

NEUROPROTECTION DURING ANOXIC STRESS IN *DROSOPHILA*
MELANOGASTER: THE ROLE OF PKG PATHWAY ON PROTECTION OF
FUNCTION AND SURVIVAL

by

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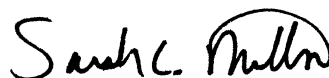
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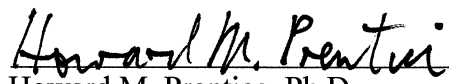
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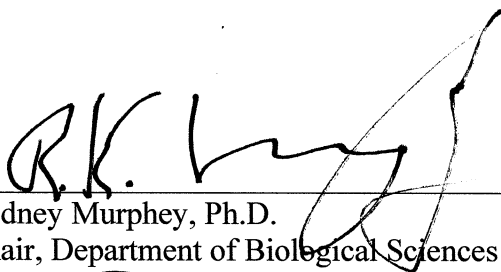
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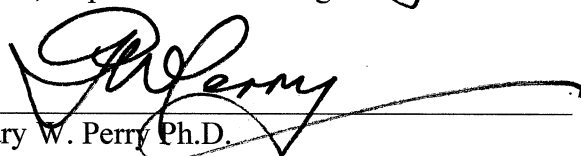
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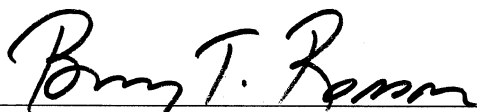
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ABSTRACT

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Anoxia is characterized by an absence of oxygen supply to a tissue (Dawson-Scully et al., 2010). Unlike humans, *Drosophila melanogaster* is an organism that can survive low oxygen levels for hours without showing any pathology (Lutz et al., 2003). Under anoxia, the fruit fly loses locomotive activity, resulting in an anoxic coma (Haddad et al., 1997). In this study we investigate the influence of five variables for anoxic tolerance in adult *Drosophila*: 1) anoxic environment (gas vs. drowning), 2) anoxia duration, 3) temperature (cold [3°C] or room temperature [21°C]), 4) age (young 2-9 days and old 35-39 days), and 5) PKG variation. Tolerance to anoxia is measured by the time of recovery and survival of the fruit fly from the anoxic coma. The results from this study show that short stress, low temperature, young age, and low PKG activity increased anoxic tolerance. Our findings will lay the foundation to investigate different variables, genes or pharmacological compounds that can modulate neuronal anoxic tolerance.

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CHAPTER I. INTRODUCTION AND STATEMENT OF PURPOSE

INTRODUCTION

Anoxia

Anoxia is a halt in oxygen supply to a specific organ or a tissue (Lutz & Nilsson, 2003). Humans are very susceptible to oxygen depletion to the brain. When the human brain encounters anoxic stress, there is an apparent functional impairment of the central nervous system within 5 seconds; there is a total loss of consciousness within 8-12 seconds, and in minutes cell death occurs (Lutz et al., 2003). Under normoxic (normal atmospheric oxygen levels) conditions, each mole of glucose is oxidized by 6 moles of O_2 , theoretically resulting in 25 moles of ATP. This means that more than 95% of the ATP in the human brain is formed aerobically (Lutz et al., 2003). On the other hand, under anoxic conditions, each mole of glucose results in only 2 moles of ATP, since anaerobic glycolysis takes place breaking down glucose into pyruvate or lactate. When oxygen supply is cut, oxidative ATP production stops, resulting in a decrease of energy supply. At the same time, ATP-dependent ion pumps, such as Sodium (Na^+) and Potassium (K^+), slow down, leading to a net outward leakage of Potassium (K^+) and a net inward flow of Sodium (Na^+) and Calcium (Ca^{2+}) that causes a progressive depolarization. This reaction produces an uncontrolled release of excitotoxins such as glutamate, dopamine and aspartate, which results in additional inflow of Calcium (Ca^{2+}) and Sodium (Na^+), ultimately leading to neuronal cell death. Neuronal death can be

caused by many factors, including activation of proteases, lipases and endonucleases, by Ca^{2+} , cell swelling due to the water that is accompanying the inflow of ions, or production of oxygen free radicals at the time of reoxygenation and reperfusion (Lutz et al., 2003).

Human brains react to anoxic conditions poorly. However, there are vertebrates that are tolerant to anoxia, such as the crucian carp, *Carassius carassius*, that survive several months of anoxia at 0°C and a day or two at room temperature by upregulating glycolysis (Nilsson & Renshaw, 2004). Also, examples are the epaulette shark, *Hemiscyllium ocellatum*, which can survive several months of anoxia at 0°C and days at elevated temperatures by increasing neuromodulatory metabolites (Lutz, et al., 2003), and the fresh water turtle *Trachemys scripta* which survives months of anoxia at temperatures below 10°C and days at room temperature; it greatly reduces brain metabolic rates to a level where energy costs are matched by anaerobic energy production (Lutz et al., 2003).

Several invertebrates are also tolerant to anoxic stress such as the land snail *Littorina littorea*; their carbohydrate metabolism is regulated in the central nervous system (CNS) leading to metabolic depression (De Fraga & Da Silva, 2010). Additionally, the pacific giant oyster, *Crassostrea gigas*, increases activation and transcription of glycogen phosphorylase, maintaining ATP supply-demand balance and upregulating glycolysis in response to anoxia (Ivanina et al., 2010). Lastly, the spider, *Arctosa fulvolineata*, behaviorally falls into a non-reactive, but non-dormant state in response to anoxia (Pétillon, Montaigne, & Renault, 2009).

The fruit fly, *Drosophila melanogaster*, is another invertebrate organism that is tolerant to anoxia, which has particular interest for this study (Haddad et al., 1997).

***Drosophila melanogaster* as a model organism to study anoxic tolerance**

Drosophila melanogaster has become a model system for the study of development and behavior (Bellen, Tong, & Tsuda, 2010). In the year 1910, Thomas Hunt Morgan identified the white gene, and at this point it became considered a model organism (Morgan, 1910). Further historic achievements have shed light on the importance of the *Drosophila*, by completely sequencing the *Drosophila's* genome, demonstrating that 75% of the human disease related genes have a *Drosophila* orthologue (Haddad, 2006).

Drosophila are an advantageous model organism because they are inexpensive and easy to maintain due to their size and their adaptability in living in the laboratory. They have a short life cycle of 60 days, which allows scientists to perform life span experiments. They reproduce in large quantities, and the progeny will become adults within ten days at 23°C (Haddad, 2006). Developmental stages are as follows: day 0, eggs are laid, in the first day eggs are hatched, in the second day it develops into the first instar larvae, in the third day it grows into the second instar larvae, in the fifth day it develops into the third instar larvae, and at day 7, pupariation occurs. At days 11 and 12, the adults eclose, where the females are able to reproduce and the life cycle repeats itself (Reaume & Sokolowski, 2006) (Figure 1). A large array of genetic tools are available, allowing us to manipulate the genome to obtain a variety of mutations (Haddad, 2006).

Adaptation is the evolutionary process by which organisms adjust to their habitat, and this is crucial for survival (Pétillon et al., 2009). Organisms have evolved to overcome unpredictable and harsh environmental conditions, such as oxygen and temperature fluxes. Insects naturally possess a variety of different strategies to handle

continually changing climates to ensure their survival. *Drosophila* is one of the insects that can adapt to extreme environment, by the means of endogenous pathways that are conserved to humans (Haddad, 2006). This is advantageous because it sheds light to understand how humans respond to extreme environments and at the same time leads to potential medical discoveries.

Drosophila melanogaster are anoxic tolerant animals that can survive very low levels of oxygen for a long period of time without showing any pathology (Haddad, 2006). The endogenous response that they implement to protect their nervous system against anoxic stress is to enter a state of quiescent coma. This anoxic coma is a state of deep reversible hypometabolism where balance is maintained between the energy demand and energy supply leading to a suppression of energy demanding functions such as the release of excitatory neurotransmitters and ion flux. This ultimately results in a complete suppression of neuronal electrical activity (Dawson-Scully et al., 2010). By measuring the recovery time from the coma, one can quantify how behaviorally tolerant they are.

The PKG Pathway

The cGMP-dependent protein kinase G (PKG) activity is encoded by the *foraging* (*for*) gene. In nature, there are two allelic variations of this gene, Rover (+PKG activity *for^R*) which encodes for high PKG activity and is present in 70%, and Sitter (-PKG activity *for^S*) that exhibit low PKG activity and is present in 30% (Osborne et al., 1997). A genetic manipulated allelic variation of this gene is *for^{s2}* (-PKG activity), which is a Sitter mutant generated on a Rover genetic background (Chen et al., 2011).

The *foraging* gene is known to be involved in different behavioral activities such as food searching strategies, learning and memory, and most importantly, in anoxic and thermotolerance (Fitzpatrick & Sokolowski, 2004) (Mery, Belay, So, Sokolowski, & Kawecki, 2007). The biochemical pathway that involves cGMP-dependent protein kinase (PKG) cascade is critical for controlling low-oxygen tolerance in the adult fruit fly, *Drosophila melanogaster* (Dawson-Scully et al., 2010). The PKG Pathway is used by *Drosophila* as an endogenous protection mechanism to survive environmental stress (Chen et al., 2011). Previously published data demonstrate that pharmacologically or genetically manipulating the PKG pathway can lead to protection of neurological function and animal survival during acute anoxic stress (Dawson-Scully et al., 2010) (Figure 2). What is very interesting is that there is an inverse relationship between function and survival when the PKG pathway is manipulated. Inhibition of the PKG pathway leads to protection of locomotion function at the cost of survival, however activation of this pathway results in protection of increased survival at the cost of prolonged function during hypoxia (Dawson-Scully et al., 2010).

The PKG pathway does not only play a role in modulating physiological tolerance under anoxic stress in fruit flies (Dawson-Scully et al., 2010), but also in mice (Armstrong et al. 2010) and tadpoles (Hsieh, Robertson, Vermehren-Schmaedick, & Balkowiec, 2010). Additionally, the PKG pathways is involved in thermotolerance (Robertson & Sillar, 2009), not only protecting neuronal function but also the physiology of the organism under stress. Inhibition of the PKG pathway protects synaptic transmission at the *Drosophila* NMJ during thermal stress; this protection is conserved in other species such as *Locusta migratoria* (Dawson-Scully, Armstrong, Kent, Robertson,

& Sokolowski, 2007). PKG seems to be a conserved mechanism for neuronal protection during acute physiological stress across species. The human *PRKG1* gene is a homolog for the *foraging (for)* gene in fruit flies. This homology lays the foundation for future investigation in how to control an animal's tolerance to physiological stressors such as low oxygen and high temperature, which in turn, helps us understand the pathological mechanism of disease progression such as stroke and neurodegenerative disorders in humans (Ogawa, Kitao, & Hori, 2007).

STATEMENT OF PURPOSE

Brain anoxia is a condition that is widespread throughout human lives. Humans are very susceptible to this and within one minute of complete oxygen depletion, cell death occurs in the human brain. *Drosophila melanogaster* is an organism that can tolerate very low levels of oxygen and has mechanisms to survive without showing pathology. At the same time, based on previous studies it is known that the cGMP-dependent protein kinase (PKG) cascade pathway is involved in modulating low oxygen tolerance in adult fruit flies (Dawson-Scully et al., 2010). Considering these two findings, this project addresses the following specific aims:

Aim #1: Investigate the behavioral effects of anoxia on recovery time and survival from an anoxic coma in *Drosophila melanogaster* taking into consideration length of stress, temperature, and age in a dry vs. wet environment.

Aim #2: Determine how natural variations of the PKG pathway alter neuronal protection under anoxic conditions (high and low PKG alleles).

Studying how *Drosophila melanogaster* protect their brains from the anoxic stress, it can help us understand different pathological mechanisms of disease progression such as ischemia/reperfusion, stroke, and neurodegenerative disorders.

Figure 1. Life Cycle of *Drosophila melanogaster*. At room temperature (23°C) generation time is about 10 days: day one embryogenesis, day two first instar larva, day three second instar larva, from day four to day to seven third instar larva and day ten pupal stage (Bellen et al., 2010).

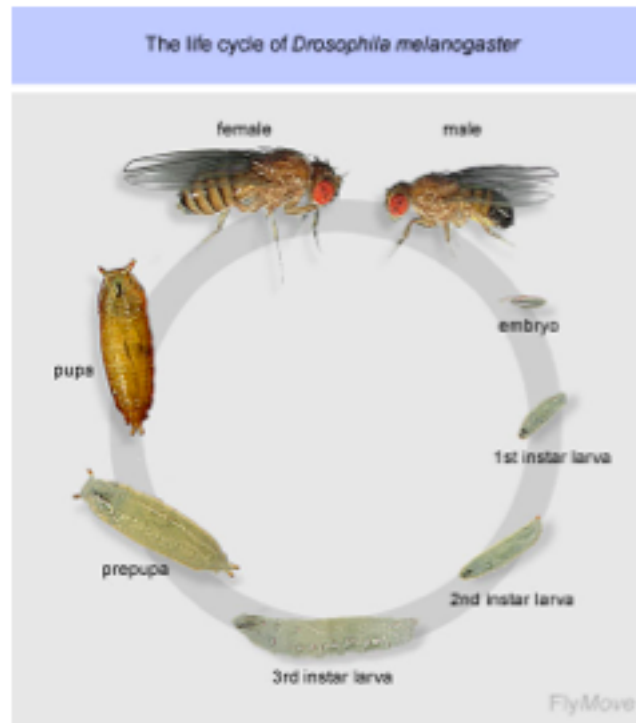
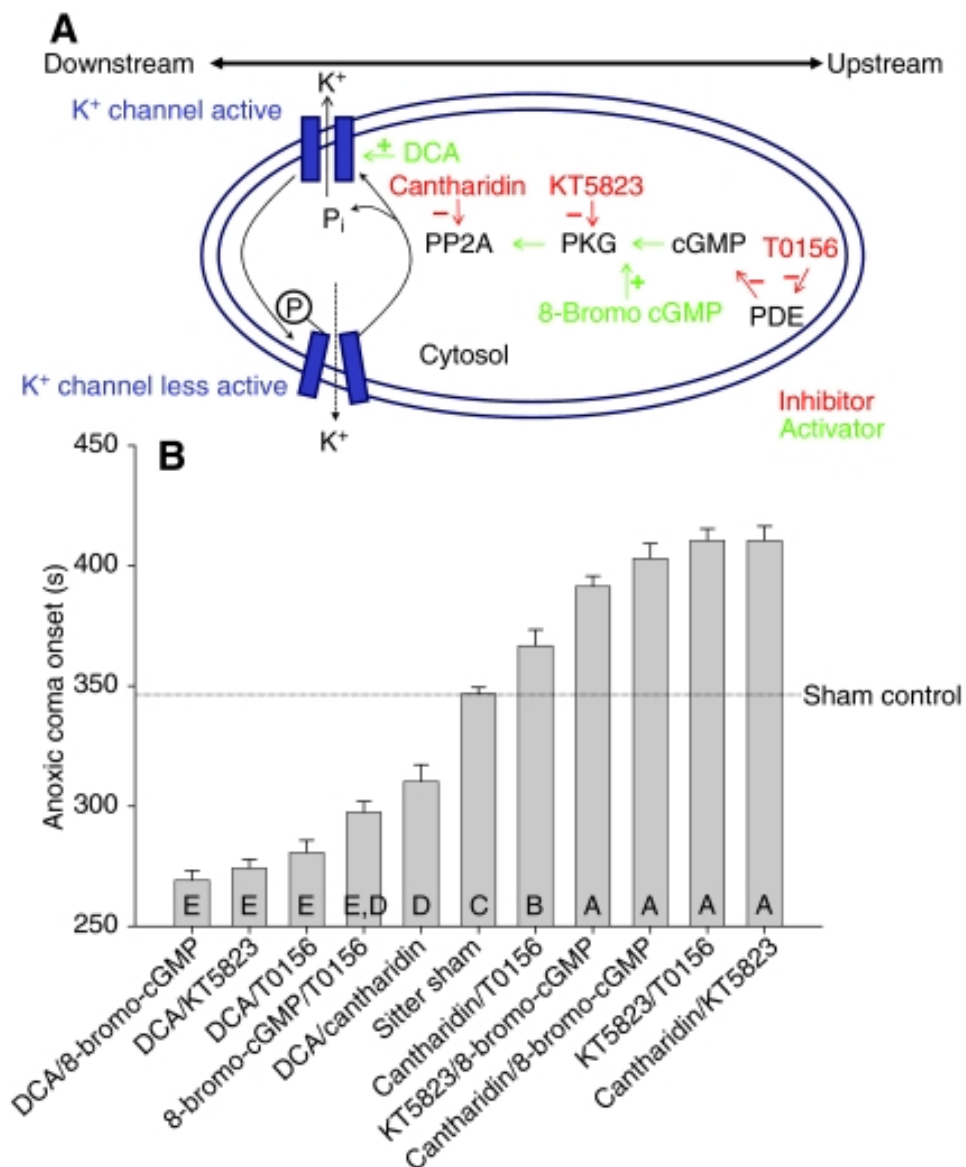


Figure 2. The PKG Pathway and the Pharmacological Agents that Modulate Anoxic Tolerance. A) The PKG signaling cascade is first activated by nitric oxide (NO), which activates cyclic-guanylnmonophosphate (cGMP). When cGMP is turned on, it activates protein kinase G (PKG) that activates protein phosphatase 2A (PP2A). The activation of this pathway ultimately leads to the dephosphorylation of the K⁺ channel which results in an increased conductance. In the pathway the targets for pharmacological intervention are represented in the figure. Inhibitory compounds are shown in red with a minus (−) sign, while activators are shown in green with a plus (+) sign. B) Different combinations of pharmacological agents are given to the adult *Drosophila*, and its resistance to anoxic coma onset during acute anoxia is measured. Significant differences are represented with the letters. Statistically P<0.05 was considered significant (Dawson-Scully et al., 2010).



CHAPTER II. MATERIALS AND METHODS

Flies

This study utilized the fruit fly, *Drosophila melanogaster*, to investigate the process that accounts for their ability to fall into a quiescent coma, which facilitates survival during anoxic stress.

Sorting flies

For this experiment, only males were used. To segregate males from female flies, the flies were anesthetized with CO₂. With the help of a microscope, the flies were sorted by visually differentiating genders. Groups of 10 males were placed into a vial and at least 24 hours passed before the experiment commenced in order to avoid any possible artifacts from the anesthesia. The females were thrown away. The flies used for this experiment were kept in an incubator at 25°C and controlled humidity with a night/day cycle.

Aging

At room temperature the *Drosophila melanogaster* generation time is about 10 days (Das, Levine, Orr, & Sohal, 2001). For about 1 day it is an embryogenesis, 1 day first instar larva, 1 day second instar larva, 2-3 days they are third instar larva, and for 5 days they are at a pupal stage. A fly becomes an adult at day 10 (Haddad, 2006). The aging process starts by clearing all the adult flies out of the bottles where flies are maintained under control conditions, this leaves only larva and pupa in the bottles. The

day the adult flies are cleared is considered “day 1” of the aging process. Four days after this, flies that have hatched are collected and separated by gender. The male flies are transferred into a vial in groups of ten where the aging process continues. Flies are tested from “day 5” until “day 9” and these flies were classified as the young flies (1 to 9 days old). A group of flies were maintained and aged until “day 35” and flies were tested between “days 35 and 39” which were categorized as old flies. After this, the flies were subjected to anoxia induced by drowning or by gas.

Drowning assay (wet anoxia)

To experimentally replicate nature, an environment accounting of all the relevant factors was created (Figure 3). A wet environment was simulated by creating a water pool and submerging the organism into this pool (drowning). To address temperature, the flies were submerged in water chambers that were either at room temperature (21°C) or cold temperature (3°C). In order to ensure that the flies that were drowned at 3°C to enter into an anoxic coma instead of a temperature induced coma, these flies were previously subjected to an anoxic environment that is created by argon for 10 minutes. To follow the natural process of aging, flies were sexed and aged under a controlled environment; 1-9 days of adult age (young) and 35-39 days of adult age (old) males were used for this study.

The assay started by sorting and aging the flies and placing them into a plastic vial with food for 24 hours. Then, the flies were transferred into the drowning containers and these were then submerged into the water chamber. The drowning container was a plastic cylinder with metallic mesh at both ends. In order to avoid any air bubbles in the surface of the container, the container was tapped. The flies were kept under water for the

varying times of anoxic stress. Once the time ended, the container was taken out of the water, opened and taken the flies with a paintbrush. These were placed on top of a kimwipe to absorb the excess water. Afterwards, the flies were placed on a plastic vial with food to wait for their recovery and their survival was assessed 24 hours after the stress (Figure 3).

Chamber assay (dry anoxia)

The previous drowning experiments were performed in order to imitate what likely happens in nature, but it is important to compare these to a controlled anoxic environment. This controlled anoxic environment was induced in a restrictive method in order to reduce variability with the objective of comparing these results with the drowning assay. Time of stress and temperature were factors that were studied in this experiment. Aging differences were not studied using this method. Only recovery time and survival 24 hours after the insult was investigated for this part of the study.

This method started by sexing and aging the flies and letting them recover from the CO₂ for 24 hours in a plastic vial with fly food. This same container was placed in an anoxic chamber (5% CO₂, 0.5% O₂ and balance N₂) in order to induce the anoxic coma. After the coma was reached, the flies were transferred into a vacuum-sealed container in which wax was placed around it to ensure complete oxygen deprivation. Then, the containers were placed in a vacuum-sealed plastic bag where the remaining oxygen was sucked out. This created a complete anoxic environment with a low amount of probability of oxygen entering the container where the flies were. Once the flies were in a complete anoxic environment, the bag with the containers and the flies were taken out and stored either in the refrigerator (3°C) or at room temperature. Once the time of anoxia was over,

the bags and the containers were opened and the flies were taken out. At this time, the flies were reintroduced into a normoxic environment. They were placed in a plastic vial with food to record their recovery from the coma (Figure 4).

Data acquisition

Function and survival of the organism determined how tolerant they were to anoxia. The effects provided by the induced coma were studied in conjunction with the effects of temperature and age. Recovery time from the coma and survival after the stress determined protection of function and endurance. As soon as the flies were taken out from the anoxic stress, they were placed in the plastic containers and immediately positioned in front of a video camera. The video camera recorded from the time the container was placed until the time the fly completely recovered from the coma. Then, this video was analyzed by recording the exact time that the video indicated. The time was recorded into minutes and seconds, which was then converted into seconds. Recovery was defined as the time the fly stood up and walked. Survival was also obtained by quantifying the number of flies that were alive 24 hours post stress. This measurement was recorded in terms of percentages, which represented the number of flies alive over the total number of flies in the container.

Statistical analysis

All statistical analyses were done using Sigma Plot. Statistical Two-Way ANOVA followed by a post-hoc Tukey's Multiple Comparison test was performed to compare more than 2 groups. Significant difference ($P < 0.05$) was represented with

capital letters. Two groups were compared using Student's t-test. Significant difference ($P<0.05$) was represented with stars.

Figure 3. Behavioral Drowning Assay. Male flies are previously aged into two groups (1-9 and 35-39 days old). Flies are then transferred to a drowning container and submerged into water with two different temperatures (21°C and 3°C). Behaviorally, protection is measured by the recovery time from the anoxic coma and can be further quantified by how many flies are alive from the total that survive chronic anoxic exposure.

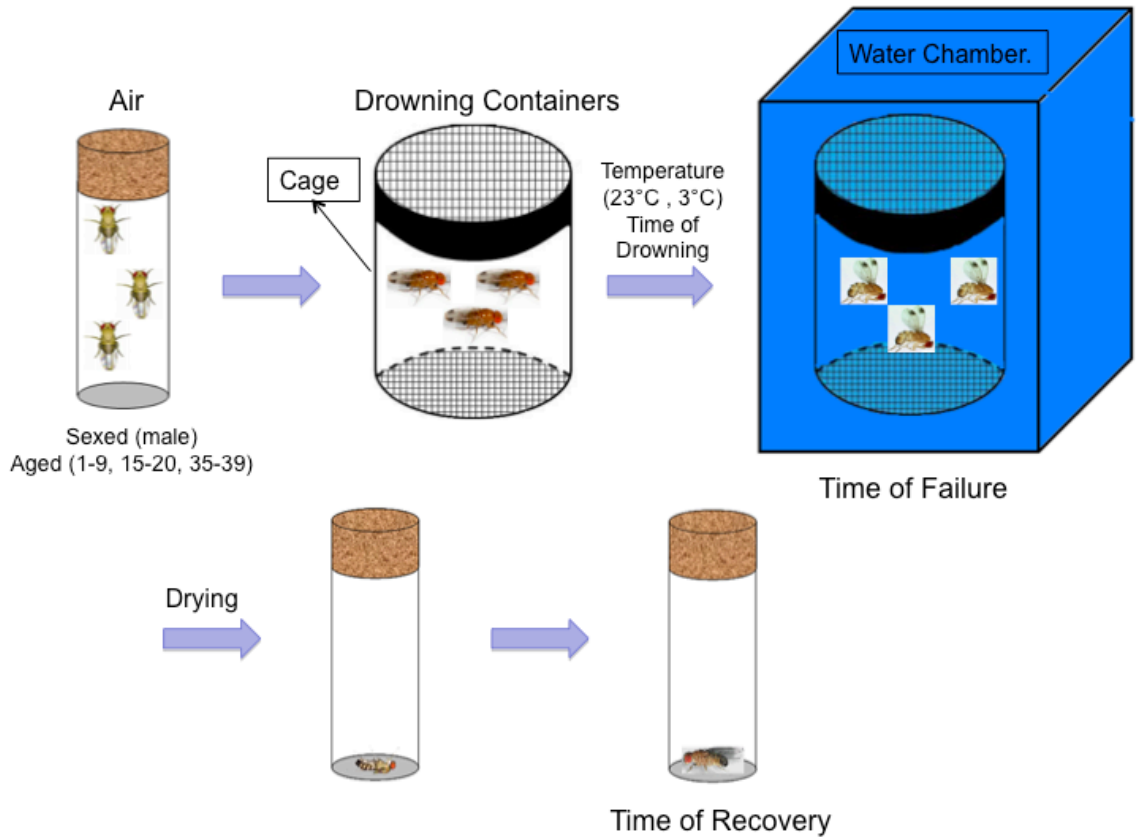
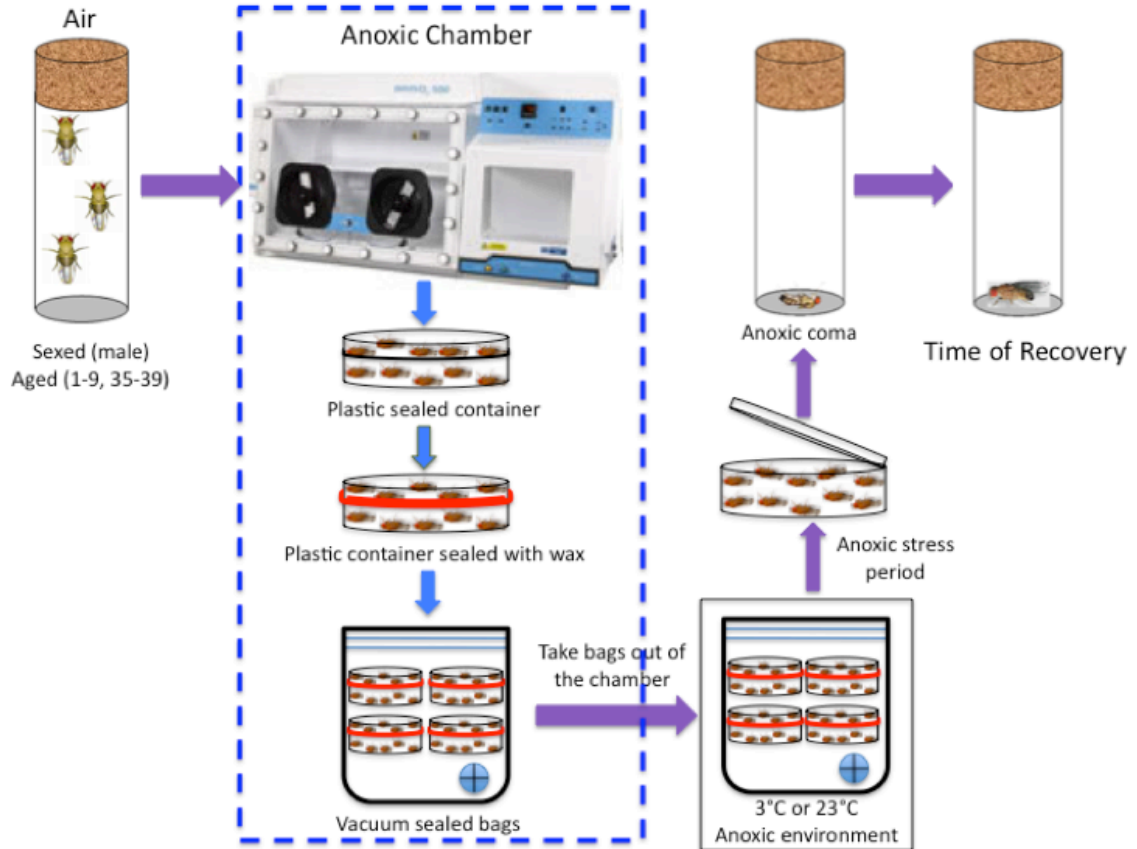


Figure 4. Behavioral Dry Anoxia Assay –Anoxic Chamber. Male flies are previously aged into two groups (1-9, 35-39 days old). Flies are then introduced into the anoxia chamber in which they become anoxic and enter into the “protective coma”. In the chamber the flies are secured in a complete anoxic environment and then taken out of the chamber and placed in two different ambient temperatures (21°C and 3°C). After the flies are in the anoxic insult for the desired time they are lastly taken out from their anoxic sealed container and placed into a regular plastic vial where its recovery and survival was taken. Behaviorally, protection is measured by the recovery time from the anoxic coma and can be further quantified by how many flies survive chronic anoxic exposure.



CHAPTER III. THE BEHAVIORAL EFFECTS OF ANOXIA ON RECOVERY AND
SURVIVAL FROM AN ANOXIC COMA IN WILD TYPE (*w¹¹¹⁸*) *DROSOPHILA*
MELANOGASTER

INTRODUCTION

Anoxia is a condition characterized by an absence of oxygen supply to an organ or a tissue (Lutz et al., 2003). In the mammalian brain during anoxia, energy supply is compromised leading to a failure of adequate neuronal activity, therefore, making humans extremely susceptible to this condition (Lutz et al., 2003) (Dawson-Scully et al., 2010). However, unlike humans, there are several organisms that are anoxia tolerant. One of these organisms is the fruit fly, *Drosophila melanogaster*, which can survive very low oxygen levels for several hours without showing any pathology (Dawson-Scully et al., 2010). Under anoxia, the fruit fly loses locomotive activity, resulting in an anoxic coma, which is a protective quiescent phase where there is a temporary cessation of neuronal and muscular activity (Haddad et al., 1997) (Nilsson & Renshaw, 2004). The onset of this coma preserves energy to avoid the consequences of complete energy loss (Van Voorhies, 2009) (Rodgers, Armstrong, & Robertson, 2010).

The phenomenon of the anoxic coma was first investigated by the Gabriel Haddad Laboratory (1997) where they subjected adult *Drosophila* to bouts of anoxia ranging from 5 to 300 minutes and recorded the time for the first fly, 50% of the flies, and 100% of the flies to enter into the anoxic coma (Haddad et al., 1997). In the same experiment

they recorded recovery, measuring the first fly, 50% and 100% of the flies to move as well as the first fly to climb the walls of the vial. They found a direct relationship between the time of stress and the recovery time where the longer the fly was under anoxia, the more it took to move and to climb the walls of the vial. Additionally, other studies determined that there is a direct but non linear relation with regards to stress exposure and recovery time (Van Voorhies, 2009). This demonstrates that there is a disproportionate increase in recovery time due to time of stress. The anoxic coma stage is attributed to loss of central nervous system function, but this is not only based on behavior but also in physiological findings (Haddad et al., 1997). This loss of electrical excitability was demonstrated by (1) the evoked muscle recordings from the thoracic ganglia of the cockroach and (2) recordings of the TTM and DLM muscle of *Drosophila melanogaster* (Haddad et al., 1997).

The ability to fall into a non reactive state is also observed in salt marsh spiders (Pétilion et al., 2009). These studies showed that time to recover and survival from anoxic stress is directly dependent of their environmental conditions including temperature variations. Taking into account previous findings, we decided to further investigate the anoxic tolerance of adult *Drosophila melanogaster* and the influence of four specific factors: 1) anoxic environment (gas vs. drowning), 2) anoxia duration, 3) temperature (cold [3°C] or room temperature [21°C]), and 4) age (young 2-9 days and old 35-39 days). Our findings should more fully characterize which have not been studied previously by pushing the limits of anoxic stress and incorporating critical variables.

RESULTS

Inducing anoxic stress by wet environment (drowning) in adult *Drosophila*

Wet environments such as rainfall surround all types of organisms. These environments can cause significant oxygen depletion, so it is critical to investigate how organisms can react when confronting this type of stress, engendering protective mechanisms. Organisms have evolved to overcome unpredictable and harsh environmental conditions, such as oxygen depletion and temperature fluxes. Temperature significantly affects behavior, physiology and survival of insects (Angilletta, Huey, & Frazier, 2010). Not only do organisms need to cope behaviorally with external influences such as temperature, but their physiology is also a factor that plays a role in coping. Aging is one of these important factors that not only affect the organisms physically, but also their function and development. The following results will measure the ability of the fly to recover from the quiescent coma and the survival rate from anoxic stress. Depending on how long the fly was subjected to anoxic stress, different outcomes were noticed. This data demonstrated that the longer the flies were under anoxia, their ability to recover and survive the insult was reduced (Figures 5, 6).

The data indicated that following one hour drowning, the fly took an average of 329.8 seconds to recover. This time of recovery was increased when the fly was exposed to anoxic drowning for six hours and furthermore 12 hours (7047.5 seconds and 12830 seconds). It was found that the stress period limit that the fly could tolerate at room temperature was between 12 and 18 hours. At the 18th hour, there was no recovery. The outcome of this study indicates that as the time of anoxia increases, the time of recovery from the anoxic coma also increases significantly (One-Way ANOVA, $F_{(3,65)} = 229.725$,

$p = < 0.001$). Survival followed a trend in which the longer the fly is under stress, the less probability of survival. One hour drowning resulted in 100% survival, 6 hours resulted in approximately 50%, 12 hours was less than 50% and 18 hours showed no signs of survival. A significant difference was observed in the survival rate between each drowning stress period (One-way ANOVA, $F_{(3,10)} = 28.873$, $p = < 0.001$).

The effects of temperature on anoxic coma in *Drosophila melanogaster*

Temperature plays a crucial role during anoxic stress. It was found that the effects of anoxia were greatly reduced by low temperature (Figures 7,8). Comparing the same stress period of drowning but at different temperatures, we can see that lower temperature increases their ability to recover from the coma. For instance, a significant difference was found at 6 hours drowning at room temperature where it took longer to recover by an average of 6336 seconds than 6 hour drowning at cold temperatures. (Student's t-test, $p < 0.05$). Thus, increasing temperature reduces tolerance levels during anoxic stress. This investigation also showed what the physiological limits of the *Drosophila* are in terms of how long they can resist and survive an anoxic insult. At room temperature, *Drosophila* survived at the 12th hour, yet there was no survival at the 18th hour. At cold temperature, these limits are significantly extended, allowing the fly to survive for 72 hours (Student's t-test, $p < 0.05$).

How the physiological factor of age alters anoxic tolerance

Aging is a factor that is embedded into the organism's natural physiology and affects its ability to tolerate stress (Martin, 2011). The older they are, the more time they take to recover from the stress. (Figure 9A) Significant difference between the recovery

time of two age groups, 1-9 days old and 35-39 days old, was noticed after a 12 hour stress period at room temperature (Student's t-test, $p < 0.05$). Additionally, it was found that lowering the temperature ameliorated the effects of age (Figure 9B). At room temperature, old and young flies survived up to 12 hours, while at 3°C young flies survived after 72 hours of stress and old flies after 48 hours (Figure 10 A, B). At room temperature, the old and young flies differed significantly in their survival rates regardless of the anoxic exposure time (Student's t-test, $p < 0.05$). At colder temperature, a unique trend is observed where a significant difference in the survival rates appeared only immediately at 1 hour anoxia and then not again until 24 hours and more (Student's t-test, $p < 0.05$). Increased age reduces tolerance level and survival during anoxic stress.

Inducing anoxic stress by dry environment (anoxic chamber) in adult *Drosophila*

The previous drowning experiments were performed in order to imitate what likely happens in nature, but it is important to compare these to a controlled anoxic environment. This controlled anoxic environment was induced in a restrictive method in order to reduce variability with the objective of comparing these results with the drowning assay. Time of stress and temperature were factors that were studied in this experiment. Aging differences were not studied using this method. Only recovery time and survival 24 hours after the insult was investigated for this part of the study.

With the dry environment experiments, similar trends to the drowning experiments were found when accounting for the variables of stress periods and temperature. Increased time of stress and temperature reduces the ability of the fly to recover and survive the insult that implied that it reduced their tolerance to anoxia (Figures 11, 12).

Studying the different influences of wet (drowning) vs. dry (gas) anoxia

When comparing controlled laboratory conditions such as the gas anoxia with simulation of drowning in nature, differences in the recovery time were observed (Figure 13 A and B). At room temperature, the only significant difference observed the drowning and the chamber anoxia at 1 hour exposure (Student's t-test, $p < 0.05$). At cold temperature, all the stress periods showed a significant difference in the recovery times between the wet and dry assay except for the 24 hour period, with the anoxic chamber showing consistently longer recovery times (Student's t-test, $p < 0.05$). For instance, at cold temperature, flies subjected to 48 hours of gas anoxia took 8545.8 seconds longer to recover than the flies drowned.

When comparing the survival rates after different anoxic exposure times in wet and dry environments, there is generally no significant difference between the two assays, even though survival is always reduced for gas chamber anoxia at room temperature and almost always at cold temperature (Student's t-test, $p < 0.05$) (Figures 14A and B). The only significant difference in survival was observed at the lower exposure period.

DISCUSSION

Studying the influence of recovery and survival according to stress duration

Throughout all cases, our findings demonstrate that while the fly is under a prolonged time of stress, it will take longer for it to recover. This supports the results of Haddad (1997) and Van Voorhies (2009), which showed a direct but non linear relationship between time of anoxic stress and recovery (Haddad, 1997) (Van Voorhies, 2009).

The subsequent increase of recovery time due to stress exposure can be explained by a previous study performed by Rodgers, Armstrong and Robertson (2010). This study consisted of the extracellular and intracellular recordings of the ventilatory neurons within the ganglion of locust to measure the change in motor pattern in relation to extracellular K^+ increase in response to different stressors such as anoxia. Their findings demonstrated a correlation with induced stress, motor pattern arrest and recovery. When anoxia is induced either by a nitrogen gas filled chamber or sodium azide, a sudden rise in extracellular K^+ is observed that correlates with motor failure, and as soon as air is reintroduced, extracellular K^+ returns to the baseline and motor patterns are restored (Rodgers et al., 2010). Based on these studies, we can infer that the longer the fly is under anoxic stress, the greater the rise in extracellular K^+ will be, consequently taking more time for the K^+ levels to return to its normal base line. Motor pattern stops when K^+ levels start to rise and recovers when extracellular K^+ returns to its normal baseline levels. We can juxtapose the recovery time from the motor patterns to the recovery time of the anoxic coma in our experiment. There is a direct proportionate correlation with the extracellular K^+ levels with regards to its recovery time, demonstrating that the longer the fly is under anoxia, the greater the extracellular K^+ increase will be, consequently the more time it takes for the extracellular K^+ to return to baseline thus, the longer time it will take for the fly to recover from the coma (Rodgers et al., 2010).

Survival results can be linked to the state of metabolic depression that the flies enter during anoxic coma, where a decrease in total energy production has previously been linked not only in *Drosophila* but also in other insects and low vertebrates (Sick, Rosenthal, LaManna, & Lutz, 1982) (Anchordoguy & Hand, 1994) (Haddad et al., 1997).

The process in which the flies enter and recover is an inverse plateau, where the first stage is metabolic depression, then maintenance and finally recovery. During the stage of metabolic down regulation, ATP consumption is decreased as well as electrical activity, ion channel function and protein synthesis among other processes requiring energy. When it reaches a basal maintenance, energy consumption is equal until replenishment is observed in the recovery stage. At the same time during this maintenance stage, some energy is used in order to sustain the fly's survival. The longer the fly is under the coma, the longer the maintenance stage will be, so more energy will be used. By the time they recover, they have less energy to survive (Lutz & Milton, 2004).

Furthermore, the limit of survival due to the time of exposure can be explained by the physiology of *Drosophila* breathing. *Drosophila* breathes through spiracles that open and close so oxygen can enter their trachea, and reach the cells, thus the organs in the insect's body. These spiracles need energy to close when oxygen is not required or available. Under anoxic stress, flies close their spiracles, where this function requires energy. When they are drowned for a long period of time, they get to a point where they deplete all their energy in order to close the spiracles, thus, the spiracle opens, allowing water to enter the trachea, shown by the fly sinking, killing the fly (Hetz & Bradley, 2005).

Identifying how temperature (cold vs. room temperature) affects neuronal protection during anoxia in *Drosophila melanogaster*

Our study shows that lowering the temperature protects the flies from anoxic stress. Flies that are subjected to cold anoxic stress take less time to recover from the

coma and have a greater survival rate in comparison to the flies that are subjected to room temperature anoxia.

These results are supported by Rodriguez and Robertson's (2011) findings, which show that during anoxia, hypothermia has a protective effect on regulating K^+ homeostasis (Armstrong et al., 2011). Their experiments show a pattern in which the increase in temperature results in an increase of extracellular K^+ . Hyperthermia (29°C) causes an increase in extracellular K^+ baseline, while hypothermia (17°C) causes the values of extracellular K^+ baseline to decrease, and room temperature (23°C) created intermediate results (Figure 3F) (Armstrong et al., 2011). Due to these results, we can hypothesize that low temperature reduces the rise of extracellular K^+ , decreasing the time of baseline levels return, consequently shortening the recovery time of the fly from the anoxic coma. At the same time, survival is possibly affected by K^+ homeostasis, but in the sense of a threshold. Once extracellular K^+ reaches a maximum point, the cell takes a longer time to recover or it may not recover at all, resulting in a longer loss of ionic homeostasis that generate cellular damage and possibly death. Since lowering the temperature leads to a much lower increase in extracellular K^+ , thus the cell is able to recover to the baseline faster, therefore less cellular damage occurs, achieving better survival. Another explanation for high temperature affecting survival rate can be explained by the fact that *Drosophila* are poikilotherms, where room temperature can alter their physiological activity, hence their lifespan (Sick et al., 1982). At high temperature, *Drosophila* live at a higher metabolic rate than normal, depleting their energy faster, leading to a decrease in survival (Sick et al., 1982). Determining how age (young vs. old) affects neuronal protection during anoxia in *Drosophila melanogaster*

Investigating the affect of age on anoxia tolerance demonstrated that age decreases protection of function and survival of the fly during and after anoxic stress. Old flies (35-39 days old) took significantly longer time to recover from the anoxic coma and their survival rate after the stress decreased in comparison to young flies (1-9 days old).

It is known that the process of aging plays an important role in neuronal protection during different types of stress such as temperature and anoxia (Martin, 2011). It has also been determined that oxidative stress is a key player on lifespan not only during normal metabolism but also under anoxic stress conditions in *Drosophila*, where the accumulation of reactive oxygen species increases along with age (Fleming, Reveillaud, & Niedzwiecki, 1992). In addition, oxygen deprivation is accompanied by reactive oxygen species formation which is exemplified by changes in metabolism known as the oxidative stress response (Blokhina, 2010). Accumulation of ROS within the cells can be detrimental and this is due to the disruption of lipids, proteins and DNA among others (Marnett et al., 1985; Fraga et al., 1990; Stadtman et al., 1992). Previous research suggests that the sum of the reactive oxygen species due to the natural process of aging plus the ones produced under anoxic stress leads to increased cellular damage, demonstrating a longer time of recovery and a decreased survival.

Other studies have shown that there is a correlation between temperature, metabolic rate and lifespan in *Drosophila* (Fleming et al., 1992). We observed that the effects of age are reduced when temperature is lowered. This can be explained by the fact that at high temperature, *Drosophila* are more metabolically active, hence they have to live in a faster and higher metabolic rate. Due to this reason, energy is used faster, reducing duration of lifespan (Fleming et al., 1992).

Comparing dry (chamber) vs. wet (drowning) anoxia

It was observed in our results that at room temperature, there is no significant difference in the recovery time from anoxic stress between dry and wet anoxia. On the other hand, at cold temperature, it was found that the flies that are subjected to the dry anoxia take more time to recover than the flies that are drowned. One of the reasons why we think this happened is that not only does anoxia play a significant role, but also desiccation creates an additional stressor in which flies that are drowned do not have to deal with. Desiccation is seen during moments of extreme dryness. The reason why we did not see a difference between dry and wet anoxia at room temperature, can be explained addressing the point that the nature of room temperature air is moist, preventing desiccation. Conversely, at cold temperatures, air is dryer, making *Drosophila* prone to desiccation. Simultaneously, when the insect is at a dormant state it may lose the potential to adjust water balance (Danks, 2000).

While analyzing survival, drowned flies demonstrate a higher survival rate than chamber flies. A critical player that possibly explains this situation is energy consumption. Organisms that are in a coma cannot recreate energy supply, but clearly energy is required during this stage. In the particular case of dry anoxia, energy is not only needed during dehydration for respiration or generation of metabolic water, but also when the organism comes back from dehydration (anhydrobiosis). Not having sufficient energy to recover both anoxic stress and dehydration causes organisms not to survive (Danks, 2000).

From our studies, we found that anoxic tolerance decreases with stress exposure, high temperature, age, and dryness. At the same time, survival limits were found to

correlate to these variables. These results filled in particular gaps regarding anoxic tolerance studies in *Drosophila melanogaster*. This is the foundation for future investigation on factors that can protect the fly under stress. As previously stated, PKG is known to be involved in anoxic stress tolerance and for this reason; a focus on PKG follows.

Figure 5. Recovery Time from Different Anoxic Stress (Drowning) Periods. W1118 1-9 days old male flies were subjected to 1,6, 12 and 18 hours anoxic stress by drowning. It is observed that the longer the flies are under anoxic stress their time of recovery increases significantly (One-Way ANOVA, $F_{(3,65)} = 229.725$, $p = < 0.001$). After 18 hours of anoxic stress no recovery was seen, showing that at room temperature the maximum time of anoxia that the fly can sustain is 12 hours.

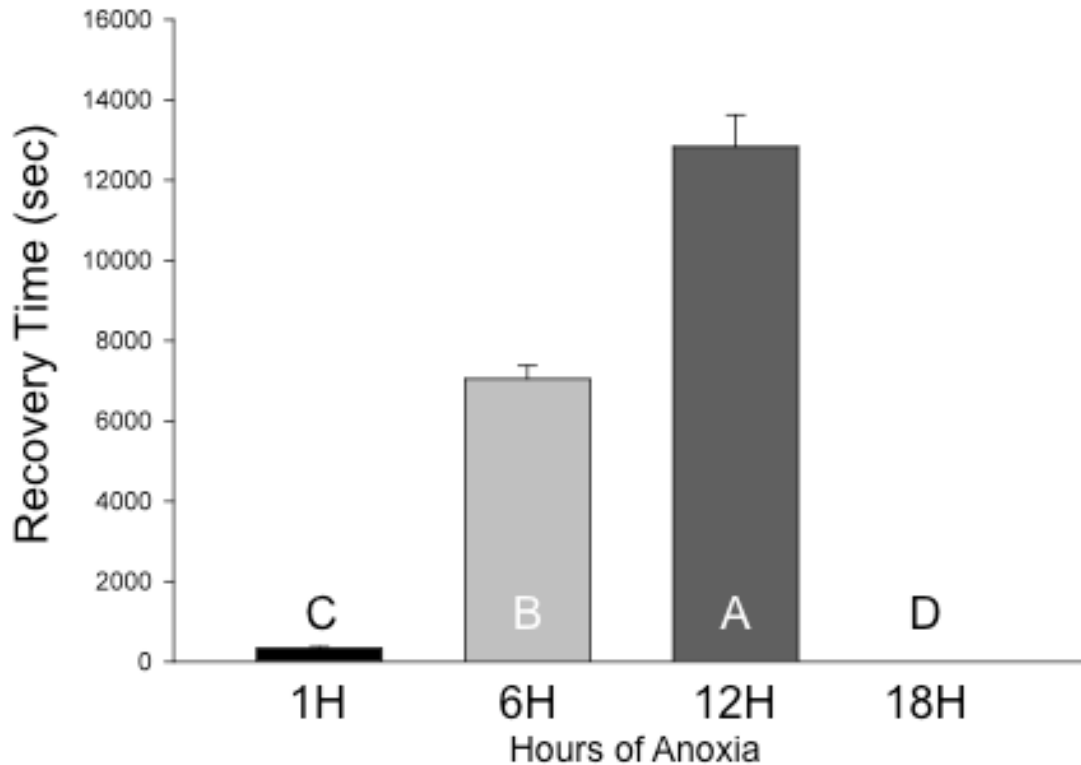


Figure 6. Survival Rate 24 Hours After Drowning. Survival rate was quantified 24 hours after the flies were subjected to the different stress periods (1,6,12,18 hours). Survival rate decreased significantly with stress exposure (One-way ANOVA, $F_{(3,10)} = 28.873$, $p < 0.001$). No recovery was observed after 18 hour drowning, setting the limit for survival at 12 hours of anoxia.

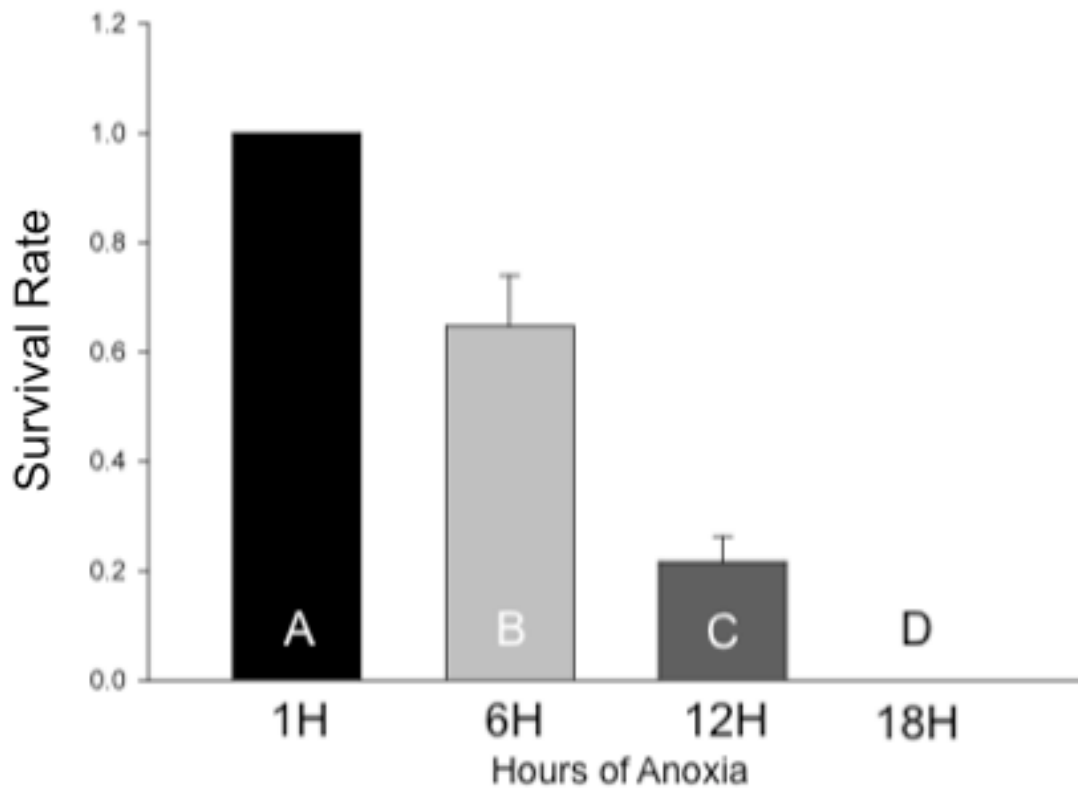


Figure 7. The Effect of Temperature on Drowning Anoxic Coma Recovery Time. W1118 1-9 days old male flies were subjected to room (23°C) and cold (3°C) temperature anoxia by drowning for different times (1,6,12,18,24,48 and 72 hours). The results indicated that there was a significantly extended recovery time for the flies that were under room temperature anoxic stress in comparison to the cold temperature anoxic stress for 1,6, and 12 hours exposure time (student's t-test, $p = <0.05$). It is also observed the limits in recovery time for room temperature drowning anoxia is 12 hours but 72 hours for cold temperature anoxia.

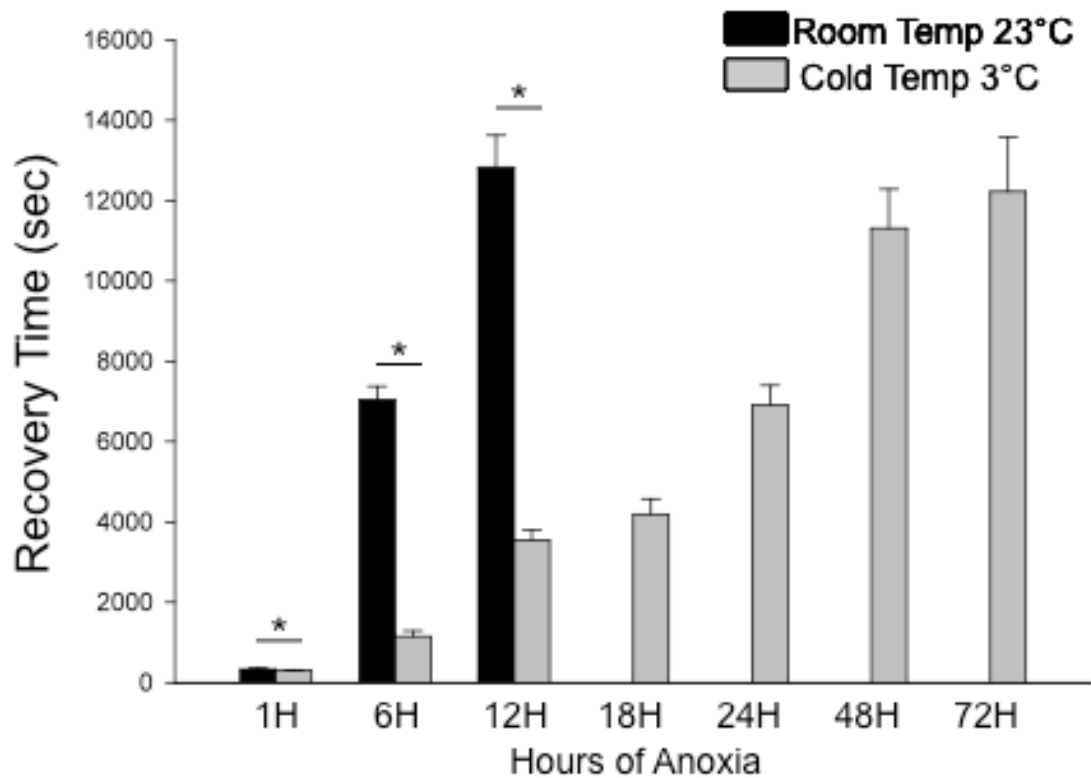


Figure 8. Survival Rate 24 Hours Post-reoxygenation after Room or Cold Temperature Drowning Anoxia. Percent survival was taken 24h after the stress, flies were subjected to 1,6,12,18,24,48 and 72 hours drowning at room or cold temperature. Significant difference between the two temperatures was found at 6 and 12 hour anoxia (student's t-test, $p < 0.05$). Maximum survival for room temperature drowning anoxia was observed at 12 hours exposure. At cold temperature anoxia survival was observed at 72 hours anoxia exposure time.

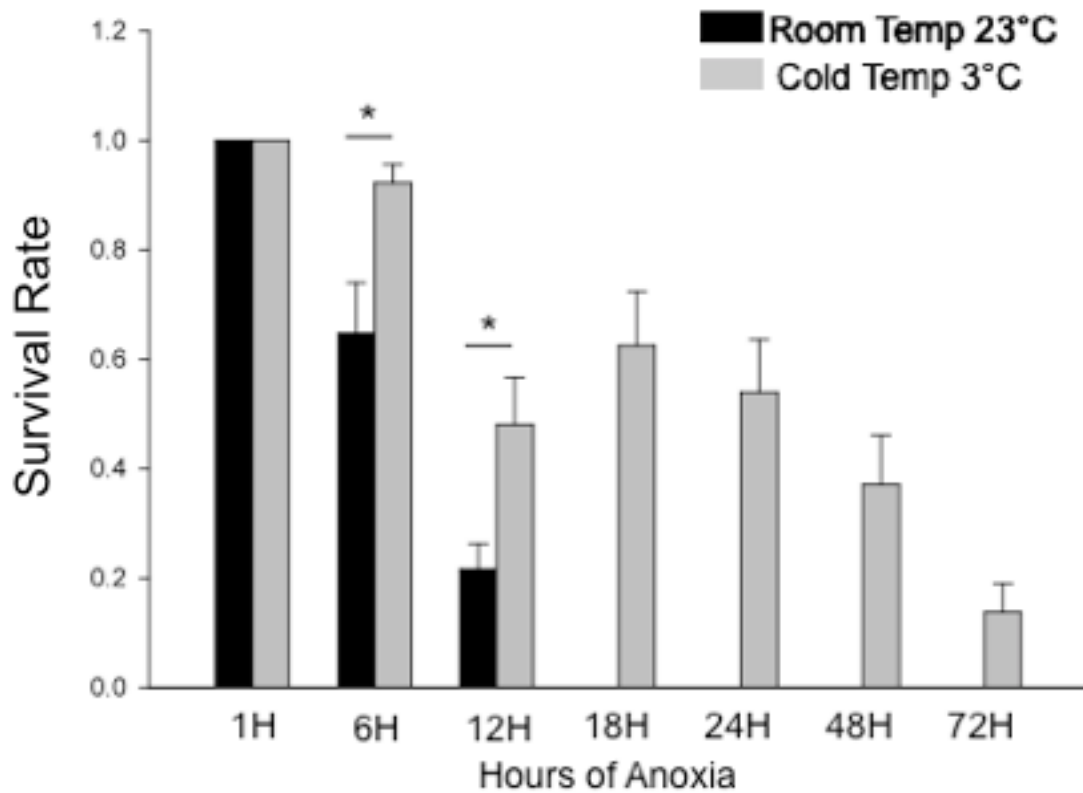


Figure 9. The Effects of Aging and Temperature on Drowning Anoxic Recovery.

W1118 male flies were aged until 1-9 and 35- 39 days old and then subjected to anoxia for 1,6,12,18 hours at room temperature, and 1,6,12,18,24,48,72 hours at cold temperature. The graphs represent time for the fly to recover vs. hours of drowning for the two different age groups. A) Flies drowned at room temperature 23°C. There is a significant difference in the recovery time between the two ages at 1 and 12 hours anoxia (student's t-test, $p < 0.05$). B) Flies drowned at cold temperature 3°C. Age difference in recovery time is ameliorated by decreased temperature. Significant difference is observed between the two age groups in all periods of anoxia except for 18 hours stress exposure (student's t-test, $p < 0.005$). Age sensitizes the flies to anoxic stress tolerance.

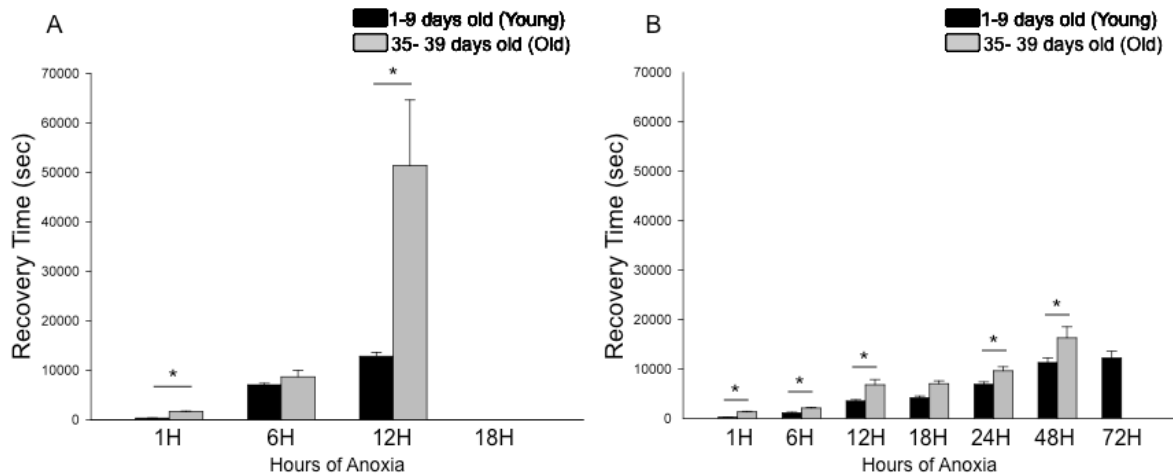


Figure 10. The Effects of Age and Temperature on Survival Rate 24 Hours After Drowning Anoxic Stress. W1118 male flies were aged until 1-9 and 35- 39 days old and then subjected to anoxia for 1,6,12,18 hours at room temperature, and 1,6,12,18,24,48,72 hours at cold temperature. The graphs represent survival rate 24 hours after the stress vs. hours of drowning for the two different age groups. A) Data for the flies drowned at room temperature 23°C. Survival rate was significantly reduced for old flies (35-39 days old) for all stress periods. (student's t-test, $p < 0.05$). B) For cold temperature (3°C) drowning, age significant difference was only observed at 1, 24 and 48 hours (student's t-test, $p < 0.05$). For the young flies the limit of survival is 48 hours of drowning anoxia, and for old flies 72 hours. These graphs suggest that survival rate decreases with aging.

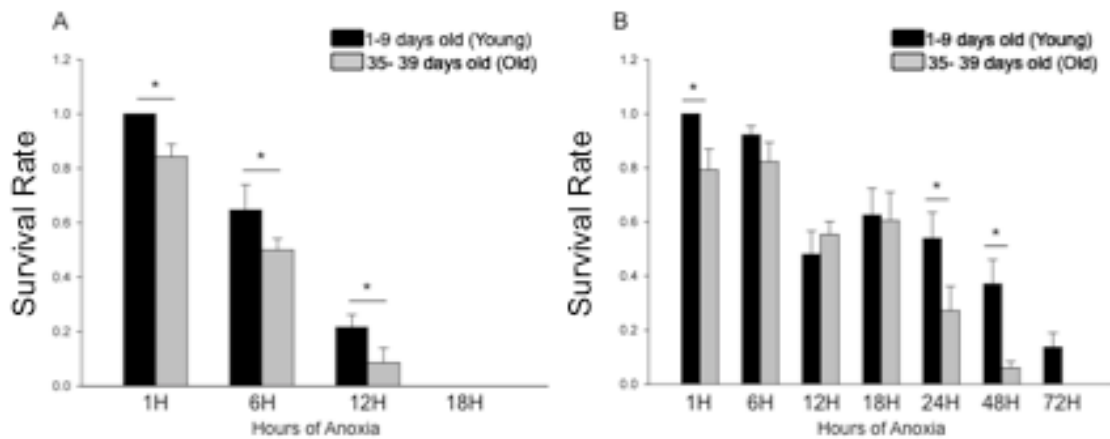


Figure 11. The Effect of Temperature on Anoxic Coma Recovery Time Induced by a Gas Chamber. W1118 1-9 days old male flies were subjected to room (23°C) and cold (3°C) temperature anoxia by gas in the anoxic chamber for different times (1,6,12,18,24,48 and 72 hours). The results indicated that there was a significantly extended recovery time for the flies that were under room temperature anoxic stress in comparison to the cold temperature anoxic stress for 6, and 12 hours exposure time (student's t-test, $p = <0.05$). It is also observed the limits in recovery time for room temperature chamber anoxia is 12 hours but 72 hours for cold temperature anoxia.

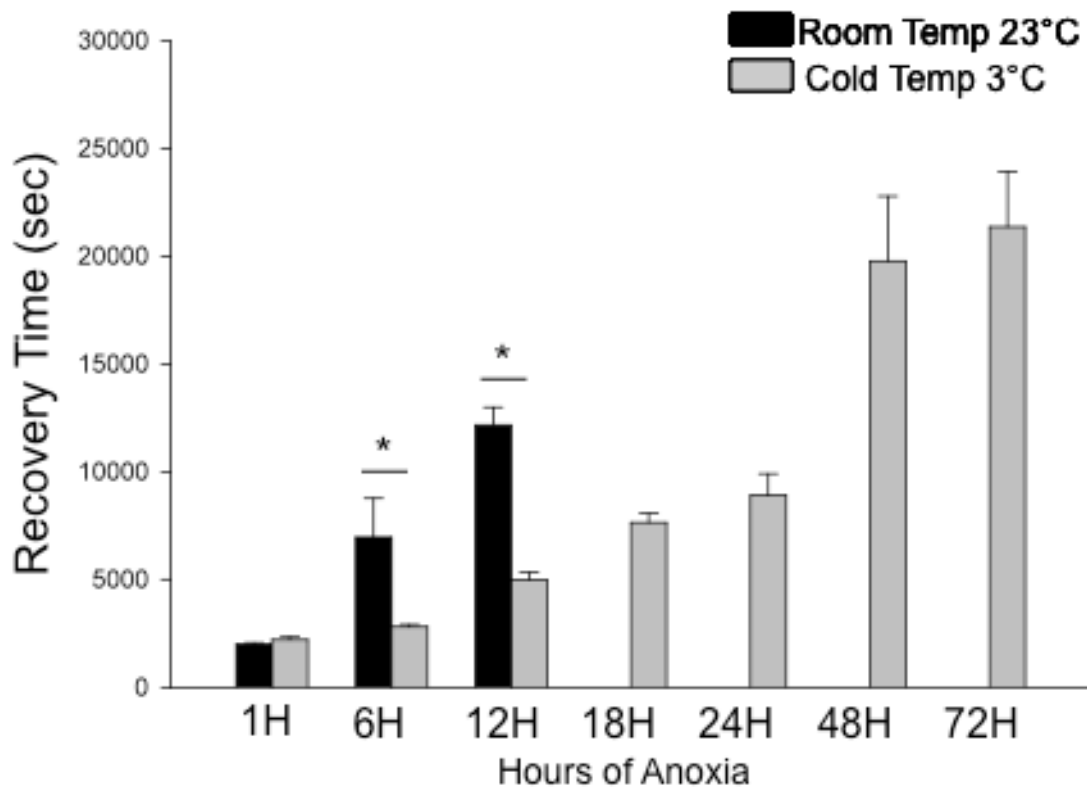


Figure 12. Survival Rate 24 Hours Post-reoxygenation after Room or Cold Temperature Gas Chamber Anoxia. Percent survival was taken 24 hours after the stress, flies were subjected to 1,6,12,18,24,48 and 72 hours drowning at room or cold temperature. Significant difference between the two temperatures was found at 6 and 12 hours anoxia (student's t-test, $p < 0.05$). Maximum survival for room temperature gas chamber anoxia was observed at 12 hours exposure. At cold temperature anoxia survival was observed at 72 hour anoxia exposure time.

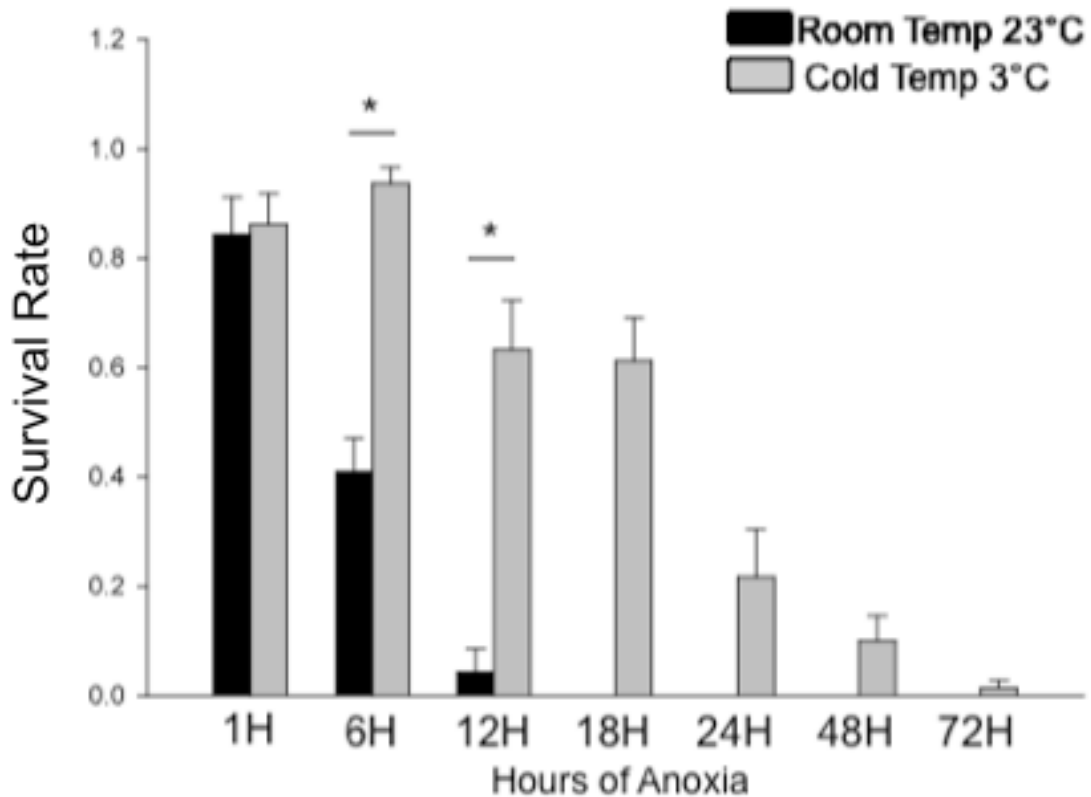


Figure 13. The Effects of Drowning (Wet) vs. Gas Chamber (Dry) Anoxia on Recovery Time From the Coma at Two Different Temperatures. W1118 1-9 day old male flies were subjected to drowning (wet) anoxia or gas chamber (dry) anoxia at room (23°C) or cold (3°C) temperature for 1,6,12,18,24,48, and 72 hours. A) Anoxia induced at room temperature. Significant difference between the two anoxic environments (dry vs. wet) is only observed at 1 hour (student's t-test, $p < 0.05$). B) Anoxia induced at cold temperature. Significant difference between the two anoxic environments (dry vs. wet) is observed in all the anoxic periods except 24 hours (student's t-test, $p < 0.05$). Flies subjected to gas chamber anoxia showed increased recovery time in contrast to drowned flies.

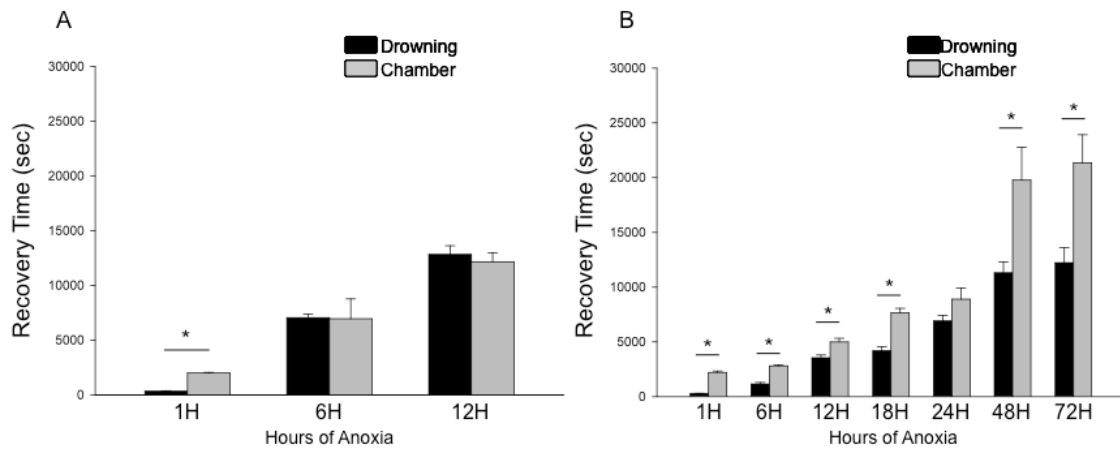
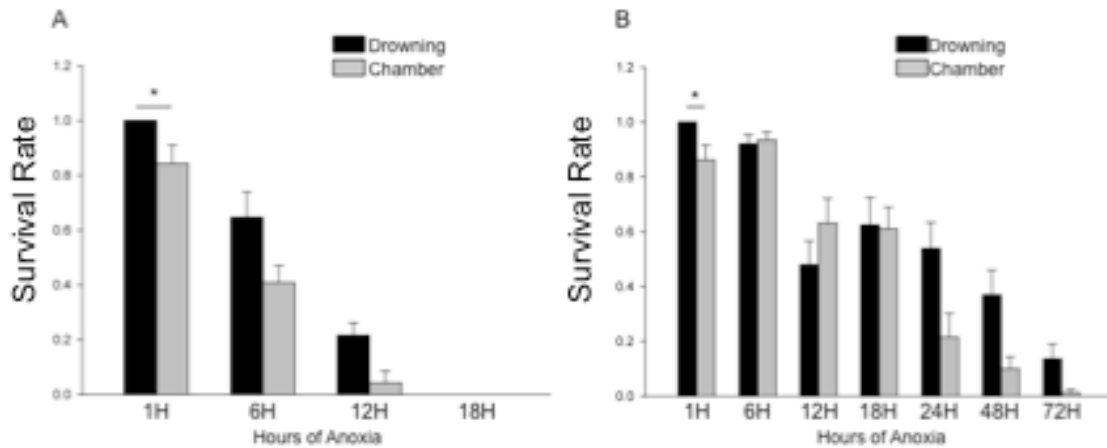


Figure 14. The Effects of Drowning (Eet) vs. Gas Chamber (Dry) Anoxia on Survival Rate From the Coma at Two Different Temperatures. Survival Rate was taken 24 hours after the flies had been subjected to drowning or gas chamber anoxia, at room (23°C) or cold (3°C) temperature for 1,6,12,18,24,48 and 72 hours. A) Room temperature anoxia data for dry and wet anoxia. Survival is reduced during gas chamber anoxia when compared to drowning, the only significant difference between the two treatments is observed at 1 hour anoxia (student's t test, $p < 0.05$). B) Cold temperature anoxia data for dry and wet anoxia. Survival rate for the flies subjected to dry anoxia is reduced for 1 hour and more in the 24 hour anoxia, the only significant difference is found at 1 hour anoxia (student's t-test, $p < 0.05$).



CHAPTER IV. ANALYSIS OF THE NATURAL VARIATIONS OF THE PKG
PATHWAY ALTERING NEURONAL PROTECTION UNDER ANOXIC
CONDITIONS (ROVER [+PKG], SITTER [-PKG], AND S2 [-PKG])

INTRODUCTION

It has been previously shown that the cGMP-dependent protein kinase (PKG) cascade pathway is involved in modulating low oxygen tolerance in adult fruit flies (Wingrove & O'Farrell, 1999a) (Dawson-Scully et al., 2010). The *foraging* gene encodes a *Drosophila* PKG, which in nature is present in two natural strains. High PKG activity is found in the Rover (*for^R*) strain flies while low PKG activity is found in the Sitter (*for^S*) strain. Induced mutations of the *foraging* gene result in a new strain (*for^{S2}*) that has a Rover genetic background with lower PKG activity than Sitter (Dawson-Scully et al., 2010). Furthermore, the effect of these alleles can be reproduced through the use of specific PKG pathway inhibitors and activators.

Up to this point, and based on previous research, more PKG activity (Rover) increases survival rates during anoxic stress in both larvae and adult *Drosophila* (Wingrove & O'Farrell, 1999b) (Dawson-Scully et al., 2010). Findings according to Wingrove and O'Farrell (1999) determined that during long periods of oxygen deprivation, PKG is an important factor in embryonic and larval survival. In their experiments, they subjected third instar *Rover* (+PKG) and *Sitter* (-PKG) larvae to 6

hours of hypoxia, and observed 83% viability for Rovers and only a 20% survival for Sitter, showing that higher levels of PKG activity protects flies from anoxic stress. With regards to behavior, they found that larvae that were subjected to 1% oxygen for 10 minutes demonstrated different feeding behaviors, in which Rovers eat less compared to Sitter. Furthermore, it was found that low PKG activity slows down behavioral hypoxia response (Wingrove & O'Farrell, 1999b).

Another study also examined the influence of PKG on anoxic tolerance by subjecting adult *Drosophila* to 6 hours of anoxia and recovery for 24 hours, measuring the time it took for the fly to enter the coma, and survival after the stress as well (Dawson-Scully et al., 2010). The results from the study demonstrate that lower PKG activity increases time for the flies to enter the anoxic coma, showing that S2 (-PKG) flies function for a longer time under anoxic stress compared to Sitter (-PKG), and subsequently Rovers (+PKG). This protection of function comes with the cost of survival since they found that low PKG activity contributes to reduce survival under 6 hours of anoxia. Rover flies have a greater percent survival than Sitter and S2. The reasoning behind these results indicate that low PKG activity protects neuronal function during hypoxia, where the nervous system has the capacity to function during moments of exacerbated stress. Notwithstanding, this protection generates an inverse relationship with survival in which mortality increases tremendously when PKG activity is reduced (Dawson-Scully et al., 2010).

What is currently unknown in terms of tolerance to anoxia through modulation of the PKG pathway are the effects of age, temperature, and functional recovery. Since we

have developed an assay to determine this, as well as a means to examine both wet and dry anoxia, we will examine these variables in the following chapter.

RESULTS

The effects of the PKG pathway variations on anoxic stress recovery, survival and how they are altered by temperature (cold vs. room temperature)

Protection of behavior from anoxia and survival of the fly given by variation of PKG (by expression of the gene *foraging*) were investigated considering different anoxic stress periods and different temperatures (cold vs. room temperature). The following data was obtained from the experiments performed using the Drowning protocol (wet anoxia).

In this experiment, tolerance to anoxic stress will be determined by recovery time from the anoxic coma and survival after anoxic stress. The following results represent the data to demonstrate the effects of the PKG pathway variations on anoxic stress tolerance taking into account temperature.

Our data show that the recovery time can be observed from Rover (+PKG), Sitter (-PKG), and S2 (-PKG) flies that were drowned at 23°C (Figure 15). At one hour drowning no significant difference was observed between the 3 fly lines (One-way ANOVA, $F_{(2,60)} = 49.885$, $p < 0.05$). At 6 hours drowning, Rover flies took a mean of 9602.3 seconds to recover, Sitter took 8194.1 seconds and S2 6821.8 seconds. A significant difference was observed between the different PKG lines (One-way ANOVA, $F_{(2,59)} = 16.006$, $p > 0.001$). The flies that were under stress for 12 hours showed similar trends to the 6 hours ones, S2 recovered first (12070 seconds), Sitter second (19353.3 seconds) and Rover last (27991.3 seconds). Rover, Sitter and S2 recovered at

significantly different times from each other (One-way ANOVA, $F_{(2,60)} = 16.006$, $p > 0.001$). Recovery was only observed for Sitter flies after they were subjected to 72 hours of anoxia.

For the protocol in which the flies were subjected to cold temperature (3°C) anoxia, no particular trend was observed with regards to the influence of PKG variation in anoxic stress tolerance (Figure 16). When the flies were drowned for 1, 6 and 12 hours no significant difference was observed between Rover, Sitter and S2 lines. At 18 hour drowning, Rover and S2 recovered around the same time (5709.5 seconds and 5652.4 seconds respectively), Sitter on the other hand recovered much more slowly (10080.16 seconds). At 24 hours drowning the opposite happened, where Sitter recovered faster than Rover and S2 (Rover 13880.8 seconds, Sitter 7172.75 seconds and S2 11695.2 seconds). Significant difference was observed between the Rover and Sitter, and between Sitter and S2 (One-way ANOVA, $F_{(2,27)} = 18.950$, $F_{(2,73)} = 18.950$, $F_{(2,68)} = 18.950$, $p < 0.001$). A similar trend for 24 hour drowned flies was reflected for 48 hour drowned flies. Rover and S2 flies took significantly more time to recover from the coma than Sitter. For 72 hours anoxic stress exposure, similar recovery time was observed between Rover and S2 flies (18455.5 and 19053.8 seconds respectively). No recovery was recorded for Sitter flies.

Survival rate was also analyzed from the drowning experiments at the two temperatures (23°C and 3°C) (Figure 17). At room temperature, one hour drowned flies did not show any statistical significant difference in survival rate (One-way ANOVA, $F_{(2,60)} = 1.22$, $p = 0.543$). At 6-hour anoxic stress S2 had a greater survival rate than Sitter, and Sitter than Rovers (Rover 0.55, Sitter 0.68 and S2 0.87). Similar trends were seen at

12-hour anoxia, where survival rate was the lowest for Rover (0.0167), the highest for S2 (0.383) and Sitter sat at the middle with a 0.085 percent survival. After 24 hour drowning, survival was only observed for Sitter flies.

The data that represents survival rate for cold temperature anoxia indicates that no significant difference was found between Rover, Sitter and S2 flies that were drowned for 1, 6 and 12 hours (One-way ANOVA, $F_{(2,60)}=2$, $p=0.368$), (One-way ANOVA, $F_{(2,60)}=1.468$, $p=4.80$), (One-way ANOVA, $F_{(2,60)}=2$, $p=0.547$). At 18-hours Rover flies had a 0.54 survival rate, Sitter at 0.758 and S2 0.812. At 24-hours Rovers also showed the lowest survival rate (0.47), S2 the largest survival rate (0.5398) and Sitter lay in the middle (0.52). No statistical significant difference was observed between Sitter and S2 survival rate (One-way ANOVA, $F_{(2,60)}=0.332$, $p=0.722$). Survival was only found in Rover and Sitter flies at 48 hours of anoxic stress. The percent survival between Rover and Sitter was significantly different. (One-way ANOVA, $F_{(2,60)}=59.511$, $p<0.001$). No survival was observed at 72 hours anoxic stress at cold temperature drowning (Figure 18).

The recovery time at 23°C showed no significant difference during the first hour among Rover, Sitter and S2. At the 6th and 12th hour, Rover took a longer recovery time compared to Sitter and subsequently S2. At the 18th hour, we only saw a recovery for Sitter. These results suggest that S2 (-PKG) seems to be more protected than Sitter (-PKG) and Rover (+PKG) during the 6th and 12th hour since it recovers much faster than the stress. Rovers, conversely, have less tolerance towards anoxic stress since they took the longest time to recover. When temperature is lowered to 3°C, time of recovery decreases in general, suggesting that temperature is protecting the flies regardless of their

PKG variation. For this reason, we see no significant difference between Rover, Sitter and S2 at the 1st, 6th and 12th hour. After 12 hours, no specific trend was found. It was surprising to find that Sitter at the 24th and 48th hour took the least amount of time to recover from the coma, where we can infer that it is more protective to the stress than Rover then S2. However, at 72 hours of drowning, we did not see any recovery for Sitter while S2 and Rovers did recover, contradicting that Sitter are more tolerant to anoxic stress than the other two PKG lines.

The survival rate at 23°C showed no significant difference in the first hour. At the 6th and 12th hours, the S2 demonstrated a greater survival rate than Sitter, and Sitter than Rovers. This shows that S2 are more protected after the stress than Sitter and Sitter are more protected than Rovers. At the 18th hour, only Sitter survived after the stress. When the temperature is lowered to 3°C, all flies regardless of the time of anoxia, show a greater survival rate in comparison to 23°C anoxia, extending survival rate up to 24 hours for S2 and 48 hours for Rover and Sitter. Due to the effects of low temperature, no significant difference between Rover, Sitter and S2 was observed all the way up to 12 hours. At 18 and 24 hours, a trend was found in which Rover has the least survival rate to Sitter, and Sitter to S2, suggesting that S2's tolerance level after the anoxic stress is higher than Sitter and Rover. Conversely, at 48 hours, S2 seems to be less tolerant to stress exposure than Sitter and then Rovers, resulting in a survival rate of 0. Rovers and Sitter follow the same trend as 18 and 24 hours, Sitter had a greater survival rate than Rovers. No survival was observed at 72 hours for any PKG variation.

When juxtaposing the recovery and survival rates at 23°C, it was found that the flies that took less time to recover had the greater survival rate. At 1 hour drowning no

difference was observed for recovery time or survival. At 6 and 12 hours, S2 (-PKG) had the least time of recovery but the greater survival rate and Rover (+PKG) took the most time to recover and the lowest survival rate. At 3°C it was difficult to find a correlation between recovery time and survival rate. The only similarity between recovery time and survival was that no significant difference was observed at the 1st, 6th and 12th hour between the three PKG lines.

DISCUSSION

Determining how natural variations of the PKG pathway alter neuronal protection under anoxic conditions (Rover [+PKG], Sitter [-PKG], and S2 [-PKG]) taking temperature into account

As mentioned before, in previous studies, low PKG activity resulted in protection of function during anoxic stress, compromising survival rate (Wingrove & O'Farrell, 1999a) (Dawson-Scully et al., 2010). In this study, different results were observed. Protection of function was measured by the time it took the fly to recover from the anoxic coma. At room temperature, 1 hour anoxia resulted in no difference between the various PKG activities. This could be explained by the fact that the stress period was very short and that *Drosophila* are already anoxic tolerant insects, resulting in minimum stress imposed on them (Haddad, 2006). At 6 and 12 hours of anoxia, low PKG activity seems to protect function of the flies, which is exemplified by a faster recovery. These results can be correlated with a portion of the data presented by Dawson Scully et al., (2010), where time to anoxic coma onset is increased by low PKG activity, suggesting protection of function (Dawson-Scully et al., 2010). It was unexpected to find Sitter recovery and

not S2 at 18 hours of anoxia, since it was previously stated that S2 are more behaviorally protected to anoxic stress than Sitter and Rovers.

Under anoxic conditions, accumulation of extracellular K^+ occurs and clearance is reduced. Thus, there is a significant increase in extracellular K^+ that results in neuronal depolarization. At this point, there is positive feedback in which voltage dependent channels are activated, increasing neuronal activity, leading to even more accumulation of extracellular K^+ . This process leads to spreading depression or in this particular case the anoxic coma. When oxygen is restored, extracellular K^+ clearance is greater than accumulation, thus reducing extracellular K^+ , consequently inactivating voltage gated channels that result in even less accumulation of extracellular K^+ . This results in recovery from the coma. It is known that reduced PKG activity decreases extracellular K^+ accumulation. For this reason, S2 flies take less time to recover than Sitter and consequently Rovers (Rodgers et al., 2010).

The results for recovery time at cold temperature drowning shows again no difference between Rover, Sitter and S2 all the way up to 12 hours of anoxia. When comparing room temperature versus cold temperature, we observed that the time in which Rovers, Sitter and S2 did not show significant difference between each other was increased when temperature was lowered. This can be supported by the reasoning that low temperature lowers metabolism, consequently slowing down the effects of anoxia all the way up to 12 hours. At 18, 24, 48 and 72 hours, no pattern was observed for different PKG activities regarding recovery time. The reason for this is unclear and some suggestions are that anoxic stress is not the only influence, but also cold temperature imposes a stress that may be affecting different steps of the pathway. Another explanation

for this is the methodology used for room and cold temperatures. At room temperature, the flies are directly subjected to drowning anoxia, in which they enter the coma in a prolonged and natural manner. At cold temperature, flies are previously subjected to nitrogen anoxia, resulting in a more rapid and uniform entry into the coma and then, they are drowned. Since the cold temperature flies enter the coma at a much faster and less variable time regardless of PKG activity, their brains are equally protected, thus demonstrating no pattern in recovery time from the coma. Human error might also be involved in these results; factors that could occur were errors in the experiment, data recording and data analysis. Within the experimental error, some of the possibilities are that the different PKG lines were mixed or switched, different food and development conditions, air bubbles present at the time of drowning due to unclear view of the experiment because of the ice, among others.

In this study, unexpected findings were observed since low PKG activity resulted in increased percent survival after chronic anoxic stress. Based on previous experiments, high PKG activity is responsible for protecting survival after anoxic stress (Dawson-Scully et al., 2010) (Wingrove & O'Farrell, 1999a). As previously mentioned and supporting our results, low PKG activity is known to decrease extracellular K^+ accumulation. For this reason, it is possible that low PKG activity organisms spend less energy trying to maintain and restore ionic homeostasis, thus they have more energy to survive the stress (Rodgers et al., 2010).

Since no difference between Rover (+PKG), Sitter (-PKG) and S2 (-PKG) was observed for recovery time at room temperature for 1 hour drowning and at cold temperature at 1, 6, and 12 hours, these results were reflected in the survival rate. Again,

no difference was observed between Rover, Sitter and S2 at 1 hour drowning for room temperature and 1, 6, and 12 hours for cold temperature. This is also explained by the fact that the effects of anoxia does not impact the organism at a short timeframe since these organisms are anoxic tolerant. These timeframes increased when lowering the temperature due to a slowdown of the effects of anoxia (Haddad, 2006).

Regarding the relationship between function and survival, the results found in this study were unexpected. These results were surprising because previous findings suggested an inverse relationship between protection of function and survival, explained by the fact that low PKG activity protects neuronal function during hypoxia, letting the brain function with increased endurance under anoxic stress. Nonetheless, this protection negatively impacts survival (Dawson-Scully et al., 2010). On the other hand, our studies suggest a direct relationship between function and survival. Low PKG activity resulted in protection of function and increased survival, and the opposite happened for high PKG activity. These results indicate that low PKG activity protects the fly's function and survival. The difference between previous research and these studies are that protection of function was measured in different parameters. In the Dawson Scully et al., (2010) studies, function was measured by time of onset to coma, which means that the flies are under stress, but for our results, we measured function by the time of recovery from the coma, when stress was already ceased. The direct relationship found in this study is possibly because both measuring parameters were taken after the stress. However, a number of factors may have influenced our current study which will need verification to further understand why the Dawson-Scully et al. (2010) and Wingrove and O'Farrell (1999) study showed high PKG leads to greater survival under 6 hours of anoxia, where

our study shows that low PKG results in greater survival than low PKG: 1) Fly genetics need to be verified, 2) animal rearing conditions differed since animals were subjected to 24 hours of light rather than a 12:12 light dark cycle, and 3) we should verify our findings via pharmacology such as was done in Dawson-Scully et al., (2010). The bottom line for our results is that low PKG activity shows neuronal function protection that is reflected in a fast recovery from the coma and a greater survival (Dawson-Scully et al., 2010).

Figure 15. Rover, Sitter and S2 Recovery Time at 23°C Anoxia. Rover, Sitter and S2 flies were drowned for different periods of time (1h, 6h, 12h, 18h) at room temperature (23°C). No significant difference was observed at 1 hour (One-way ANOVA, $F_{(2,60)} = 49.885$, $p > 0.001$). At 6 and 12 hours, Rover flies took more time to recover than Sitter, and the S2 (6 hours Rover 9602.2 seconds, Sitter 8194.1 seconds, and S2 6821.8 seconds-12 hours Rover 27991.3 seconds, Sitter 19353.3 seconds, and S2 12070.08 seconds). At 18 hours, only Sitter flies recovered from anoxic stress.

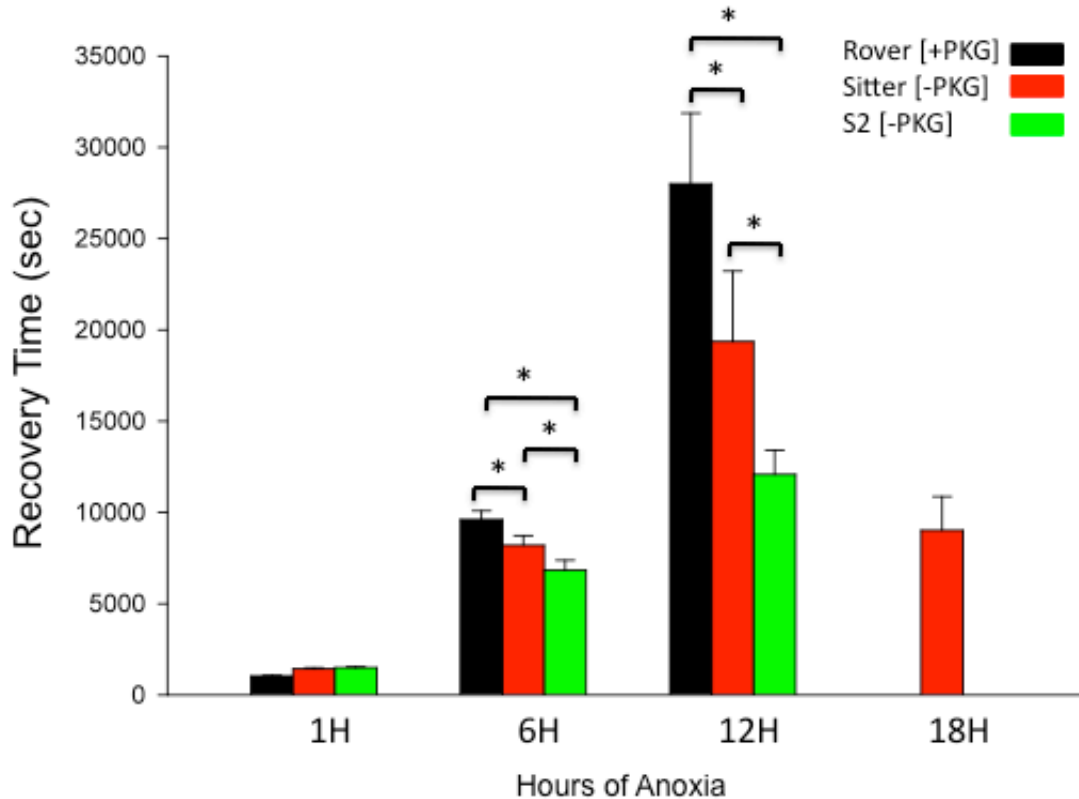


Figure 16. Rover, Sitter and S2 Recovery Time at 3°C Anoxia. Rover, Sitter and S2 flies were drowned for different periods of time (1h, 6h, 12h, 18h) at cold temperature (3°C). No significant difference was seen during the 1st, 6th and 12th hour (One-way ANOVA, $F_{(2,60)} = 65.22$, $p > 0.001$) (One-way ANOVA, $F_{(2,60)} = 49.885$, $p > 0.001$). One-way ANOVA, $F_{(2,60)} = 2.213$, $p = 0.331$. At 18 hours, Rover and S2 recovered at about the same time but faster than Sitter. Conversely at 24 and 48 hours, Rover and S2 took more time to recover than Sitter. Significant difference was found between Rover and Sitter, and Sitter and S2 for 24 and 48 hour. (One-way ANOVA, $F_{(2,27)} = 18.950$, $F_{(2,73)} = 18.950$, $F_{(2,68)} = 18.950$, $p < 0.001$). (One-way ANOVA, $F_{(2,24)} = 12.134$, $F_{(2,44)} = 12.134$, $F_{(2,16)} = 12.134$, $p = 0.002$). At 72 hours, similar recovery times were recorded for Rover and S2. No recovery was observed for Sitter.

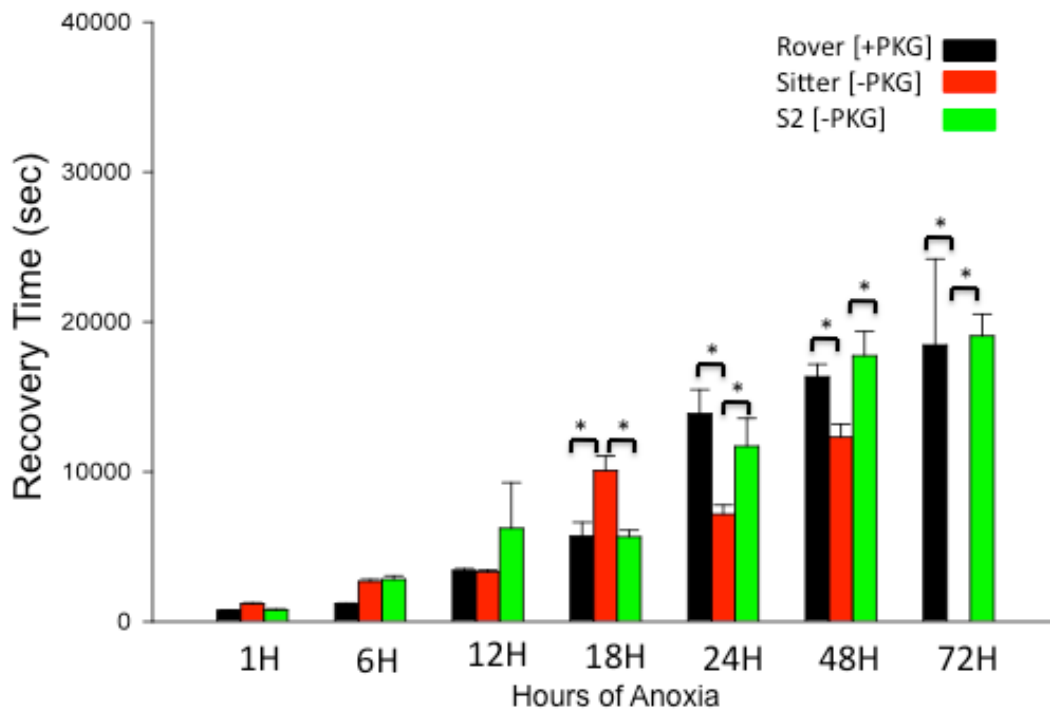


Figure 17. Rover, Sitter and S2 Survival Rate 24 Hours After. Percent survival was recorded 24 hours after anoxic stress. For this experiment, the flies were drowned at 23°C for periods of 1 hour, 6 hours, 12 hours, and 18 hours. At 1 hour drowning, Rover Sitter and S2 flies recovered around the same time. At 6 and 12 hours, Rover showed the least percent survival followed by Sitter and S2. Significant difference was observed at both durations of stress between Rover, Sitter and S2 (One-way ANOVA, $F_{(2,60)} = 13.322$, $F_{(2,60)} = 13.322$, $p=0.001$). (One-way ANOVA, $F_{(2,60)} = 6.750$, $F_{(2,60)} = 6.750$, $p=0.034$). There was only recovery observed for Sitter at 18 hours.

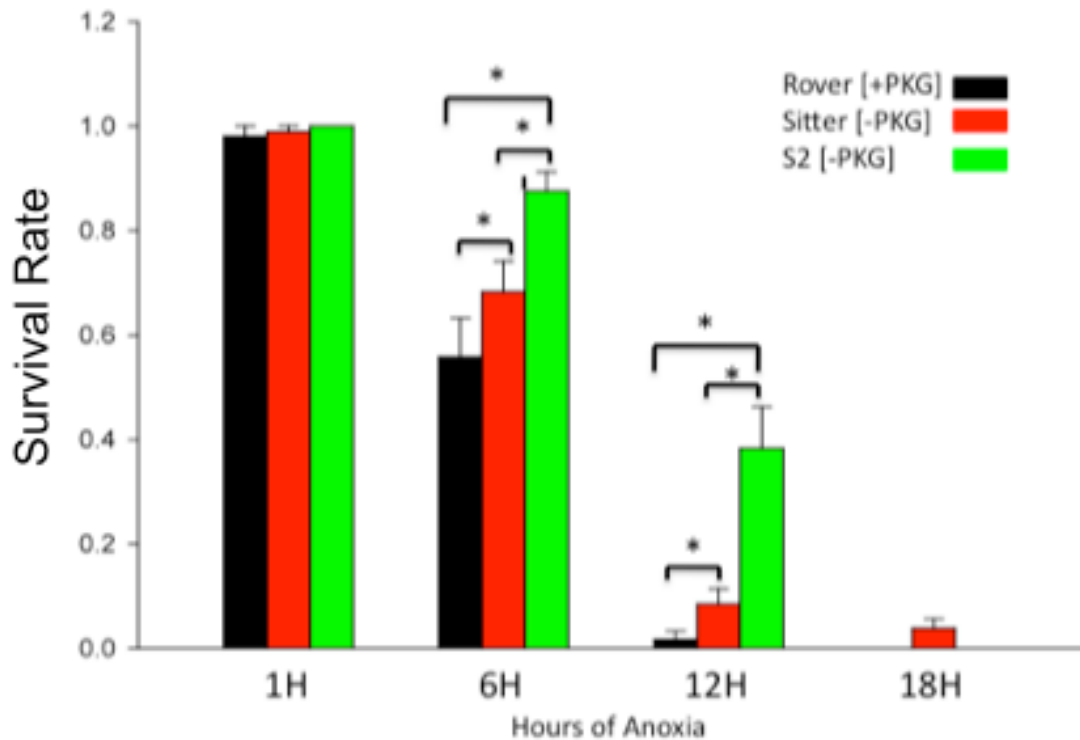
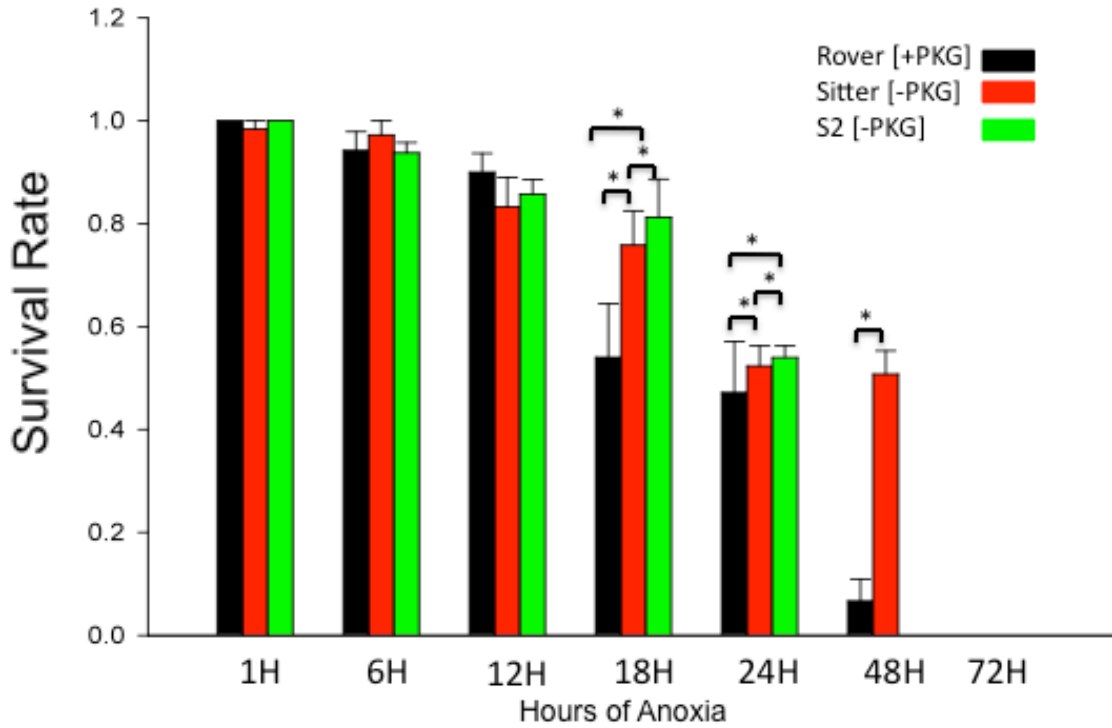


Figure 18. Rover, Sitter and S2 Survival Rate 24 Hours After. Percent survival was recorded 24 hours after anoxic stress. For this experiment, the flies were drowned at 3°C for different stress periods (1, 6, 12, 18, 24, 48, 72 hours). Similar and high percent survival was recorded for 1, 6, and 12 hour drowning. At 18 and 24 hours drowning, S2 demonstrated greater survival rate in comparison to Sitter and Rover subsequently. At 48 hours, the same trend was observed but only for Rovers and Sitter since S2 flies did not survive. No survival was observed at 72 hours of stress exposure.



CHAPTER V. INVESTIGATING THE BEHAVIORAL EFFECTS OF ANOXIA ON
RECOVERY AND SURVIVAL FROM AN ANOXIC COMA IN WILD TYPE (*W1118*)
DROSOPHILA MELANOGASTER TAKING INTO ACCOUNT INFLUENCING
VARIABLES

DISCUSSION

After performing our investigation on anoxic tolerance in *Drosophila melanogaster*, we demonstrated how the variables of time of stress, temperature and age affected tolerance to anoxia. This unprecedented combination of variables opens the doors to a world in which we can actually have a complete notion of how organisms are impacted by anoxia in nature. Thus, very interesting results were found.

Tolerance to anoxia is reduced with stress exposure. The longer the fly was under anoxia, the more time it took to recover, reducing the ability of the fly to survive the insult. This was observed in both the wet and dry anoxia. It was also found that even though the flies are tolerant to the stress, they reach a survival limit in which they can no longer withstand it. These findings can be explained with the principle of ionic homeostasis, specifically with the rise of extracellular K^+ , which is a consequence of stress exposure and its return to baseline levels (Rodgers et al., 2010). The rise was correlated to locomotory failure (coma) and the return to recovery time. The longer the fly was under anoxia, the larger the rise of extracellular K^+ , the longer it took the extracellular K^+ to return to baseline, thus the longer the fly it took to recover.

Low temperature significantly ameliorates the effects of anoxia. It was surprising to see how striking the influence temperature was on anoxic stress. At room temperature, flies survived up to 12 hours but at cold temperature, they were with no oxygen for 72 hours and still recovered and survived the insult. The low temperature great effect was not only observed in survival, but also in the considerable reduction of recovery time of the fly from anoxic coma. The effects of low temperature are reflected in the fact that metabolic rates as well as energy depletion slow down, at the same time there is a reduction of ROS and other metabolic products (Fleming et al., 1992). The rise of extracellular K^+ due to stress is also reduced, making the cells return ionic homeostasis faster, reducing the recovery time of the fly (Rodgers et al., 2010).

In addition, it was also observed how age plays a crucial role with regards to anoxic stress tolerance. The older the fly is, the more time it takes to recover and their survival rate is decreased, demonstrating that age reduces tolerance level to anoxia. It was striking to observe how the effects of age were reduced and almost disappeared once the temperature during the anoxic stress was lowered. It is known that oxidative stress influences lifespan, generally due to the accumulation of reactive oxygen species (Fleming et al., 1992). Reactive oxygen species increases along with age, and when exposed to stress conditions, it increases even more (Fleming et al., 1992). This accumulation results in decreased tolerance of the fly to anoxia. Since low temperature slows down metabolism, ROS accumulation due to stress is reduced, resulting in increased survival. The effects of temperature on recovery time are explained as mentioned before due to the loss of ionic homeostasis and its return (Rodgers et al., 2010).

Wet vs. dry anoxia both follow the same trends in terms of the time of exposure and temperature for recovery and survival. It was very interesting to find that recovery time between the two anoxic treatments at room temperature demonstrated no significant difference, while at cold temperature the flies subjected to the chamber always took more time to recover than the flies subjected to drowning. What was even more striking was with regards to survival, since the effects were reversed, and significant difference was observed at room temperature between the two treatments, while during the cold temperature no significant difference or any pattern was found. There is no clear explanation for these results, but it is possible that dryness is playing a role on decreasing anoxic stress tolerance.

There have been numerous studies regarding the recovery and survival of the *Drosophila* to anoxia. This investigation pushed the *Drosophila* even further, by understanding what were its limits of survival under stress, and at the same time analyzed how temperature, age and environment affected this investigation. These outcomes are the foundation for further investigation of other factors that can influence anoxic stress tolerance. As previously stated, the PKG pathway is highly involved in modulation of anoxic stress tolerance, thus demonstrating its importance on the focus of PKG.

Determining how natural variation of the PKG pathway alters neuronal protection under anoxic conditions (Rover [+PKG], Sitter [-PKG] and S2 [-PKG])

The results obtained when studying the influence of PKG on anoxic stress tolerance were completely unexpected, based on previous published data (Dawson-Scully et al., 2010). At room temperature, Rover flies (+PKG) took the longest recovery time and the lowest survival rate. At cold temperature, no pattern was observed between PKG

variations and recovery time but the lowest survival rate was also observed in Rovers. These results show that high PKG activity decreases tolerance to anoxic stress, seen in the reduction of survival and increased in recovery time.

Surprisingly, the opposite was found regarding the relationship between protection of function and survival, dictated by Dawson Scully et al., (2010). He explains that low PKG activity protects the fly when it is under anoxic stress, compromising survival after the stress. In our studies, we found a direct relationship in which low PKG activity protects the fly from anoxic stress for both behavior and survival (Dawson-Scully et al., 2010). This is observed by the fast recovery of the fly from the anoxic coma and the increased survival rate after the stress. One of the reasons why these results contradict each other is because in the Dawson Scully et al., (2010) paper, protection of function is measured when the fly is introduced in the stress environment (locomotory failure was measured), but in the case of our study locomotory recovery is measured when the fly is taken out of the stress environment. This means that we are measuring stress tolerance using completely different parameters.

In order to clarify the influence of PKG activity on anoxic stress tolerance, it is important to further manipulate the pathway pharmacologically and genetically. Nonetheless, these results are remarkable and insightful towards the achievement of striking findings in science pertaining to protection of the brain during anoxic stress.

FUTURE STUDIES

Since contradicting results were found between this data and previous studies, it is imperative to replicate the experiment on this past research. It is suggested to reproduce the experiments performed by Dawson Scully et al., (2010) but using the same PKG lines

tested in our studies. If results correlate with Dawson Scully et al., (2010) paper, it is an indication that something went wrong in our fly lines. Then the step to follow is to first collect new PKG lines and repeat all our experiments. This way we will rule out any possible genetic and development variation. At the same time, it is important to repeat our experiments with new PKG lines to rule out any human and experimental error.

Molecular studies including genetic or pharmacological manipulation targeting each player of the PKG pathway will dissect the specific component that influences anoxic stress tolerance. At the same time, manipulation of upstream and downstream factors of the PKG pathway will allow us to understand its relationship between this pathway and other processes. Also, it should be interesting to knock down PKG activity using tools such as RNAi in specific tissues.

Electrophysiological studies will shed light in order to understand what is happening in the brain at a more specific approach. The technique of Giant fiber system recording is a great model to find this information. The Giant fiber System is a well studied neuronal circuit that mediates the escape response of the *Drosophila melanogaster* (Allen & Godenschwege, 2010). For this technique, the neurons are stimulated directly in the brain and the response is recorded from the tergotrochanteral jump muscle (TTM) and dorsal longitudinal fly muscle (DLM). The use of this technique will explain how synaptic transmission responds to anoxic stress, specifically how synaptic transmission recovers from the anoxic coma. It has been previously shown that behavioral responses under N₂ anoxia correlates with the electrophysiological changes (Haddad et al., 1997). The longer the fly is under anoxia, the longer it takes the flies to

recover behaviorally and physiologically. Adding to this, it will now be interesting to test the variables of PKG activity, temperature and age using this protocol.

Lastly, for a better understanding of cell survival after anoxic stress, it will be of interest to chemically induce anoxia in neuronal cell culture, including all the variables taken into account for this study. This will explain a specific relation between PKG activity and neuronal cell death that will inform us if our results were correct regarding survival.

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