± 34.5	332.7 ± 29.8

C

Applied Frequency	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
Anesthesia Dose (Min)	7.5	7.5	7.5	7.5	7.5	7.5
Achieved Heart Rate (BPM)	207.9 ± 11.2	299.3 ± 0.9	329.4 ± 10.3	312.8 ± 14.8	283.7 ± 12.0	283.5 ± 7.7

D

Applied Frequency	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
Anesthesia Dose (Min)	10	10	10	10	10	10
Achieved Heart Rate (BPM)	198.7 ± 11.9	270.6 ± 16.4	301.3 ± 21.7	278.5 ± 24.2	275.3 ± 25.7	269.0 ± 22.4

Figure 3.10 shows maximum heart rate achieved during electrical pacing for each of the anesthesia levels for 9 day-old flies.

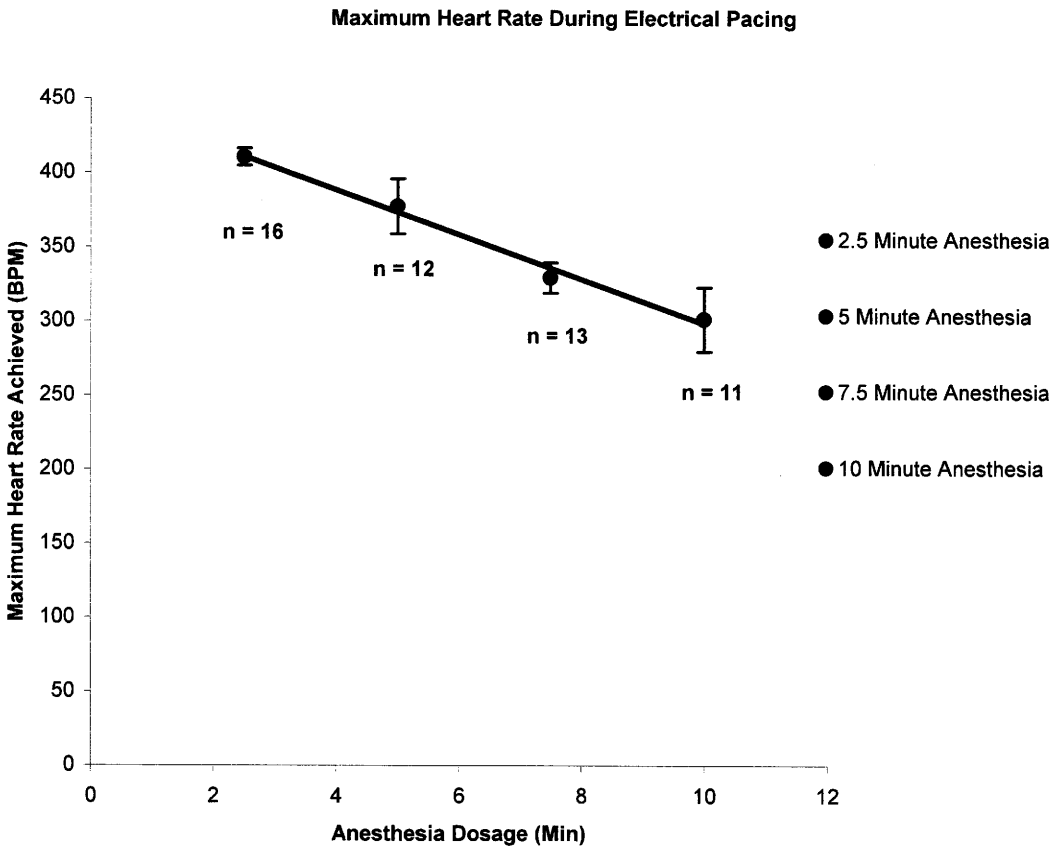


Figure 3.10 Maximum heart rate achieved for 9 day-old flies while under electrical stimuli

This stress test was also performed on older flies. 30 day-old flies were stressed under each of the four anesthesia levels. The 53 day-old flies, however, were only stressed at the 2.5-minute level due to the small number of flies that lived to this age.

As demonstrated earlier with 9 day-old flies, anesthesia affects the heart's ability to respond to stress. This trait is conserved in older, 30 day-old flies (Figure 3.11 and Table 3.9) and appears to be more pronounced. Without electrical stimuli, there weren't any significant differences (Table 3.10) found between the 5 and 7.5-minute anesthesia levels or the 7.5-minute and 10-minute anesthesia levels. There was a significant

difference in achieved heart rates between the 2.5 and 5-minute anesthesia levels. Under 5 Hz, significant differences were found in the achieved heart rates between the 2.5-minute and 5-minute anesthesia levels as well as the 5 and 7.5-minute anesthesia levels. There wasn't any significant difference found between the 7.5-minute and 10-minute exposures. Under 6 Hz of electrical stimuli, a significant difference was found between the 2.5-minute and the 5-minute anesthesia exposures, but no significant differences were found between the 5 and 7.5-minute or the 7.5-minute and 10-minute combinations. Tests of both the 7 Hz and 8 Hz frequencies resulted in no significant differences between the 2.5-minute and 5-minute exposures as well as between the 5 and 7.5-minute exposures and between the 7.5-minute and 10- minute exposures. Tests using a frequency of 9 Hz resulted in significant differences found between the 2.5-minute and the 5-minute exposures as well as between the 5 and the 7.5-minute exposures. No significant differences were found between the 7.5-minute and 10-minute anesthesia levels.

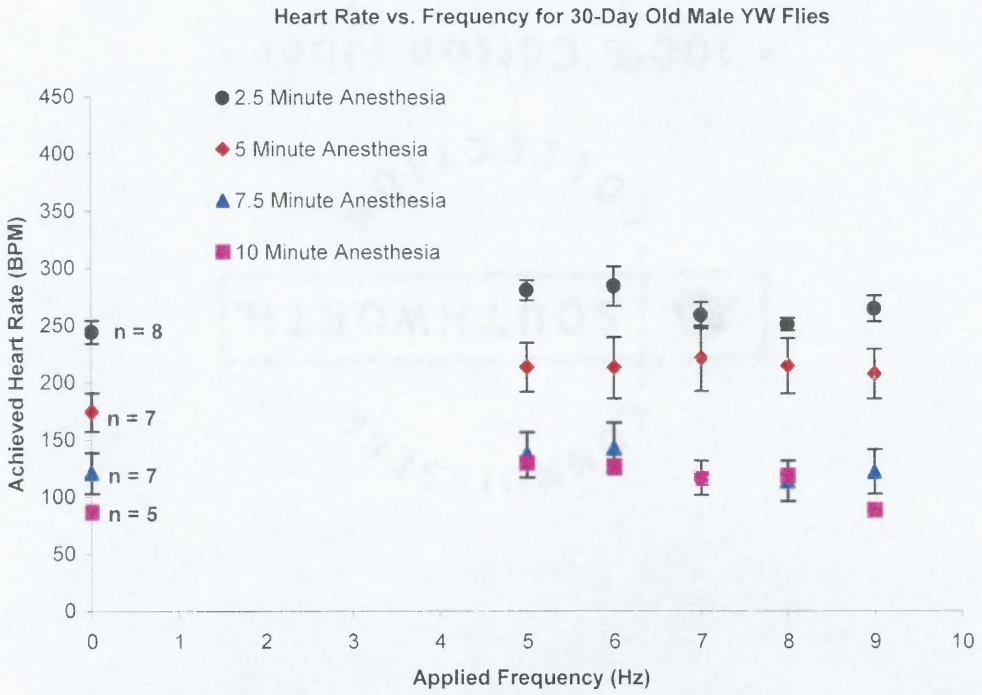


Figure 3.11 30 day-old electrical pacing

A table of the achieved heart rates under different pacing frequencies and anesthesia levels was constructed.

Table 3.10 Achieved Heart Rates for 30 Day-old Flies

Anesthesia Level	Frequency (Hz)					
	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
2.5	244 ± 10.1	280.6 ± 8.8	284.3 ± 17.2	258.8 ± 11.5	250.9 ± 5.5	264.6 ± 11.4
5	174 ± 16.7	213.3 ± 21.4	212.9 ± 26.7	221.1 ± 28.6	214.5 ± 24.3	207.4 ± 21.8
7.5	120.8 ± 18	136.6 ± 19.7	142.3 ± 22.5	116.6 ± 15	113.7 ± 17.6	121.9 ± 19.5
10	86.4 ± 16	128.9 ± 47.1	126 ± 58.1	115.4 ± 57.7	117.9 ± 59.4	88 ± 36.8

Table 3.11 Results of the student's t-test ($p<0.05$) for 30 day-old flies under the 2.5, 5, 7.5 and 10- minute anesthesia levels

Comparisons (Measured By Frequency)	2.5 & 5	2.5 & 7.5	2.5 & 10	5 & 7.5	5 & 10	7.5 & 10
0	T = 0.0047	T = 0.0002	T = $6.0627 * 10^{-5}$	T = 0.0509	T = 0.0037	T = 0.1847
Conclusion	Significant	Significant	Significant	Not Significant	Significant	Not Significant
5	T = 0.0120	T = 0.0001	T = 0.0308	T = 0.0220	T = 0.1567	T = 0.8846
Conclusion	Significant	Significant	Significant	Significant	Not Significant	Not Significant
6	T = 0.0474	T = 0.0003	T = 0.0505	T = 0.0673	T = 0.2260	T = 0.8039
Conclusion	Significant	Significant	Not Significant	Not Significant	Not Significant	Not Significant
7	T = 0.2567	T = $8.4376 * 10^{-6}$	T = 0.0665	T = 0.1273	T = 0.1516	T = 0.9839
Conclusion	Not Significant	Significant	Not Significant	Not Significant	Not Significant	Not Significant
8	T = 0.1890	T = 0.0001	T = 0.0885	T = 0.0064	T = 0.1888	T = 0.9488
Conclusion	Not Significant	Significant	Not Significant	Significant	Not Significant	Not Significant
9	T = 0.0443	T = $9.4700 * 10^{-5}$	T = 0.0066	T = 0.0129	T = 0.0279	T = 0.4452
Conclusion	Significant	Significant	Significant	Significant	Significant	Not Significant

As shown previously for the 9 day-old flies, a graph displaying the maximum heart rates achieved for the flies while being electrically paced and under different anesthesia dosages was constructed for 30 day-old flies (Figure 3.12). Clearly there is an anesthesia effect on the heart's ability to respond to stress.

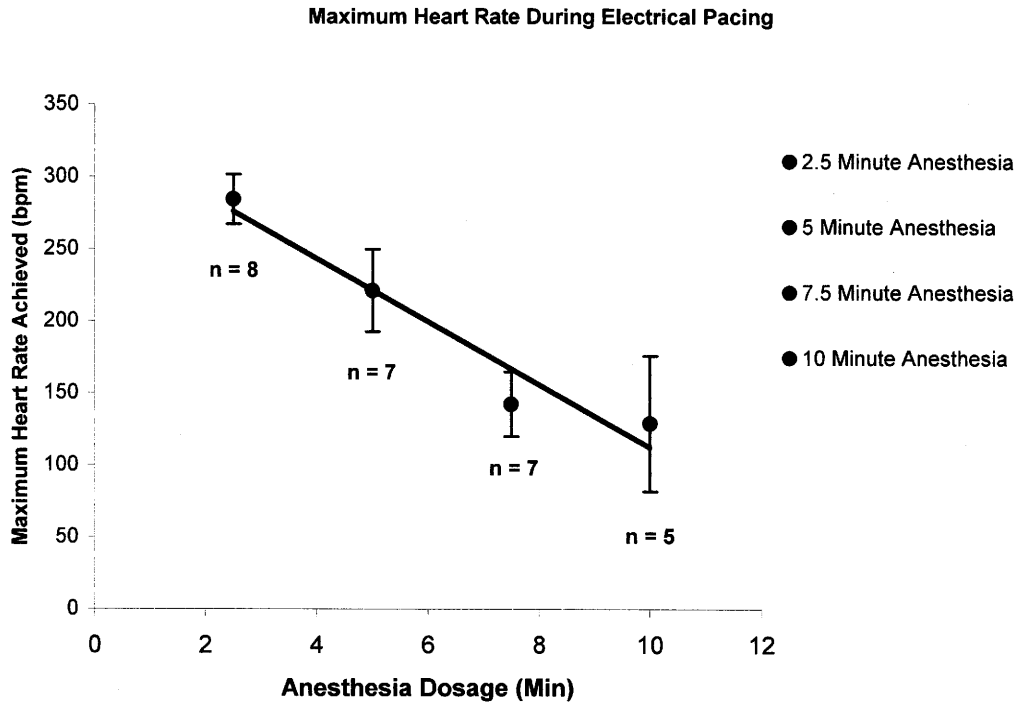


Figure 3.12 Maximum Heart rate achieved for 30 day-old flies while under electrical stimuli

Lastly, as mentioned earlier, electrical pacing tests were performed on 53 day-old flies as well. These were only conducted under the influence of 2.5-minutes of anesthesia due to the limited quantity of 53 day-old flies. Figure 3.8 shows the results of this study. As demonstrated, 53 day-old flies are not able to achieve higher frequencies.

Table 3.5 was constructed to show the heart rates that were achieved under each of the frequencies used to pace these flies.

Table 3.12 Achieved Heart Rates for 53 Day-old flies

Applied Frequency	0 Hz	5 Hz	6 Hz	7 Hz	8 Hz	9 Hz
Anesthesia Dose (Min)	2.5	2.5	2.5	2.5	2.5	2.5
Achieved Heart Rate (BPM)	149.8 ± 13.8	124.7 ± 22.1	133.7 ± 19.8	139.8 ± 18.7	142.3 ± 17.3	133.6 ± 10.7

CHAPTER 4

DISCUSSIONS, CONCLUSIONS, AND FUTURE WORK

4.1 Discussion

There have been methods used to measure the average heart rate in *Drosophila melanogaster*, each with their respective limitations.

Paternostro [2] and Wessells [10, 11] used a video imaging technique to determine an average heart rate, which is a simple method to view the heart contracting, allowing the investigator to determine average heart rate through counting of beats. The limitation of this technique is the amount of time needed for data analysis. Measurement of a number of flies through the counting of beats ("the gold standard") requires a tremendous amount of time, which delays the interpretation of results and therefore decisions on the direction of a study. We also found this method to be the most reliable, although it required a large amount of time to calculate heart rate.

Using this technique, we found that the average heart rate for 9 day-old flies was 285.2 ± 5.8 BPM. An average heart rate of 221.7 ± 8.1 BPM was determined for 30 day-old flies and an average heart rate for 53 day-old flies was found to be 195.1 ± 9 BPM. These values compare well to the values that Paternostro et. al [2] reported for average heart rate. He showed that 10 day-old flies had an average heart rate of 286 ± 3 BPM, 31 day-old flies had an average of 249 ± 5 BPM, and 54 day-old flies had an average of 220 ± 3 BPM.

We used an improved technique based on Paternostro's [2] methods. Where he applied a rough volume of 25 – 40 μl using the wand and the dosage time varied, depending on when the flies fell asleep (roughly two minutes).

The differences seen in our average heart rate measures in older flies (30 days and 53 days) compared to those of Paternostro [2] could be explained by the differences in fly strain, where we used yw wild type flies and Paternostro used Oregon-R flies. Moreover, differences could be due to the imprecise method of anesthetizing flies.

Wessells [10, 11] on the other hand used the same strain of yw wild type fly; howevers used an anesthetizing method described in [11], where he applied 1 ml of FlyNap® in a Carolina Anesthetizing Chamber. The average heartrate that was observed for a 1-week old fly was 174 BPM. A 5-week old fly was found to have an average heartrate of 144 BPM. The differences between the values we observed and those observed by Wessells could also be due to the amount and exposure time of anesthesia that the flies experienced.

Johnson et. al [14] measured the heartrate of fruit fly pupa using an EKG method. He used a Canton-S strain. A heartrate of 2.28 ± 0.05 Hz (136.8 ± 3 BPM) was observed under the lowest temperature the flies were exposed to. At the highest temperature the flies were exposed to (37°C) the heartrate observed was 3.33 ± 0.05 Hz (199.8 ± 3 BPM). The differences in heartrate here could be explained by the fact that the studies were conducted on pupa, not adult flies. Furthermore, the differences in the strains of the flies may also account for this lower heartrate.

Automated data analysis methods have been established [2,10,13] based on image analysis techniques. This innovation has sped up determination of average heart rate, at

the same time sacrificing accuracy. Therefore, the original counting method remains the most accurate way to determine average heart rate and is considered the gold standard in the field. Visual hand counting is necessary, however, to check the automated system and ensure that it works properly. This method, therefore, requires more time.

A limitation of the data acquisition method is in the 30-frames/sec camera used, which only allows average heart rate to be determined in a fly whose heart is beating at approximately 280 BPM. Therefore, heart rate variability cannot be determined with such a low frame rate camera and such a fast beating heart. However, there exist high frame rate cameras that can image at over 1000 frames/sec. Use of high frame rate cameras could allow determination of heart rate variability, but the large number of frames would require an automated method to measure the heart rate motion of the fly.

Sláma et. al [14] used the following four techniques to measure heart rate: two optocardiographic methods, contact thermography , and a visual-electric method. These methods determined average heart rate through analysis of a transmitted pulse. A limitation of this technique involves the quality of data acquired. The pulse of light passing through the fly tissue results in out-of-focus images, which can lead to several inaccuracies when analyzed.

Johnson et al. [12] used a phototransistor that measured changes in light intensity as a method of determining an average heart rate. A limitation of methods involving measurement of light intensity involves how to properly calibrate the signal, as well as how to take into account changes in light intensity due to the effects of their physiology (fluid movement, etc.). Therefore, this method may misinterpret acquired data, leading to inaccurate results.

Johnson et al. [15] used s sharpened tungsten electrode to measure the EKG in restrained anesthetized fruit flies. The EKG is then analyzed to determine average heart rate. This method allows average heart rate as well as heart rate variability to be determined. Therefore, it is an excellent method; however, a major limitation is technically how to insert the electrodes. This involves a costly micromanipulator to properly position the EKG probes. Moreover, a large amplifier is needed to amplify the low voltage signal obtained from the fruit fly. Therefore, the challenging and costly nature of this technique makes it impractical for most laboratories. In fact, when we attempted this method, we found that although we were able to insert the electrodes, we achieved nothing but a noisy electrical signal after several attempts. In contrast, the imaging technique employed produced results the first time we conducted the experiment.

Although we did attempt this method, we found it very difficult to place the electrodes into the flies' bodies. Use of a very skilled surgeon or a mechanical manipulator would be needed to place the electrodes accurately, without causing harm to the flies.

Zornik et al. [8] used a microscope to optically detect heart rate. A signal recorded the opening and closing of the heart walls for ten minutes. This was good in that it recorded the flies' heart rate for an extended period of time and then took an average.

Wolf et al. [5] developed a technique involving optical coherence tomography (OCT) that was able to measure the heart rate of a conscious fly. A major advancement here is that as all the other previously mentioned techniques measure the changes in wall

dimensions, while this group's technique involves measuring changes in actual chamber sizes similar to what is seen in echocardiography. This allows for specific disease-related changes in certain mutant flies to be measured. The limitation of this method includes the inability to pace the fly hearts because flies are restrained in an agarose plate. Moreover, although the measurements are conducted in conscious flies, the animal is still restrained, which can lead to inaccuracies due to change in cardiac function resulting from attempts to free itself.

The OCT method resulted in mean heart rate of 271 ± 13 at a temperature of 22°C for a 7 day-old, wild-type fly [5]. This value compares well to our room temperature measurement of 285.2 ± 5.8 BPM for a 9 day-old fly under 2.5-minutes of FlyNap®. This indicates that our level of anesthesia is an appropriate dosage to provide to flies so that an accurate, conscious heart rate value can be achieved, any decrease in heart rate due to the anesthesia itself. Wolf et. al's method [5] also indicates that our anesthesia chamber effectively anesthetizes the flies without causing their hearts any stress.

Paternostro et. al [2] demonstrated that by using video imaging techniques, you can also conduct conscious fly measures by resting the fly on the slide. Therefore, conscious measures are not unique to the OCT method. In fact, we were also able to image the hearts of conscious restrained flies, which are characterized by moving antennae and legs [OCT]. However, we found that this could produce variability compared to tranquilized flies because the fly movement can produce inaccurate images and increase or decrease heart rate due to struggling.

Wessells et. al [10] developed a technique to electrically stimulate the heart of *Drosophila melanogaster*. This method was great in that it effectively paced the heart.

The voltage used, however, was 40V. We were able to pace our flies at approximately 4V, which is significantly lower than previous studies. This is a very high voltage. In addition, the voltage output was not monitored nor was the temperature under which the flies were being stressed.

Using a much lower voltage of 4 volts, we were effectively able to stress the fly heart. In fact, the maximum frequency at which some of our 9-day old flies were able to reach was 8 Hz, with a maximum heart rate of 388.8 ± 19 BPM. Nearly all of these flies were effectively paced at 7 Hz, resulting in a maximum frequency of 410.6 ± 5.8 BPM.

FlyNap®, containing triethylamine, was an anesthesia that was used by Paternostro et. al [2] and Wessells et. al [10, 11]. Neither reported a significant anesthesia effect on the heart rate of the flies used, indicating that this agent is cardio-protective, although it has previously been reported that other anesthesia methods affect heart rate [20-22]. Furthermore, Paternostro et. al [2] and Wessells et al [10, 13] showed that there is an effect of aging on the cardiac function of *Drosophila melanogaster.* We too observed this aging effect in our flies. However, the results indicated that there was not only an effect of aging on the cardiac function, but an anesthesia effect as well. Incremental doses of anesthesia resulted in a much lower heart rate, indicating that FlyNap® is not cardio-protective, but cardio-depressive. For 9 day-old flies, under 2.5 minutes of anesthesia, we found an average heart rate of 285.2 ± 5.8 BPM. Under 5 minutes of anesthesia, an average heart rate of 218.9 BPM was discovered. Under 7.5 minutes, an average heart rate of 211 BPM was found. And lastly, under 10 minutes of anesthesia, an average heart rate of 187.2 BPM was found. These anesthesia affect was conserved in both the 30 day-old flies as well as the older, 53 day-old flies.

Another major drawback to the anesthetic FlyNap® is that the wands supplied by the manufacturer are said to collect between 35µl and 40µl in the amount of FlyNap® that they can absorb. The wands also do not provide a centralized distribution of gas when inserted on the side of the cotton vial. The developed anesthesia chamber allowed a precise volume of anesthesia to be applied, and provided a more centralized distribution of the FlyNap® vapor.

4.2 Conclusion

Consistent with previous studies on measuring heart rate, a visual hand-counting method seemed most reliable to count the heartbeats of *Drosophila melanogaster.* Although not as fast as possible image analysis software, visually counting by hand is easiest and allows for less error in calculations.

We employed a pacing method of stressing the *Drosophila* heart. For our experimental purposes, electrical stimuli seemed an easier, more cost-effective stress measurement. It also emulates physical stresses to the body as a result of labor. When studying stresses to cardiac functions, electrical stimuli seemed to be the best method.

As demonstrated by other researchers, aging does have an effect on the heart's ability to respond to stress. Our studies have confirmed this and shown that not only does aging affect the heart's ability to respond to stress, but so does anesthesia.

Previous studies considered triethylamine as cardio-protective, even insisting that large doses can be applied with no consequences. However, our studies have shown that anesthesia itself *is* a form of stress. Therefore, when conducting a test of physiology, the anesthesia dosage and exposure time does matter. Because an anesthesia dose response is

observed, physiology studies should try to analyze flies that are near the conscious condition.

A major drawback, however, is that the chief component in FlyNap® is triethylamine. It is not well understood how this product or drug affects the flies. The pathway that the drug undergoes in anesthetizing the fly is not well understood.

4.3 Future Work

In the future, an image analysis program could be developed that will accurately count heart rate, leading the way for using a high frame rate camera for heart rate variability studies. It would need to be able to adjust the contrast and brightness, however, of the images so that the heart walls (which in older flies become more difficult to distinguish) can be easily identified and observed. This program would also need to account for the positioning of the fly because not every fly is going to be in the exact same position when placed onto the microscope slide. It would also need to consistently calculate an accurate average heart rate.

A faster camera could be purchased and used as well. This, however, would necessitate creating an accurate, automated heart rate detection system. This would be needed because a faster camera would produce more images in a given amount of time. To calculate heart rate by hand for a large number of flies would be extremely insufficient.

Next, it has previously been found that AC5 is involved in regulating lifespan in mice. A homologous fly could be developed that shows a similar phenotype. Also, a fly

that does not show the phenotype of lower heart rate with age could be developed and studied.

In summary, a system was developed to monitor fly cardiac physiology. This includes a characterized method of anesthesia, an average heart rate monitoring method, and a system to stress the heart (electrical pacing). This system is a great tool for future work involving cardiac studies in fruit flies.

Lastly, the pathway that is involved between exposing the fly to triethylamine and actually anesthetizing the fly is not clear. A possible future work could involve exploring the role of this anesthetic more deeply. This may involve mutant flies being developed that have mutations involving their ion channels. The flies would then be exposed to FlyNap® and it's effects on these ion channels could be observed.

APPENDIX A

EVOLUTIONARILY CONSERVED CARDIAC DISEASE GENES

Many human cardiac disease genes that have been found to be conserved in *Drosophila melanogaster* [6]. These genes and their phenotypes are shown in the table below.

Table 1
Cardiac disease genes within nondevelopmental disease categories that have *Drosophila* counterparts

Disease category	Disease	OMIM #	e-Value	Fly genes	Alleles	Protein function
Cardiomyopathy	Cardiomyopathy, familial hypertrophic, 1	160760	e-300	*mhc*	70	Cardiac myosin heavy chain-β
	" "	" "	e-300	*zip*	20	" "
	Cardiomyopathy, hypertrophic, midventicular chamber type (3)	160790	2e-34	*Mlc-c*	1	Essential light chain of myosin
	Cardiomyopathy, hypertrophic, midleft ventricular chamber type (3)	160781	3e-42	*sqh*	15	Myosin ATPase
	Cardiomyopathy, familial hypertrophic, 9	188840	e-300	*bt*	39	Titin: myosin light chain kinase
	Cardiomyopathy, familial hypertrophic,4	600958	e-70	*CG18242*	pl(3)j1D7	Cardiac myosin binding protein C
	Cardiomyopathy, familial hypertrophic, 3	191010	2e-63	*Tm1*	1	Tropomyocin
	Cardiomyopathy, familial hypertrophic, 2	191045	5e-12	*up*	14	Troponin complex
	Cardiomyopathy, familial hypertrophic, 3	191044	e-10	*wupA*	24	Troponin I, cardiac form
	Cardiomyopathy, idiopathic dilated	102540	e-300	*Act57B, 79B, 87E*	1 each	Smooth muscle actin
	" "	" "	e-300	*Act42A*	pEP(2)2096	" "
	" "	" "	e-300	*Act5C*	6	" "
	" "	" "	e-300	*Act88F*	80	" "
	Becker muscular dystrophy, cardiomyopathy, dilated, X-linked, Duchenne muscular dystrophy	310200	e-300	*Dys*	1	Dystrophin
	Cardiomyopathy (1), myopathy, desminopathic	125660	3e-37	*Lam*	8	Desmin
	Barth syndrome	302060	3e-61	*tafazzin*	3	Phospholipid and glycerol acyltransferase?
Conduction	Jervell and Lange–Nielsen syndrome	192500	e-64	*CG12215*	pEP(2)2074	Potassium channel
	Long QT syndrome-2	152427	e-300	*sei*	11	Potassium channel
	Long QT syndrome-3	600163	e-300	*para*	42	Sodium channel
	Artial septal defect with atrioventricular conduction defects	600584	2e-26	*vnd*	36	Transcription factor
	Stress-induced polymorphic ventricular tachycardia	604772	e-300	*Rya-r44F*	5	Ryanodine receptor (release of internal Ca++ stores)
	Arrhythmogenic right ventricular dysplasia	600996	e-300	*Rya-r44F*	" "	" "
Hypertension	Hypertension, salt-resistant	108962	3e-43	*CG3216*	1	Natriuretic peptide receptor C (receptor guanylate cyclase)
	Hypertension, essential, susceptability	139130	e-164	*Gbeta13F*	pEP(x)1071	Guanine nucleotide-binding protein, β-3
	Hypertension, essential	106165	4e-24	*AR-2*	1	Angiotensin/allatostatin receptor
	Hypertension, essential	145505	e-38	*AcCoAS*	pl(3)00217	Acetyl CoA synthetase
	Preeclampsia/eclampsia	199900	e-300	*Nos*	1	Nitric oxide synthetase
	Hypertension, pregnancy induced	163729	e-300	*Nos*	1	Nitric oxide synthetase
	Hypertension due to apparent mineralocorticoid excess	218030	8e-15	*CG8888*	1	Hydroxysteroid dehydrogenase
Atherosclerosis	Atherosclerosis, susceptibility	131210	6e-50	*fw*	25	Cell adhesion: selectin
	Myocardial infarction	106180	e-153	*Ance*	5	Angiotensin I converting enzyme
	Coronary artery disease	152200	4e-35	*CG10663*	1	Serine endopeptidase
Vascular defects	Venous malformations	600221	6e-63	*htl*	21	Receptor tyrosine kinase
	Cavernous angiomatous malformations	116860	7e-18	*CG11848*	1	Ras-interacting protein

APPENDIX B

PROCEDURE FOR FLY FOOD PREPARATION

The preparation of the medium on which flies were housed is discussed in detail below.

For the making of 100 bottles- by Jiyeon Choi [39]

1. Adjust the motor in the kettle deep enough but not to contact the bottom.
2. Add 3.6 liter of tap water and 44 grams of fly food agar to the kettle.
3. Turn on the mixer and set the heat to 8 and motor speed at 60.
4. Once the pressure reaches 20, maintain it for 10-minutes.
5. Add 480 ml of molasses into the kettle.
6. Turn the heat down to 5 and mix for 10-minutes.
7. Prepare the "mix" as follows while avoiding lumps (using a mass cylinder)

   1200 ml tap water

   600 ml Cornmeal

   264 ml Yeast
8. Put the mix in the kettle and turn the heat down to 3. Mix for 10-minutes.
9. Add 30 ml propionic acid.
10. Add 60 ml of tegosept (anti-fungal agent)
11. Turn the heat off and mix for 10-minutes.
12. Dispense it into the vials and bottles with the mixer on.
13. Turn of f the main power of mixer
14. Put the cloth on the dispensed fly food and leave it over night.
15. Plug the vials with cotton balls and wrap the whole box with plastic bag.
16. Label and store at 4°C.

Table B.1 summarizes the amount of each ingredient that goes into the fly food.

Table B.1: Ingredients of fly food [33]

Ingredients	**Batch Size**	**250 Bottles**	**100 Bottles**	**50 Bottles**
Tap Water		9000 ml	3600 ml	1800 ml
Fly Food Agar		110 g	44 g	22 g
Molasses		1200 ml	480 ml	240 ml
Mix	Tap Water	3000 ml	1200 ml	600 ml
	Cornmeal	1500 ml	600 ml	300 ml
	Yeast	660 ml	264 ml	132 ml
Propionic Acid		75 ml	30 ml	15 ml
Tegosept		150 ml	60 ml	30 ml

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