ABSTRACT

THE ACUTE EFFECTS OF AN ENVIRONMENTAL NEUROTOXIN L-BMAA ON WALKING BEHAVIOR OF *DROSOPHILA MELANOGASTER* – A MODEL TO STUDY NEURODEGENERATIVE DISEASES

Humans who have been exposed to Beta-methylamino-L-alanine (L-BMAA) developed amyotrophic lateral sclerosis Parkinson-dementia complex, (ALS-PDC). The structure of L-BMAA is similar to glutamate, making it a glutamate agonist. Since glutamate is an excitatory neurotransmitter in both insects and vertebrates, *Drosophila melanogaster* is an ideal model to study the effects of L-BMAA toxicity. Previous studies on fruit flies that ingested L-BMAA showed heterogeneous walking behaviors–some flies were more affected.

The research outlined in this thesis studied the acute effects of L-BMAA on the locomotory behaviors of fruit flies (*Canton S.)* when injected with L-BMAA. The study focused on three main goals: (1) test whether injection is a better method of delivering the toxin than ingestion because it might reduce heterogeneity, (2) quantify the acute effects of injecting L-BMAA on locomotory behavior compared with the effects of more prolonged exposure by ingestion, and (3) search for evidence of sequestration or other coping mechanisms by monitoring flies for evidence for transient effects. Comparison of the two introduction routes resulted in no difference in the observed behavioral changes. Fruit flies injected with L-BMAA did not show any observable transient effects. However, this thesis project was able to shed light on the possibility of a sequestering mechanism in fruit flies to control for L-BMAA intoxication.

Athena Goodarzi
August 2012

THE ACUTE EFFECTS OF AN ENVIRONMENTAL NEUROTOXIN L-BMAA ON WALKING BEHAVIOR OF *DROSOPHILA MELANOGASTER* – A MODEL TO STUDY NEURODEGENERATIVE DISEASES

by

Athena Goodarzi

A thesis

submitted in partial

fulfillment of the requirements for the degree of

Master of Science in Biology

in the College of Science and Mathematics

California State University, Fresno

August 2012

APPROVED

For the Department of Biology:

We, the undersigned, certify that the thesis of the following student meets the required standards of scholarship, format, and style of the university and the student's graduate degree program for the awarding of the master's degree.

Athena Goodarzi
Thesis Author

Ulrike K. Muller (Chair) Biology

John V.H. Constable Biology

Joy J. Goto Biology

For the University Graduate Committee:

Dean, Division of Graduate Studies

AUTHORIZATION FOR REPRODUCTION

OF MASTER'S THESIS

X I grant permission for the reproduction of this thesis in part or in its entirety without further authorization from me, on the condition that the person or agency requesting reproduction absorbs the cost and provides proper acknowledgment of authorship.

_____ Permission to reproduce this thesis in part or in its entirety must be obtained from me.

Signature of thesis author:_____

ACKNOWLEDGMENTS

This thesis was supported by the Natural Science Foundation through a grant to Dr. Ulrike K. Müller (DBI-0821820). I am extremely grateful and indebted to Dr. Ulrike K. Müller, my research advisor and mentor, for her confidence and trust in me. I especially acknowledge her expert, sincere, and valuable guidance and encouragement extended to me. Thanks also go to my committee members, Dr. Joy. J Goto and Dr. John V.H. Constable. Dr. Goto provided me with necessary facilities and her expert guidance. Thanks to Dr. Constable for his insightful comments on my thesis and his guidance as my graduate coordinator. I would also like to take this opportunity to thank Nalong T. Mekdara, my friend and colleague, for his unceasing support and tremendous help, as well as his continuous encouragement. I also thank Prasong J. Mekdara for his unconditional help. Lastly, I would like to express my gratitude to my family for their endless love and support.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

ALS – A Tragic Neurodegenerative Syndrome Looking for a Cure

Neurodegeneration is the progressive loss of structure and function of neurons, which ultimately causes neuronal demise (Lobner et al., 2007; Edwards and Meinertzhagen, 2010). Common neurodegenerative diseases include Parkinson's (PD), Alzheimer's (AD), and Multiple Sclerosis (MS, and Amyotrophic Lateral Sclerosis (ALS). Neurodegenerative diseases all result in neuronal cell death (Koo and Kopan, 2004). What causes this cell death is often unknown; as a result neurodegenerative diseases are frequently defined by their symptoms (cell death) rather than their cause, making them syndromes rather than diseases (Cox et al., 2003; Karamyan and Speth, 2008). Research into the possible causes of neurodegeneration focuses on two main types of causes: genetic and sporadic causes. Sporadic causes are by definition non-genetic and include environmental factors, diet, and lifestyle.

Amyotrophic lateral sclerosis syndrome (ALS), also referred to as Lou Gehrig's disease, is the most common form of motor neuron disease (Guiloff and Goonetilleke, '95; Del Aguila et al., 2003). The cause of this disease has remained unknown; epidemiological studies show that 90 to 95% of the cases are sporadic (Costa et al., 2010), implicating environmental causes (diet, life style, etc.). ALS has a high incident rate worldwide with 2-3 new cases out of 100,000 people (NTP-NIEH report, 2008). However the occurrence of ALS in the United States is higher compared with other countries- 5 per 100,000 of the total population (Majoor-Krakauer et al., 2003). Patients diagnosed with ALS initially manifest muscle weakness, often in their arms and hands. The disease progresses rapidly, leading to complete paralysis and death due to respiratory failure (Cardoso et al.,

2002). ALS has no known cure; the disease inevitably leads to death within 2 to 3 years after the initial diagnosis (Cardoso et al., 2002).

ALS – A Case Study on a Small Island Points the Finger at an Environmental Neurotoxin

Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex (ALS/PDC) is a part of the ALS syndrome family that has an incidence rate of 50-100 times higher (Cox et al., 2003) among the Chamorro people of Guam compared with incidence rates of ALS elsewhere (Spencer et al., '86). As genetic factors do not appear to be causal, researchers have focused on diet as a possible cause (Cox et al., 2003).

The Chamorro people of Guam had two unique staples in their daily diet: flour made from the seeds of cycad palms and flying foxes, which also ate the cycad palm seeds (Murch et al., 2004). The most commonly consumed cycad species was *Cycas circinalis* (NTP/NIEH report, 2008), and studies showed that its seeds contained significant amounts of Beta-N-methylamino-L-alanine (L-BMAA) (1-9 μg/g) (Cox et al., 2003). L-BMAA is produced by root-symbiotic *Nostoc* species of cyanobacteria in the cycad palm (NTP/NIEH report, 2008). Uniqueness of L-BMAA in the diet was hypothesized to be the cause of ALS /PDC in Guam (Duncan, '90), yet this hypothesis was rejected by the scientific community (Karamyan and Speth, 2008). Later studies showed that L-BMAA is bio-magnified in the local food chain in Guam: flying foxes contain up to 400 times more L-BMAA than the cycad seeds (9 versus 3556 μg/g) (Cox et al., 2003). These findings revived the hypothesis that ALS/PDC is caused by L-BMAA intoxication (Cox et al., 2003). New studies of ALS/PDC stated that L-BMAA was present in the brain tissues of Chamorros in Guam who died of this neurodegenerative disease (7 μg/g; Cox et al., 2003); however L-BMAA was not

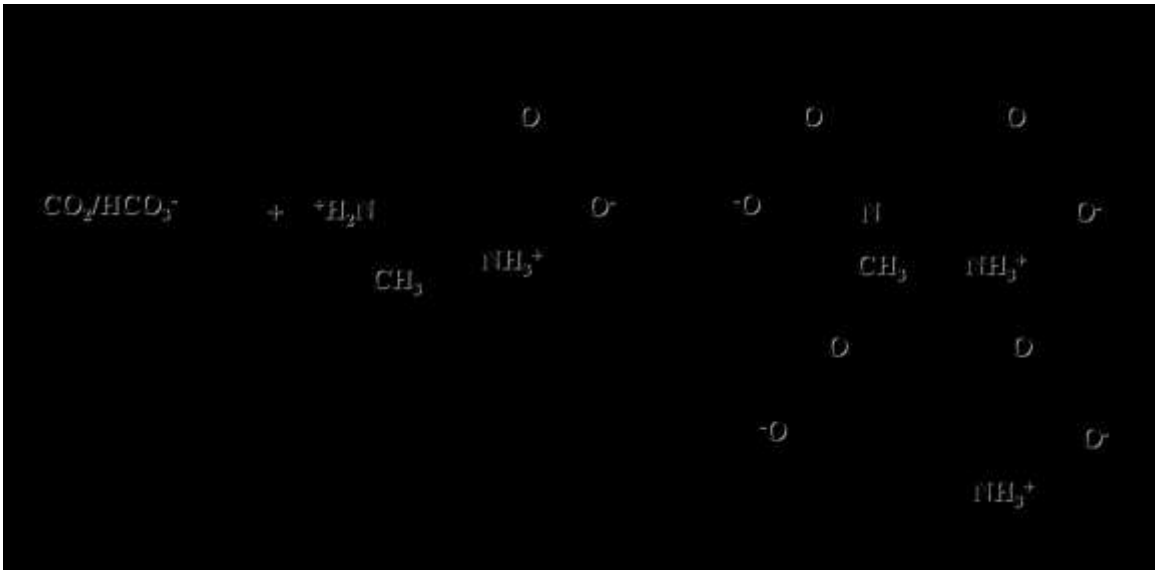
present in brain samples of Chamorros in Canada who died of causes unrelated to neurodegenerative diseases (Murch et al., 2004).

However, not just the Nostoc genus produces this neurotoxin, but also many other marine and fresh water cyanobacteria (Banack et al., 2007; Brand et al., 2010). This suggests that L-BMAA intoxication due to chronic L-BMAA exposure is not just localized on the island of Guam. Over the past 60 years, the number of cases of L-BMAA intoxication has been rising globally due to increased presence of cyanobacteria colonies in oceans, lakes, rivers and streams (Banack, 2010). Large algal blooms are usually present only in tropical and subtropical regions, but with the increase in global temperatures they have been showing up in temperate regions (Jonasson et al., 2010). Studies conducted in south Florida have shown that presence of L-BMAA causes death and disease in both terrestrial and aquatic animals around coastal regions in Florida (Banack, 2010; Brand et al., 2010).

L-BMAA has been linked not only to ALS-PDC, but recent studies suggest that it might also be related to sporadic incidences of Alzheimer's and Parkinson's disease (Brand et al., 2010). However, because neurodegenerative diseases have a long latency period, studies examining their direct causes are often controversial (Becker and Meier, 2009). With global climate change promoting algal blooms, and with scientific studies suggesting a link between chronic exposure to L-BMAA and neurodegenerative diseases, governmental funding agencies are encouraging research into the mechanisms of L-BMAA intoxication in order to develop preventive measures and treatments (NTP-NIEH report, 2008).

L-BMAA – A Glutamate Agonist Might Act as a Neurotoxin

L-BMAA becomes a glutamate agonist when it is carbamylated in the presence of carbon dioxide or bicarbonate. Carbamylated L-BMAA binds to glutamate receptors in the nervous system and causes stimulation (Weiss et al., '89; Brand et al., 2010). An overstimulation causes degradation of neuronal cells leading to neurodegenerative diseases in both the mammalian (Choi et al., '87) and insect (Feaney and Bender, 2000) models.



L-Glutamate is the most abundant endogenous amino acid in the mammalian central nervous system (McEntee and Crook, '93). Being a major excitatory neurotransmitter, L-Glutamate is involved in many aspects of normal brain functions including cognition, memory and learning (Danbolt, 2001). However, at concentrations of 50-100 μM (2 to 5 times normal) L-Glutamate can become neurotoxic in mammals (Choi et al., '87). According to Lipton and Rosenberg 1994, overstimulation of glutamate receptors might contribute to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ALS. Mammals have two different types of glutamate receptors: ionotopic (iGluR)

and metabotropic (mGluR) (Ozawa et al., ‘98). Ionotropic glutamate receptors are excitatory and involved with locomotor responses (Ozawa et al., ‘98); metabotropic glutamate receptors are principally used for learning and memory activities (McEntee and Crook, ‘93).

L-BMAA-related ALS – Finding an Animal Model

One of the goals of this project was to develop an animal model that can be used to examine the causal mechanism for neurodegenerative diseases such as ALS, which might be influenced by L-BMAA neurotoxicity. Since ALS is a progressive neurodegenerative disease, mammalian models such as mice and rats (Karamyan and Speth, 2007) may take years to develop symptoms, slowing our understanding of the disease process. In the study of late-onset neurodegenerative diseases, *Drosophila* has become a more suitable model due to its short lifespan of 60 to 120 days, which makes one “fly day” roughly equivalent to one human year resulting in more rapid and economical studies.

Glutamate is an important neurotransmitter both in vertebrates and invertebrates, yet the role it plays is different. In insects, glutamate is the main excitatory neurotransmitter at the neuromuscular junctions (Jan and Jan, ‘76). In vertebrates, glutamate is mainly a neurotransmitter in the central nervous system. In the context of this study the key component is that both vertebrate and invertebrate ionotropic glutamate receptors have similar protein structures (Marrus et al., 2004).

Acute Effects of L-BMAA – Understanding Our Insect Model

The fruit fly has glutamate receptors in both the central and peripheral nervous system (Usherwood, ‘94). Within the central nervous system the central

complex, consisting of the central pattern generator (CPG), controls activity parameters such as stepping frequency, walking bout frequency and bout duration that determines walking speed through glutamate receptors in central pattern generator (Strauss and Heisenberg, ‘93). The central pattern generator controls stepping frequency, which in turn determines walking speed; insects change walking speed mainly by adjusting stepping frequency, not step length (Strauss and Heisenberg, ‘93; Martin et al., ‘99). Studies on the central pattern generator suggest that it controls activity periods (Featherstone et al., 2005), but not inactivity periods (Martin, 2004). According to Martin, over-stimulation of the central complex should increase walking activity by increasing walking bout frequency without increasing walking bout duration and should also increase the likelihood of initiating a walking bout.

OBJECTIVES

Acute Effects of BMAA – Improving Our Experimental Design

A natural way to introduce L-BMAA to flies is through food as seen in the Guam incident. A joint study conducted between two laboratories (Dr. Goto's and Dr. Muller's laboratories) (Mekdara et al., 2012) examined the possible effects of over-excitation of glutamate receptors in both central nervous system (CNS) and peripheral nervous system (PNS) by ingestion of L-BMAA. That study documented that low doses of L-BMAA cause hyperactivity, high doses and prolonged food uptake of low doses cause significant loss of motor ability; however among individuals the study observed variations in walking ability, particularly variation in walking speed, walking bout frequency, and total distance of walking. A possible explanation for this heterogeneity within the treatment groups is differences in the amount of L-BMAA ingested: while some flies ingested sufficient toxin to develop severe loss of motor ability, others might have consumed less toxin and therefore showed only mild symptoms.

Other published studies conducted on vertebrate and invertebrate models have shown similar variability in results. Brand et al. (2007) examined different blooms of cyanobacteria in South Florida, and the L-BMAA content of animals living in those areas. They found considerable variation in L-BMAA concentrations in muscle tissues of deceased individuals. They presented a range of hypotheses to account for their results, including "differential uptake and excretion" rates among studied animals. They also found variation in survival rate, which also can be due to different levels of toxin exposure, time of exposure, and amount consumed by the individual. These observations suggest that L-BMAA

intoxication is affected by level of concentrations present in tissue samples (Cardoso et al., 2003; Brand et al., 2007).

To avoid any differential uptake and excretion experienced by individual flies, one of the aims of this project was to introduce a consistent amount of L-BMAA through abdominal injection. The expected outcome was that fruit flies injected with a known amount of L-BMAA experience similar locomotor dysfunction as the flies that ingest the toxin, but with less variation in behavior. Furthermore, by injecting a known amount of BMAA directly into the hemolymph, the behavioral effects of injected L-BMAA can be compared with the behavioral dose effects seen in flies that ingested three different concentrations of the toxin. This comparison might be able to predict how much of the ingested L-BMAA makes it from the intestines into the flies' hemolymph. To explore the acute effects of L-BMAA on *Drosophila*, this thesis project looked at a new route for administering the toxin.

Acute Effects of L-BMAA – Improving Our Understanding of L-BMAA as a Glutamate Agonist

The hemolymph of fruit flies is known to contain relatively high levels of glutamate (McDonald, '75, Augustin et al., 2007). Exposure of neurons to glutamate concentrations above normal can cause neuron degradation and decreased life expectancy (Edwards and Meinertzhagen, 2010). Fruit flies have an excitatory amino acid transporter, dEAAT1 that removes excess glutamate from the neuron extracellular matrix (Besson et al., 2000; Chen et al., 2009). Information on the function of this transporter in the presence of L-BMAA is unknown. Administering L-BMAA through injection should result in an acute response if the dEAAT1 transporter is responsive. Within our research team we

had pilot data from decapitated fruit flies suggesting that the flies exhibit transient effects to acute L-BMAA exposure, mainly severe loss of motor ability. Within minutes flies exposed to L-BMAA started to regain motor control of the effects and even started to walk. However, flies that had just been decapitated without any exposure had no locomotor activity. This suggests that L-BMAA stimulates the central nervous system, which results in walking of decapitated fruit flies. To advance this research, my project injected flies to check for transient effects that might tell us more about possible (extracellular) sequestration mechanisms.

HYPOTHESES & PREDICTIONS

Injection Effects (Saline and 50mM L-BMAA)

Question 1. Does injecting BMAA affect the walking behavior of fruit flies? Are these effects due to L-BMAA or due to artifacts induced by the physical trauma of injecting?

Hypotheses 1.1. Injection of saline will not cause any change in the walking behavior.

Prediction 1.1.1. If flies are injected with saline, their walking behavior is not significantly different from uninjected flies.

Hypotheses 1.2. Injecting L-BMAA will increase the walking speed and walking activity of fruit flies relative to saline injected flies as L-BMAA stimulates the CPG.

Prediction 1.2.1. If L-BMAA acts as a glutamate agonist in the Central Complex, then injecting L-BMAA should increase walking speed compared with controls.

Prediction 1.2.2. If L-BMAA acts as a glutamate agonist in the Central Complex, then injecting L-BMAA should increase walking activity (e.g., bout frequency, total time spent active) to increase compared with controls.

Prediction 1.2.3. If L-BMAA acts as a glutamate agonist in the Central Complex, then injecting L-BMAA should affect only walking bout frequency, but not walking bout duration.

Hypotheses 1.3. Injecting L-BMAA will increase the stumble frequency and decrease the walking speed of fruit flies because L-BMAA causes loss of motor ability by over-stimulating the NMJs.

Prediction 1.3.1. If L-BMAA acts as a glutamate agonist in the neuromuscular junctions, then injecting L-BMAA acts as a glutamate agonist in the neuromuscular junctions, then injecting L-BMAA should cause walking speed to decrease compared with controls.

Comparison of the Data for Injected vs. Ingested L-BMAA

Question 2. Does injecting L-BMAA reduce the variability in the response of the fruit flies to the toxin?

Hypothesis 2. The variation in injected fruit flies is less than in fruit flies ingesting L-BMAA.

Prediction 2.1. If differences in feeding rate and therefore differences in the total amount of toxin taken up by the flies are the main cause for this heterogeneity in locomotor activity (more variation than can be explained by chance), then injected flies should have a less heterogeneity in all quantified performance parameters.

Question 3. Do the flies respond to a lower injected dose with smaller changes in locomotor performance parameters?

Hypothesis 3.1. Injected fruit flies will show less severe behavioral changes than fruit flies that ingested the toxin because the ingested dose is

between 50% and 300% higher than the injected dose. This hypothesis makes three assumptions. First, the response of the flies is dose dependent. Second, flies have no mechanisms to remove or otherwise disarm the toxin so that any toxin added to the fly will remain active for the entire duration of the experiment. Third, ingestion and injection of the same dose of toxin results in the same behavioral effects.

Predictions concerning the effects on the Central Complex.

Prediction 3.1.1. If injecting lower doses of toxin causes less severe behavioral effects as ingesting, then the flies that ingested the toxin should show a significantly greater increase in walking speed than injected flies.

Prediction 3.1.2. If injecting lower doses of toxin causes less severe behavioral effects as ingesting, then the flies that ingested the toxin should show a significantly greater increase in walking activity than injected flies.

Prediction 3.1.3. If injecting lower doses of toxin causes less severe behavioral effects as ingesting, then the flies that ingested the toxin should show a significantly greater increase in bout frequency, but bout duration should be unaffected.

Hypothesis 3.2. Injected fruit flies will show less severe behavioral changes than fruit flies that ingested the toxin in stumbles and walking speed because the injected dose is between less than the ingested dose.

Predictions concerning the effects on the neuromuscular junctions

Prediction 3.2.1. If injecting lower doses of toxin causes less severe behavioral effects as ingesting, then the flies that ingested the toxin should show a significantly greater increase in stumble frequency than injected flies.

Prediction 3.2.2. If injecting lower doses of toxin causes less severe behavioral effects as ingesting, then the flies that ingested the toxin should show a significantly stronger decrease in walking speed than injected flies.

Transient Effects of Injected L-BMAA

Question 4. Can we observe not only an acute onset of the toxic effects, but later a gradual decline in the strength of the response? We know for example that flies can sequester excess glutamate (Besson et al., 2000; Chen et al., 2009), and that they excrete toxins and osmolytes at a rate of 10.4 mM / L $-^{1}h^{-1}$ (Etienne et al., 2001).

Hypotheses 4.1. The sequestering mechanisms of fruit flies can remove sufficient amounts of L-BMAA within 50 minutes, which causes less severe toxic effects towards the end of the observation period.

Prediction 4.1.1. If the sequestering mechanisms can cope with L-BMAA intoxication, then stumble frequency will be less towards the end of the observation period compared with the beginning or middle of the observation period.

METHOD

Insects

Experiments were performed on the laboratory strain of *Drosophila melanogaster* using only virgin females to eliminate more complex social behaviors, such as mating and aggression. Flies were raised on a glucose-based food gel composed of 6% glucose, 3% sucrose, 1% yeast, 1% agar, water, and green food color at room temperature. To collect age-matched flies for the experiments, bottles of *D. melanogaster* were emptied of any adults with only larvae and pupae remaining. The cultures were then placed in an 18°C incubator for 18 hours to avoid mating among emerging flies. After the 24-hour period, adult flies were anesthetized with CO_2 and sorted by gender. The selected females were placed in food vials and were kept for two days in an incubator at 22°C with a 12:12 light:dark cycle. The flies were removed from the vials for experiments approximately three days after eclosion, to ensure that sufficient time had passed between CO_2 anesthesia and injection to return the CO_2 levels in the flies back to normal. Each experiment used two body size matched flies, which were selected immediately prior to the experiment to be matched in body size. A total of 32 flies were used for 16 experiments.

Treatment: L-BMAA

To determine the effects of L-BMAA on the insect nervous system, 0.2 μL of a 50 mM BMAA solution (1.18 mg L-BMAA: 1 mg body weight of fruit fly) was injected in the fly's abdomen. The control flies were injected with 0.2 μL of an isotonic saline solution containing no L-BMAA. The saline solution was made by mixing 0.129 M NaCl, 4 mM KCl, 2 mM CaCl2, 35 mM Tris HCl to 30 mL of DI water. A 500 mM solution of L-BMAA was made by mixing 0.0022g of

BMAA and 28 μL of saline solution. Serial dilution was made from the 500 mM stock solution: 50 mM = 1:10 ratios. For the injections, 40 injection needles were pulled from 1-5 μL micropipettes (Drummond Scientific Company). To pull the needle, a P-97 micropipette puller (Sutter Instruments Co.) was used at the following arbitrary instrument settings: pressure = 500, heat = 515, velocity = 60 and time = 200. For each experiment, two needles were loaded with 0.2 μL of the two solutions using capillary action and stored in closed Petri dishes to safely transport the needles for up to one minute before the actual injections. To transfer the 0.2 μl of L-BMAA into the injection needle without apparent loss, the needle was inserted into the opening of the pipette tip and all fluid was transferred into the injection needle by capillary action. All injection needles were inspected visually to ensure that they contained the same amount of fluid (meniscus of the fluid was 2±0.5 mm from the first marker on the capillary).

To prepare the flies for injection, the flies were anesthetized by putting them in a vial and then inside an ice bath for 24 seconds and transferred to an ice plate underneath a dissecting microscope. Flies were injected with the aid of a dissection microscope (Olympus). The two flies were injected within a 40 second period; each fly took 7 seconds to inject and 10 seconds to transfer it into the filming arena. Each fly was filmed in its own lenticular-arena to quantify their walking ability (Lenticular arena: Mekdara et al., 2012).

General Filming Protocol

The lenticular-arena assay was used to assess the flies' walking ability. The arena had a lenticular concave shaped floor with a flat top. The diameter of the arena was 75 mm, the height of the arena at the deepest point in the center of the arena was 8 mm. The incline of the floor was 0 ° in the middle and 15 ° at the

edge. The floor of the arena was made from a watch glass (75 mm diameter, Catalog number 02-610C, Fisher Scientific) that was glued inside a PVC plumbing adapter (NIBCO 3″ × 1-½″ reducer bushing, model number 5801-2F). The arena was painted in matte black and a circular flat anti reflection cover glass (Edmund Optics) was used to keep the flies in the arena. To record two treatment groups simultaneously (one control and one treatment) one arena was placed under one camera. A hemispherical plastic diffuser was used to cover each arena. The diffuser had an opening at the top to let the camera view the arena. The arena was lit through eight red LEDs (Luxeon Star with collimating optics, 1 W, wavelength 660 nm) on the outside of the diffuser.

The walking behavior was recorded starting at 14:00 hr. using two Photron PCI high-speed cameras set at a frame rate of 10 Hz with a 50 mm lens (Carl Zeiss, Planar T 1.4 operating at f/5.6). Each camera was placed directly above a walking arena, recording for 45 minutes at a resolution of 512 by 512 pixels. One filming set up was recording one L-BMAA treatment of 50 mM and second camera was recording one control experiment of injection with saline solution.

Data Analysis

Both manual and automated tracking were used to quantify the change in walking behavior of treated flies. Ctrax (Branson et al., 2009) software was used to automatically track the x and y position of the flies for each frame recorded. The gross position of the fly (floor versus ceiling of the arena) was scored manually because the automated tracking software could only distinguish flies moving in the z axis. So manual scoring, using excel spreadsheet involved recording the number of the frames in which a fly changes position from floor to ceiling or *vice versa*.

The manual and automatic position data were used to compute the following parameters for each fly: (1) instantaneous walking speed, (2) duration of walking bouts, (3) number of walking bouts, (4) number of stumbling events (the fly loses its footing and rolls towards the center of the arena), (5) total distance walked in 45 minutes.

A custom Matlab routine, **executeFruitFlyStatsEngine.m,** was used to determine walking speed (mm/s), bout frequency (count value), bout duration (s), time spent active(% of time spent walking), and stumbles (count value). The program determined walking behaviors through confined conditions:

1. It calculated *walking speed* by dividing the distance traveled, mm, by the time spent in a walking episode (s).
2. For a *walking bout* to qualify as a true bout, it had to meet the following two conditions: the fly must be in motion for at least 5 frames and have a minimum average walking speed of 2 mm/seconds. A walking bout was considered as terminated if the fly was stationary for 5 frames.
3. To determine *stumbles*, the program detected a sudden change in speed that must be greater than +/- 4 standard deviations from the original walking speed. The frame of the stumbling episode was then removed from the walking bout; if the fly continued walking after the stumble, then the two walking bouts were patched together, if the fly stopped for at least 5 frames, the walking bout was counted as ending at the stumble.
4. From the raw data, *walking bout duration* was determined by dividing the average walking bout duration (calculated in frames) by 10 frames/second.
5. *Time spent active* was calculated by determining the sum of all walking bout durations and dividing it by the total number of recorded frames

(=26111 frames) to get % time active during 45 minutes of film. Refer to Appendix IV for program code.

Statistical Analysis

All treatments (injected and ingested) were subjected to tests of normality by using Kolmogorov-Smirnova and Shapiro-Wilk tests along with an N-N (linear distribution) test. For randomness of the sample a Q-Q (quintile) test was used. Skewness and Kurtosis tests were also used to assess the distribution of the means. Significance between treatment groups were assessed with both parametric and non-parametric analyses. To determine how different injected and ingested groups were from control groups, test for heterogeneity, a standard t-test with Levin's homogeneity of variance test was used. Data processing and statistical analyses were carried out using Matlab and SPSS (SPSS, Inc., Chicago, IL).

Parametric analysis: Analysis of variance (ANOVA) was used to determine statistical significance of the sample. ANOVA makes three assumptions: (1) the distribution of the data must be normal and random; (2) the distribution must display homogeneity (Levin's Test); (3) the distribution must be independent and fit a linear-additive model. Unfortunately, the data gathered for both injected and ingested L-BMAA violated ANOVAs' assumption of homogeneity of variance. However, F-test ANOVA with post-hoc test was still used for all experiments. Post-hoc tests included Tukey's HSD, Games-Howell, Dunnet T-3, and Scheffe test.

Non-parametric analysis: Non-parametric statistics still retains the assumption that distribution must be independent and fit a linear-additive model. The data points for testing the effects of L-BMAA comparing injected and ingested data did not normally distribute, it could not be transformed using logarithmic or power

transformations and violated the assumptions required for a parametric test (ANOVA). Kruskal-Wallis non-parametric ANOVA with pair-wise comparison (Mann-Whitney U) was used to assess whether there was a difference between injected and ingested groups.

T-Test: The standard t-test compares one group to another, and makes two assumptions: (1) the distribution of each group in normal, (2) the two groups are independent of each other. To determine the level of heterogeneity, each treatment group (injected and ingested) were compared with the saline control groups using Levin's test to test the level of differences – the two control groups were not statically different.

RESULTS

Question 1

Research question 1 follows: Does Injecting L-BMAA Affect the Walking Behavior of Fruit Flies? Are These Effects Due to L-BMAA or Due to Artifacts Induced by the Physical Trauma of Injecting?

Addressing Hypothesis 1.1. Uninjected flies did not differ significantly in any of the behavioral parameters from flies injected with saline. So injection itself does not cause any observable changes in locomotion. There were also no significant differences between the control flies of the injection experiments and the control flies of the ingestion experiments (ingestion data gathered by Nalong Mekdara). (Prediction 1.1.1; tables 1 A-E, refer to Figures 1-5 for distribution).

Addressing Hypothesis 1.2. Contrary to our predictions, injection of L-BMAA did not increase walking speed and activity. Instead, injection of the 50 mM L-BMAA resulted in a significant decrease in walking speed compared with the control flies (Prediction 1.2.1; table 1D) and no significant increase in walking activity (Prediction 1.2.2; table 1C). However, prediction 1.2.2. concerning bout frequency was confirmed - injection of 50 mM L-BMAA, resulted in a significant increase in bout frequency (Prediction 1.2.2; table 1A). Prediction 1.2.3 was also confirmed - flies injected with 50 mM L-BMAA did not differ significantly from the control flies in bout duration (Prediction 1.2.3; table 1B & Figure 4).

Addressing Hypothesis 1.3. Flies injected with 50 mM L-BMAA showed a significant increase in number of stumbles per 10 minutes compared with the control groups (Prediction 1.3.1; table 1E & Figure 5). Injected flies also showed a significant drop in walking speed, confirming both prediction 1.3.1. and prediction 1.3.2 The trend in walking speed contradicted the prediction about

walking speed effects being dominated by CNS effects; instead it is dominated by NMJ effects.

Question 2

Research question 2 follows: Does injecting L-BMAA reduce the variability in the response of the fruit flies to the toxin?

Addressing Hypothesis 2. Heterogeneity of variance within treatment groups was determined for all walking parameters using an independent sample t-test. Flies that were injected with 50 mM of L-BMAA were not consistently more homogeneous than flies that ingested L-BMAA, which contradicts prediction 2.1. Of the five metrics, bout duration and stumble frequency were the only two that were homogeneous for all four treatment groups. In the other three metrics, at least one of the four treatment groups was heterogeneous. Overall, the data sets for injected flies were homogeneous in 3 out of 5 metrics. As for the data sets for flies that ingested L-BMAA, 3-4 out of 5 metrics were homogeneous. This showed that injecting did not reduce heterogeneity of variance compared ingestion as the delivery mode (Table 2).

Question 3

Research question 3 follows: Do the flies respond to a lower injected dose with smaller changes in locomotor performance parameters than to a higher ingested dose?

Addressing Hypothesis 3.1. As far as central-nervous-system effects are concerned, there were no significant differences between flies that ingested the toxin versus were injected with the toxin in the three locomotory metrics that reflect CNS effects (bout frequency, bout duration, walking activity). Given that there were significant differences between treated and control flies, these results

contradict predictions 3.1.2 and 3.1.3 (Table 1 & Figures 1-3). Prediction 3.1.1 is void because walking speed was dominated by NMJ effects.

Addressing Hypothesis 3.2. Concerning the effects of L-BMAA on the NMJ, Fruit flies that were injected with L-BMAA only showed significant difference in number of stumbles when compared with flies that ingested 12.5 mM. This suggests that flies that were injected are most similar to flies that ingested a high dose of L-BMAA, contradicting our prediction that the injected flies should show weaker effects due to the lower total dose. Flies that were injected with L-BMAA also showed significantly lower walking speeds compared with flies that ingested L-BMAA (Prediction 3.2.2; table 1D & Figures 4-5), again contradicting our expectation that the lower injected dose should cause weaker effects on locomotion.

Question 4

Research question 4 follows: Will sequestering mechanisms of fruit flies cause a transient effect when L-BMAA is injected?

Addressing Hypothesis 4. To look for transient effects in stumbles, the 45-minute filming period was split into nine 5-minute sections. Flies injected with L-BMAA stumbled more often than control flies during the entire observation period. The control flies showed a shallow peak in stumbles early on in the recording period (from 0 to 10 minutes).

The treated flies showed an increase in number of stumbles initially (from time 0 to 20), with no indication of a decrease for the remainder of the observation period. The data suggest that there was no transient effect because we saw an increase in stumbling but we did not see a significant decrease towards the end of the observation period (Prediction 4.1, Figure 6).

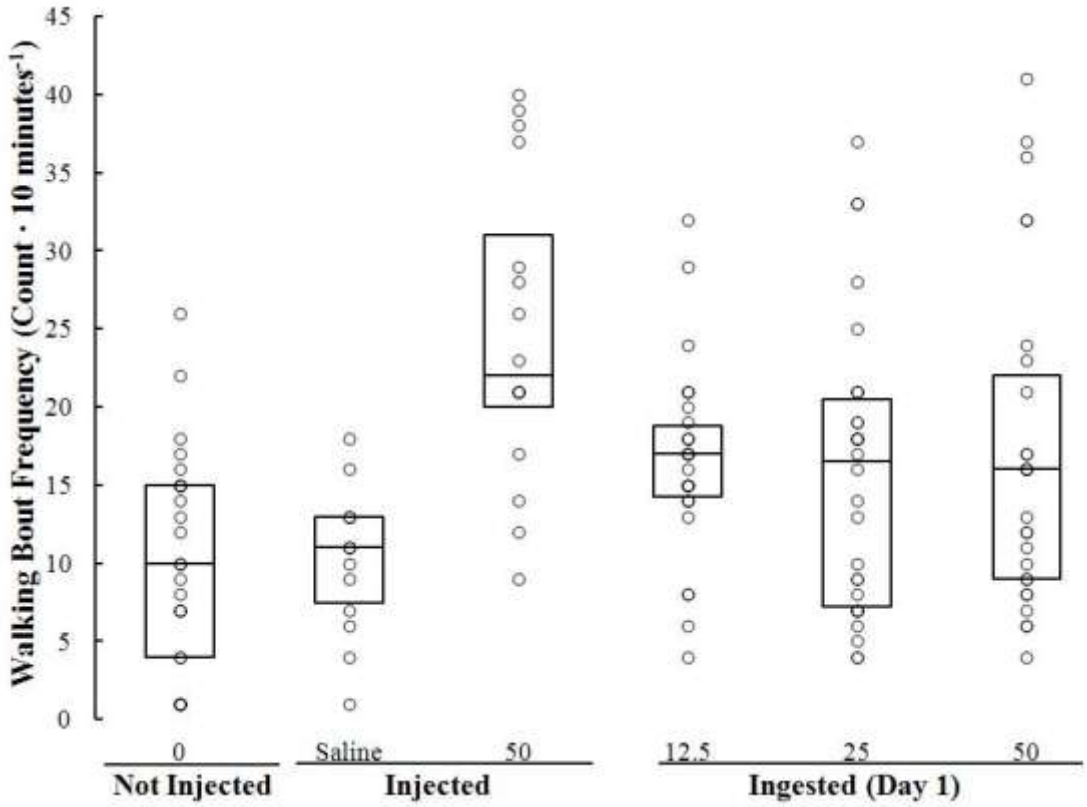


Figure 1. Average walking bout frequency of flies treated with L-BMAA, injected vs. ingested (ingested data by Mekdara et al. not yet published). Box and whisker plots were used to present the data with the distribution of all flies represented by the circles running vertically through the plot.

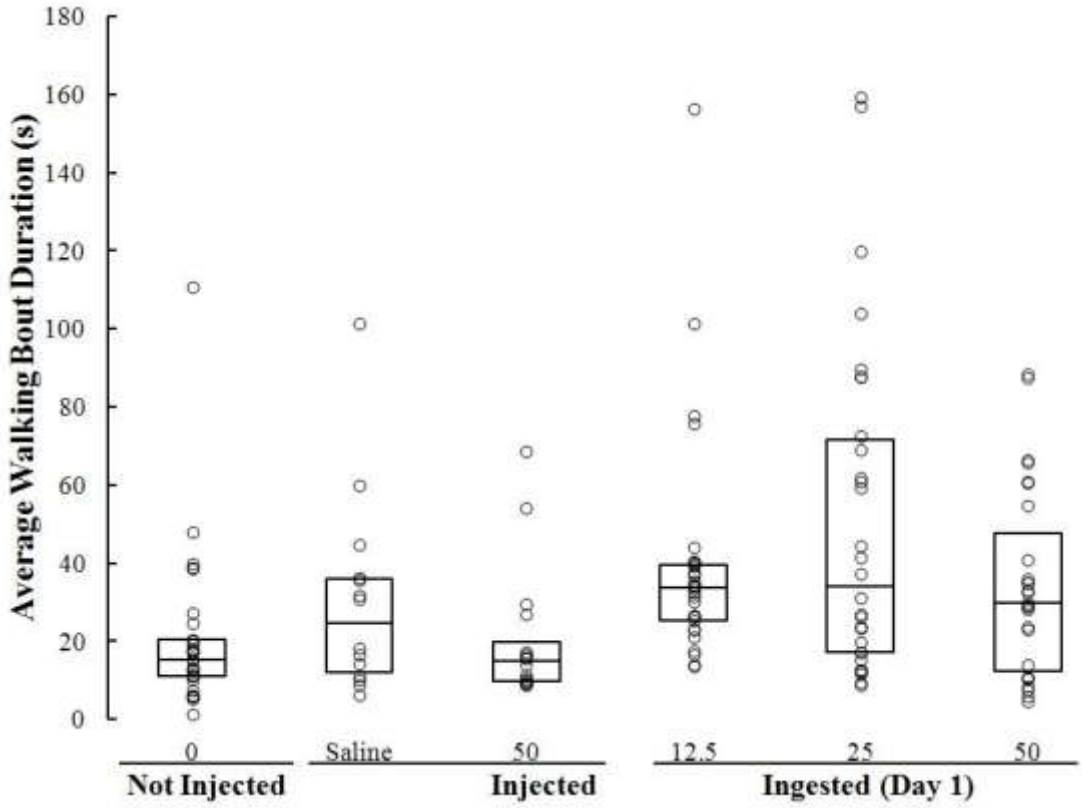


Figure 2. Average walking bout frequency of flies treated with L-BMAA, injected vs. ingested (ingested data by Mekdara et al. not yet published). Box and whisker plots were used to represent the data with individual fly distribution being represented by vertical circles.

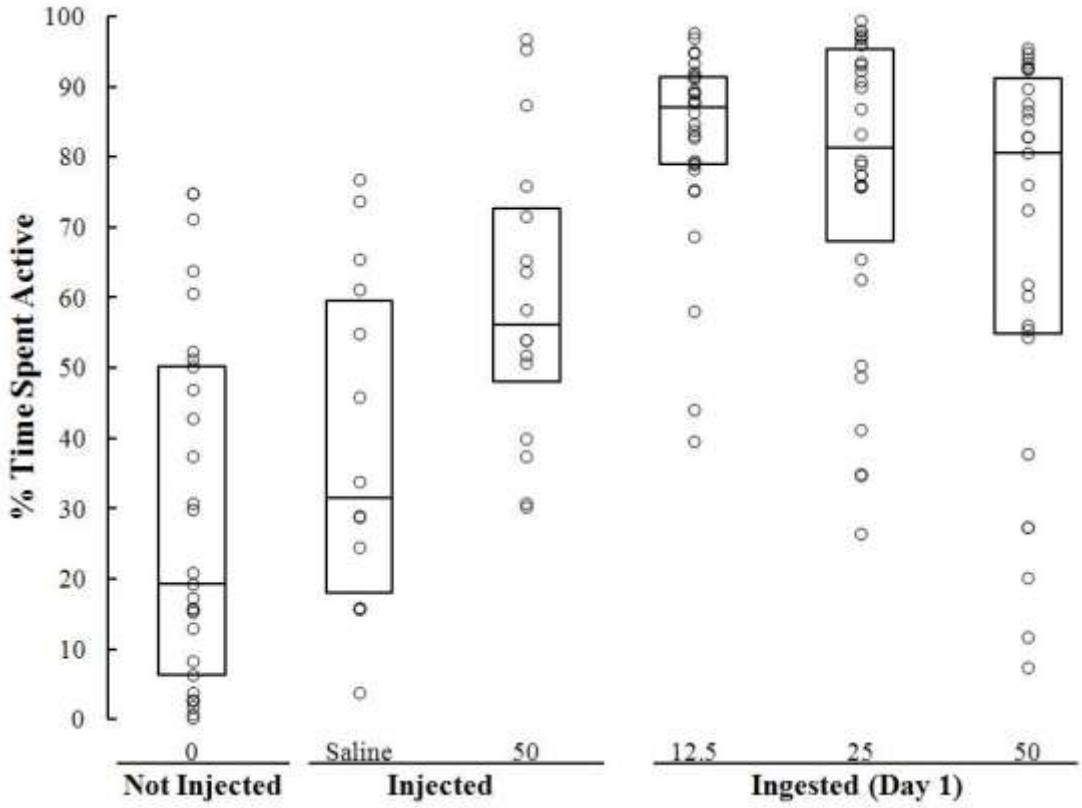


Figure 3. Activity levels of flies treated with L-BMAA, injected vs. ingested (ingested data by Mekdara et al. not yet published). Box and whisker plots were used to present the data with the distribution of all flies represented by the circles running vertically through the plot.

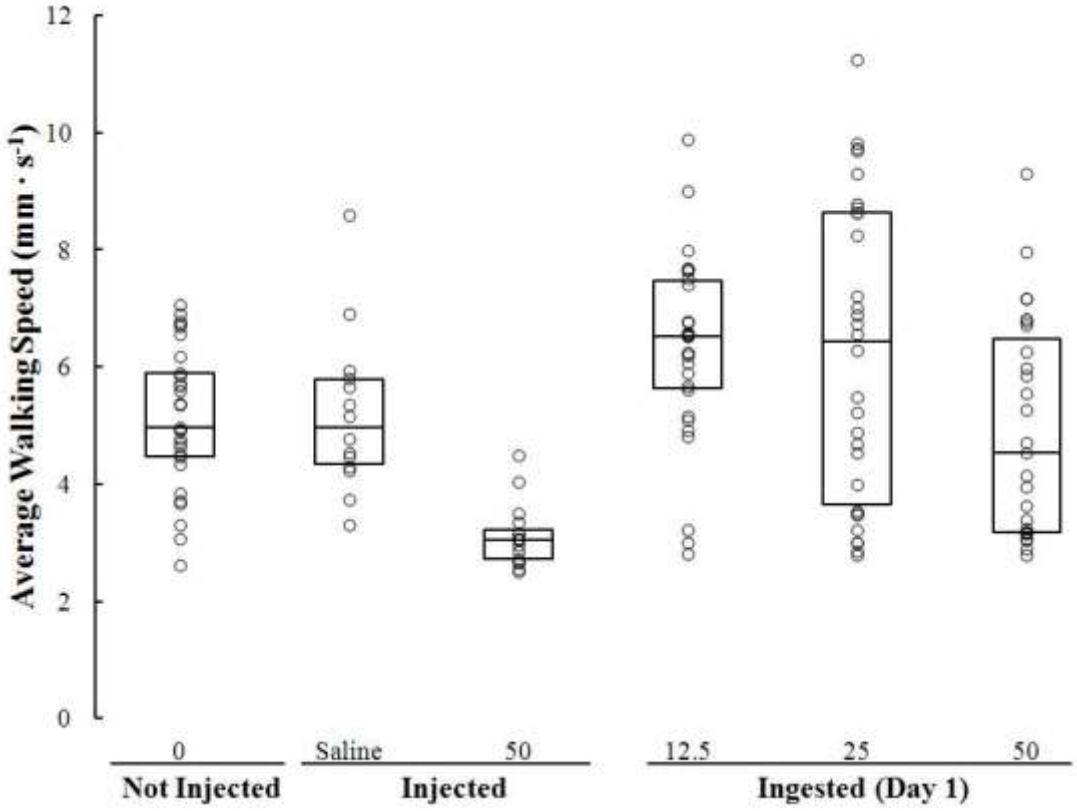


Figure 4. Average walking speed of flies treated with L-BMAA, injected vs. ingested (ingested data by Mekdara et al. not yet published). Box and whisker plots were used to present the data with the distribution of all flies represented by the circles running vertically through the plot.

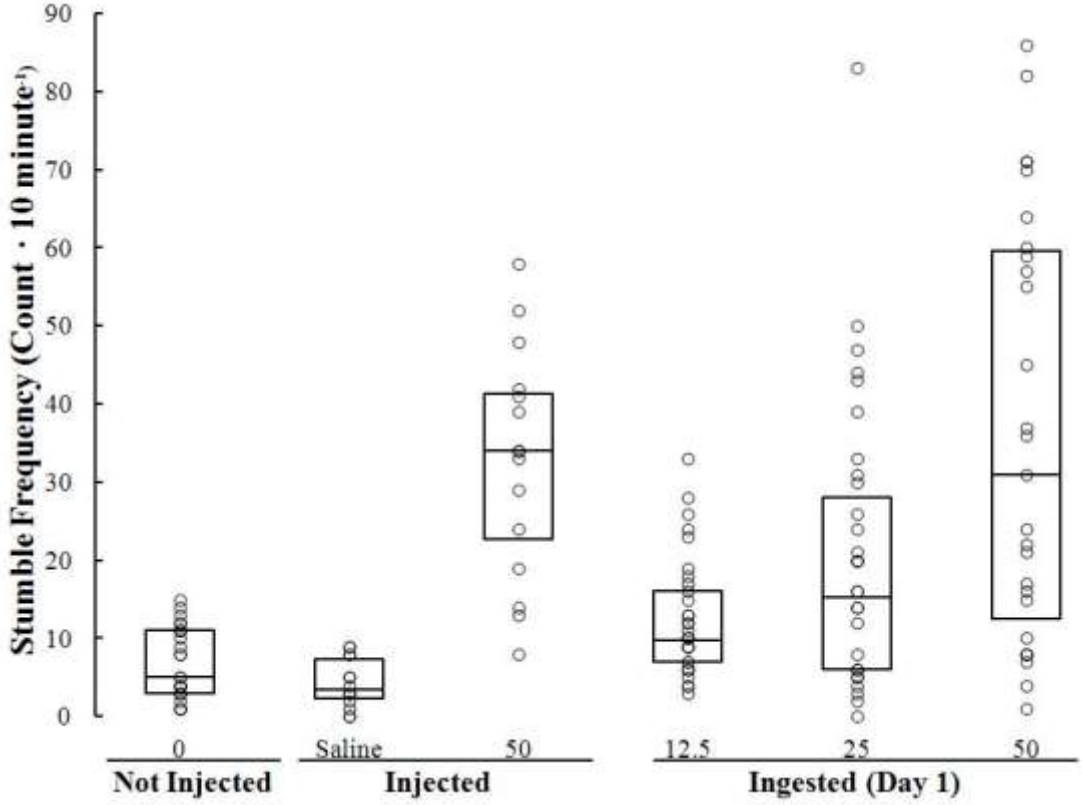


Figure 5.Stumble frequency of flies treated with L-BMAA, injected vs. ingested (ingested data by Mekdara et al. not yet published). Box and whisker plots were used to present the data with the distribution of all flies represented by the circles running vertically through the plot.

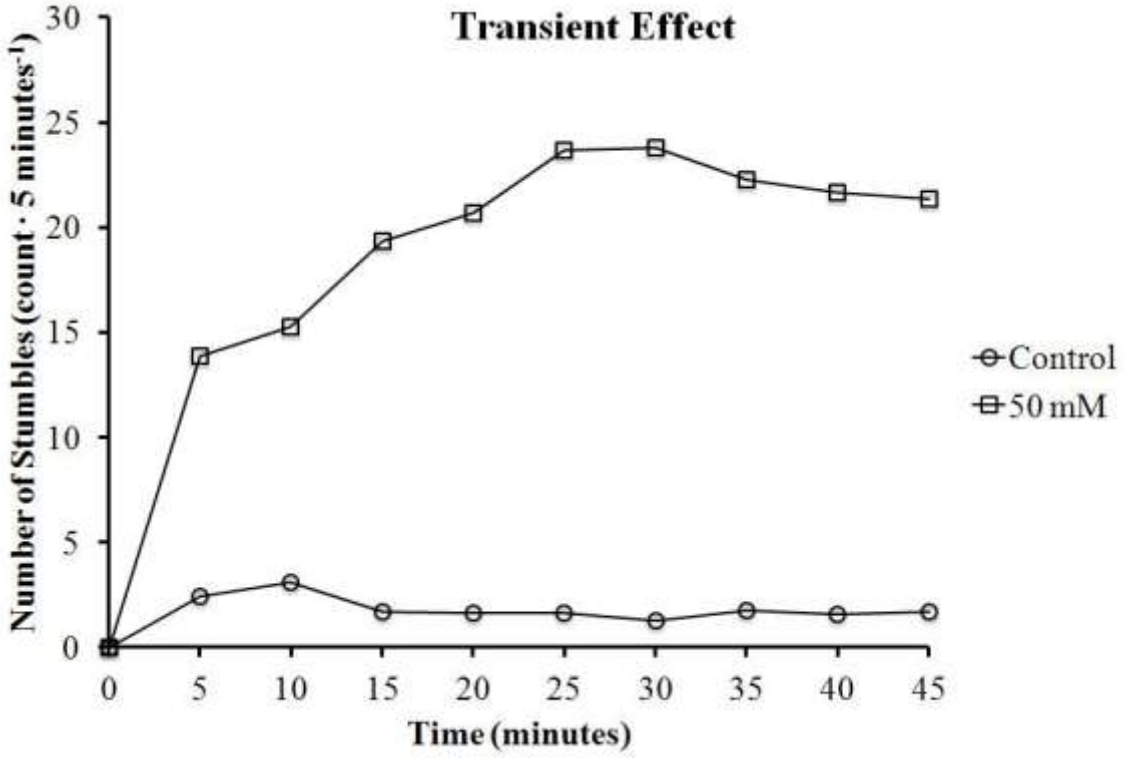


Figure 6. Transient Effect: Distribution of stumbles over 45 minutes (intervals of 5 minutes) were used to assesses the possibility that fruit flies injected with 50 mM L-BMAA would show a transient effect over the time period (ingested data by Mekdara et al. not yet published).

Table 1: Injected vs. Ingested L-BMAA. The data is presented with mean and ± S.D., $p < 0.05$. N-values for injected and ingested data are different due to the number of trials conducted. Ingested data provided by Nalong Mekdara.

(A) Treatment	**Dosage (mM)**	**Walking Bout Frequency (Count · 10 minutes^{-1})**
Not injected	0	9.69 ± 6.93 (29)^{a}
Saline Injected	0	10.2 ± 4.58 (16)^{ab}
Injected	50	24.8 ± 9.79 (16)^{c}
Ingested	12.5	16.6 ± 5.85 (30)^{bc}
Ingested	25	15.7 ± 9.09 (30)^{abc}
Ingested	50	17.0 ± 10.5 (27)^{abc}
(B) Treatment	Dosage (mM)	Walking Bout Duration (s)
Not injected	0	20.8 ± 20.7 (29)^{a}
Saline Injected	0	30.4 ± 25.7 (16)^{abc}
Injected	50	20.5 ± 17.3 (16)^{abc}
Ingested	12.5	39.6 ± 29.1 (30)^{c}
Ingested	25	50.7 ± 42.8 (30)^{c}
Ingested	50	34.5 ± 24.1 (27)^{abc}
(C) Treatment	Dosage (mM)	% Time Spent Active
Not injected	0	28.8 ± 24.9 (29)^{a}
Saline Injected	0	38.9 ± 23.9 (16)^{a}
Injected	50	60.2 ± 21.0 (16)^{ab}
Ingested	12.5	82.4 ± 14.0 (30)^{b}
Ingested	25	77.1 ± 21.8 (30)^{b}
Ingested	50	67.4 ± 28.2 (27)^{b}
(D) Treatment	Dosage (mM)	Speed (mm s^{-1})
Not injected	0	5.13 ± 1.22 (29)^{a}
Saline Injected	0	5.20 ± 1.36 (16)^{a}
Injected	50	3.12 ± 0.54 (16)^{b}
Ingested	12.5	6.32 ± 1.60 (30)^{a}
Ingested	25	6.26 ± 2.57 (30)^{a}
Ingested	50	4.92 ± 1.86 (27)^{a}
(E) Treatment	Dosage (mM)	Stumble Frequency (count · 10 minutes$^{-1)}$
Not injected	0	7.00 ± 4.41 (29)^{ab}
Saline Injected	0	4.28 ± 3.17 (16)^{a}
Injected	50	32.6 ± 14.3 (16)^{b}
Ingested	12.5	12.8 ± 18.6 (30)^{bc}
Ingested	25	21.6 ± 27.0 (30)^{c}
Ingested	50	36.5 ± 27.0 (27)^{bc}

Table 2: Determining statistical heterogeneity of treated groups compared with control (Saline) groups. Check marks indicate that the data is homogeneous and X means that the data is heterogeneous when comparing the parameters of walking ability. An independent sample t-test was used to determine significance of walking parameters, p = 0.05 (ingested data by Mekdara et al. not yet published).

Parameters	50 mM injected	12.5 mM	25 mM	50 mM
Frequency	√	x	√	√
Duration	√	√	√	√
Activity	x	√	x	x
Speed	x	x	√	√
Stumble	√	√	√	√

Table 3: Distribution of walking parameters for flies treated with L-BMAA. The numbers in the table were from the ingested groups (Mekdara et al.), and those in the boxes were from the injected groups.

Day1

Walking Parameters	12.5	*25*	50
Frequency [/10 min]	17	*16*	17 **25**
Duration [s]	40	51	35 **20**
Activity [%]	0.82	0.77	0.7 **0.6**
Speed [m/s]	6.3	6.3	4.9 **3.1**
Stumbles [/10 min]	13	22 **32**	36

Day 2

Walking Parameters	12.5	25	50
Frequency [/10 min]	15	21	20 **25**
Duration [s]	57	41 **20**	12
Activity [%]	0.77 **0.6**	0.58	0.38
Speed [m/s]	5.7	4.1 **3.1**	2.9
Stumbles [/10 min]	28 **32**	40	44

Day 3

Walking Parameters	12.5	25	50
Frequency [/10 min]	14	15	16 **25**
Duration [s]	75	33 **20**	14
Activity [%]	0.76	0.60 **0.6**	0.35
Speed [m/s]	6.1	4.2	3.1 **3.1**
Stumbles [/10 min]	21	24 **32**	43

DISCUSSION

Injection: A Method to Reduce Heterogeneity in Response to L-BMAA in Fruit Flies

The first objective of this thesis project was to develop an alternative procedure to apply L-BMAA to fruit flies in order to reduce the heterogeneity observed in the ingested data. Previous experiments with flies that ingested the toxin with their food showed high heterogeneity in most of the behavioral metrics (Mekdara et al., 2012). We suspected that this heterogeneity might be caused by differences in food uptake between flies. Flies consume roughly 1-3.5 µl of food per day (William Ja, pers. comm.). This implies that the consumption rate of the toxin might have varied by a factor of 3.

To reduce the heterogeneity, fruit flies were injected with a known concentration of L-BMAA, with saline injections as a control. Given that injections are a more invasive procedure that requires more time and experience from the experimenter, ingestion is a more attractive procedure for applying the toxin. Compared with ingestion, injections come with much higher risks also for the flies. The process of injection results in both a physical and mechanical stress. The injection creates a needle puncture the abdomen that may have led to physical trauma inside the body. Furthermore, since the fluid was injected, the increased volume of body fluid might have caused an increase in pressure on the exoskeleton leading to mechanical stress. However, the data showed that flies injected with saline showed no significant difference compared with untreated flies. So in answer to Question 1 of our Objectives: The physical trauma and mechanical stress from injecting fruit flies did not change their behavior.

However, in answer to Question 2 of our Objectives and against our expectations, injecting fruit flies with L-BMAA did not reduce heterogeneity

compared with flies that ingested the toxin. The fact that heterogeneity did not decrease in the injected flies could be explained either by experimental error or be due to genuine heterogeneity within our fly population. Concerning experimental error, these unexpected results might have been due to experimental errors in the injection process (volume errors) or in the process of making the solution (concentration errors).

To reduce volume errors, a 2.5 µl Eppendorf pipette was used to pipette 0.2 µl of the BMAA solution. The pipetted volume was then transferred into the injection needle without any loss, by inserting the needle into the opening of the pipette tip and transferring the fluid by capillary force. All injection needles were inspected visually to ensure that they contained the same amount of fluid (meniscus of the fluid was 2±0.5 mm from the first marker on the capillary). The precision of the volume was ±0.5 mm of fluid column, which corresponds to ±0.035 µl. So variation in toxin predicted from volume errors is much smaller (17.5%) than the variation in toxin predicted from the range of ingestion rates (300%).

To reduce concentration errors, solutions were made fresh from frozen stock to eliminate evaporative losses or amino-acid deterioration as possible causes for changes in concentration. The time between defrosting the stock of L-BMAA (which was always kept at -80 °C to avoid any evaporative losses) and going through steps of making 50 mM of L-BMAA was always between 20-25 minutes, which left little chance of evaporation differences between different days. To avoid errors due to poor mixing, all diluted solutions were vortexed after the dilution step to ensure that the solution was mixed properly. Therefore the errors that might have occurred during this process should be negligible.

Given that experimental error is not sufficiently large to explain the heterogeneity in the data, it was concluded that the flies were the source of the heterogeneity. When comparing the data collected from both injection and ingestion processes, the levels of heterogeneity were similar. Where there was heterogeneity in flies that have ingested L-BMAA, there was also heterogeneity in the injected data. This conclusion implies that the variation cannot come from the experimental errors because the errors were an order of magnitude smaller than the variation predicted from ingestion rates. Therefore, the heterogeneity likely resulted from variations within the fly. This means that injections are not a better way of administering precise toxins doses than ingestion. It also means that future experiments should aim to identify the source for the heterogeneity within the fly population.

Injection Versus Ingestion: Do We See the Same Overall Trends in the Effects on Locomotion?

This thesis project has shown that injecting L-BMAA affects locomotory behavior of fruit flies at both the CNS and PNS. Previous studies on the CNS control of locomotion led to the following predictions: over excitation of the CNS should cause an increase in activity level, walking bout frequency, walking bout duration, and speed (Strauss and Heisenberg, 2003; Martin, 2004). Over stimulation of the PNS should cause an increase in walking stumbles and a decrease in walking speed, but it should not affect activity levels, bout duration and bout frequency as long as the fly is still able to walk. Flies injected with L-BMAA showed an increase in bout frequency, consistent with the prediction for CNS effects. The effects on bout duration and activity levels showed the predicted trend (increase), but this increase was less than in the flies that ingested the toxin. This caused the injected flies to be not statically different from both controls and

flies treated by ingestion – in contrast, the flies that ingested the toxin were significantly different from controls. Flies that were injected with BMAA also showed significant effects on their PNS – their walking speed was significantly lower than that of control flies and flies treated by ingestion. Stumble frequency of injected flies was again intermediate between controls and flies that ingested the toxin, resulting in no significant differences between injected flies and controls or flies ingesting the toxin, whereas the controls were significantly different from the flies that ingested the toxin.

Injecting the toxin caused the same type of behavioral defects as ingesting the toxins. Injecting the toxin affected both the CNS and PNS, just like ingesting the toxin. None of the findings in this study fundamentally contradicted any of the predictions.

Injection Versus Ingestion: What Can We Learn About the Role of the Absorption Process Through the Intestinal Wall?

As stated in Question 3 of the Objectives, data on injected flies were compared with data from flies that ingested the toxin to explore possible differences in dose effects and the elucidation of the absorption process of ingested L-BMAA. If the digestive process does not affect L-BMAA (e.g. no chemical break-down of BMAA by digestion and complete absorption of the ingested L-BMAA into the hemolymph), then a similar injected dose should cause similar behavioral effects as an ingested dose. The flies were injected with 0.2 µl of 50 mM L-BMAA. Given that ingestion rates range from 1 to 3.5 µl of standard food per day, flies receiving L-BMAA in their food should ingest between 1 and 3.5 µl of 50 mM of BMAA per day, which is 5 to 18 times more toxin than the flies received through injection. Although the total amount of toxin delivered is

one order of magnitude higher, the rate of toxin delivery is much lower – instantaneous in the case of the injections, over the duration of 24 hours in the case of ingesting the toxin. A quantitative comparison of the behavioral effects from Day 1 showed that flies injected with the toxin showed more severe effects than the flies that ingested the highest dose (50 mM) in four of five behavioral metrics (Table 3, Day 1). Only one parameter showed effects that were between 25 mM and 50 mM on Day 1 (Table 3, Day 1). When comparing the injected data with ingested data on Day 2, the effects of injecting 50 mM of L-BMAA were centering around the effects of ingesting 25 mM of the toxin (Table 3, Day 2). Flies that ingested 50 mM of L-BMAA on Day 1 and 25 mM of L-BMAA on Day 2, should have consumed the same amount of toxin in total and about 10 times more toxin than flies that were injected with 0.2 µl. Yet similar behavioral effects were shown in the collected data. In answer to Question 3, injection causes 10 times more severe effects on locomotory behavior than ingesting L-BMAA of the same concentration. Therefore, it could be concluded that the concentration of L-BMMA in the hemolymph that resulted from ingesting about 1-3.5 µl of 50 mM BMAA was similar to that of injection of 0.2 µl. This finding suggests that the glutamate channels in flies' intestine prevent 90% of L-BMAA from passing through the intestinal wall and only 10% is absorbed and enters the hemolymph.

Injection and Transient Effects: What Can We Learn About Sequestrations Processes by Starting to Observe Flies Immediately After L-BMAA Was Applied?

Drosophila are known to respond to excess extracellular glutamate by trafficking glutamate receptors out of the membranes at the neuromuscular junctions (Chen et al., 2008), however, these experiments on the responses to excess glutamate were on larvae. There are far fewer studies on how adult fruit

flies respond to high levels of extracellular glutamate. Adult fruit flies have excitatory amino acid transporters (EAATs) in the glial cells of the neuromuscular junctions (dEAAT 1; Rival et al., 2006) and the central nervous system (dEAAT 1; Sinakevitch et al., 2010) that are important in sequestering extracellular glutamate. The same mechanisms that respond to excess glutamate might also respond to excess L-BMAA, causing glutamate receptors to be moved and L-BMAA to be sequestered. If any of these responses require genes to be switched on and proteins to be synthesized, then flies injected with excess L-BMAA should show a transient response with the debilitating effects of BMAA on locomotory behavior weakening over the duration it takes the dEAATs to become effective. Studies on gene function show that the time from signal over switching on a gene and protein production to seeing an effect is typically on the order of minutes to 1-2 hours rather than seconds (Guzowski et al., 2005). So in this study, genetically controlled sequestration mechanisms should only be observable towards the end of the observation period, in contradiction to pilot observations (Goto, pers. comm.). The main mechanism for transient effect would be akin to the trafficking of receptors observed in larval fruit flies (Chen et al., 2008). An unknown factor is the time it takes for the injected L-BMAA to be distributed through the body by the hemolymph.

However, fruit flies injected with BMAA did not show any observable transient effects. This might be because the observation period was too short to observe genetic effects, or the time between injection and filming (ca. 10 seconds) was too long to observe trafficking effects. In any case, the injection experiments did not reproduce the effects observed in the pilot study with decapitated flies. In the light of these contradictory observations, it might be worthwhile to repeat the decapitation experiments and quantify the behavior of the decapitated flies.

Conclusion

The main goal of this thesis project was to develop fruit flies as a model system to study L-BMAA. The injections showed that indeed fruit flies respond to L-BMAA, however, as a model this study found responses that were qualitatively similar to ingestion, so injection did not improve the heterogeneity of variance among fruit flies. From the study point of view, the injection method was not a useful method to administer the neurotoxin, however the study found that the response to injection was stronger than to ingestion. This important finding suggests that the glutamate channels in flies' intestine prevent 90% of L-BMAA from entering the hemolymph. From what we see in fruit flies, is it possible for humans to have the similar sequestering mechanism in their intestine? Future research should look at a similar mechanism inside the intestine of mammalian models in the search for finding a reliable treatment to reduce the symptoms of ALS.

LITERATURE CITED

LITERATURE CITED

Augustin H, Grosjean Y, Chen K, Sheng Q, Featherstone DE. 2007. Nonvesicular release of glutamate by glial xCT transporters suppresses glutamate receptor clustering in vivo. J Neurosci 27: 111–123.

Banack SA, Johnson HE, Cheng R, Cox PA. 2007. Production of a neurotoxin BMAA in cyanobacterium. Marine Drugs 5:180-196.

Banack SA, Caller TA, Stommel EW. 2010. The cyanobacteria derived toxin beta-n-methylamino-l-alanine and Amyotrophic Lateral Sclerosis. Toxins 2(12): 2837-2850.

Becker C, Meier CR. 2009. "Statins and the risks of Parkinson disease: An update on the controversy." Informa health care, Volume 8, No 3, pp. 261-271.

Besson MT, Soustelle L, Birman S. 2000. Selective high-affinity transport of aspartate by a Drosophila homologue of the excitatory amino-acid transporters. Curr Biol 10, pp. 207-210.

Brand LE, Pablo J, Compton A, Hammerschlag N, Mash DC. 2010. Cyanobacteria bloom and the occurrence of the neurotoxin, beta-N-methylamino-L-alanine (BMAA), in South Florida aquatic food webs. Harmful Algae 9:620-635.

Branson K, Robie AA, Bender J, Perona P, Dickinson MH. 2009. High-throughput ethomics in large groups of *Drosophila*. Nature Methods 6: 451–457.

Cardoso RM, Thayer MM, DiDonato M, Lo TP, Bruns CK, et al. 2002. Insights into Lou Gehrig's Disease from the structure and instability of the A4V mutant of human Cu, Zn superoxide dismutase. J Mol Biol 324:247–56.

Chen KY, Augustin H, Featherstone D. 2009. Effect of ambient extracellular glutamate on *Drosophila* glutamate receptor trafficking and function. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 195:21–29.

Choi DW, Maulucci Gedde M, Kriegstein AR. 1987. Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357-68.

Choi J, Levey AI, Weintraub ST, Rees HD, Gearing M, Chin LS, Li L. 2004. Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idio-pathic Parkinson's and Alzheimer's diseases. J Biol Chem 279, 13 256–13 264.

Cox, PA, Banack, SA, Murch, SJ. 2003. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proc. Natl Acad Sci USA 100, 13380 -13383.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol. 65:1-105.

Del Aguila MA, Longstreth WT Jr, McGuire V, Koepsell TD, van Belle G. 2003. Prognosis in amyotrophic lateral sclerosis: a population-based study. Neurology. 60(5):813-819.

Edwards TN, Meinertzhagen IA. 2010. The functional organisation of glia in the adult brain of *Drosophila* and other insects. Prog Neurobiol 90: 471–49.

Etienne R, Fortunat K, Pierce V. 2001. Mechanisms of urea tolerance in urea-adapted populations of *Drosophila melanogaster*. J of Exp Biology 204: 2699-2707.

Featherstone DE, Rushton E, Rohrbough J, Liebl F, Karr J, Sheng Q, Rodsech CK, Broadie K. 2005. An essential *Drosophila* glutamate receptor subunit that functions in both central neuropil and neuromuscular junction. J. Neurosci 25:3199-3208.

Guiloff RJ, Goonetilleke A. 1995. Natural history of amyotrophic lateral sclerosis observations with the Charing Cross Amyotrophic Lateral Sclerosis Rating Scales. Adv Neurol 68:185-198.

Guzowski JF et al. 2005. Mapping behaviorally relevant neural circuits with immediate-early gene expression. Curr Opin Neurobiol 15, 599–606.

Jan LY, Jan YN. 1976. L-Glutamate as an Excitatory Neurotransmitter at the *Drosophila* Larval Neuromuscular Junctions. J Physiol 262:215-232.

Jonasson S, Erikasson J, Berntzon L, Spcil Z, Ilag LL, Ronnevi L. 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggensts pathways for human exposure. PNAS 107 (20):9251-9257.

Karamyan VT, Speth RC. 2008. Animal models of L-BMAA toxicity: a critical review. Life Sciences 82: 233-246.

Koo EH, Kopan R. 2004. Potential role of presenilin-regulated signaling pathways in sporadic neurodegeneration. Nat Med. 10 (Suppl.), S26–S33.

Lipton, SA, Rosenberg, PA. 1994. Excitatory amino acid as a final common pathway for neurologic disorders. N Engl J Med. 330, 613-622.

Lobner D, Piana PMT, Salous AK, Peoples RW. 2007. β-N-Methylamino-L-alanine enhances neurotoxicity through multiple mechanism. Neurobiology of Diseases 25(2):360-366

Majoor-Krakauer, D, Willems, P, Hofman, A. 2003. Genetic epidemiology of amyotrophic lateral sclerosis. Clin Gen 63: 83-101.

Marrus SB, Portman SL, Allen MJ, Moffat KG, DiAntonio A. 2004. Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. J Neurosci 24(6): 1406-1415.

Martin J-R. 2004. A portrait of locomotory behavior in *Drosophila* determined by a video-tracking paradigm. Behav Proc 67: 207-219.

Martin J-R, Ernst R, Heisenberg M. 1999. Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*. J Comp Phys 185:277-288.

McDonald TJ. 2008. Neuromuscular pharmacology of insects. Annu Rev Entomol 20:151-166.

McEntee W, Crook T. 1993. Glutamate: Its role in learning and memory, and the aging brain. Psychopharmacological 111(4):391-401.

Mekdara NT, Goto JJ, Choudhury S, Mekdara PJ, Yingst N, Cao Y, Berg O, Müller UK. 2012. A Novel Lenticular Arena to Quantify Locomotor Competence in Walking Fruit Flies. J Exp Zool. doi:10.1002/jez.1731.

Murch SJ, Cox PA, Banack SA. 2004. A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. Proc Natl Acad Sci. USA 101, 12228-12231.

NTP (National Toxicology Program). 2008. A National Toxicology Program for the 21st Century: Chemical Information Review Document for L-β-Methylaminoalanine. Available:http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/LbetaMethylaminoalanine_508.pdf [accessed 21 April 2010].

Rival T, Soustelle L, Cattaert D, Strambi C, Iche M, Birman, S. 2006. Physiological requirement for the glutamate transporter dEAAT1 at the adult Drosophila neuromuscular junction. J Neurobiol 66, 1061-1074.

Spencer PS, Nunn PB, Hugon J, Ludolph A, Roy DN. 1986. Motorneurone disease on Guam: possible role of a food neurotoxin. Letters to the Editor. Lancet, 1(8487):965.

Strauss R, Heisenberg MA. 1993. Higher control center of locomotor behavior in the Drosophila brain. J Neurosci 13: 1852-1861.

Usherwood PNR. 1994. Insect Glutamate Receptors. Advances in Insect Physiology 24:309-341.

Weiss JH, Christine CW, Choi DW. 1989. Bicarbonate dependence of glutamate receptor activation by β-N-methylamino-l-alanine: Channel recording and study with related compounds. Neuron 3(3): 321-326.

APPENDICES

APPENDIX A: VIRTUAL DUB

Virtual Dub Instructions:

1. Virtual Dub version: 1.8.8(Build 30091/release) by Avery Lee.
2. File Open original AVI file of movie located:
3. Find Audio on top menu and select “No Audio.”
4. Find Video on top menu and select:

    -Full Processing Mode.

    -Compression:

-“ (Uncompressed RGB/YCbCr).”

    -Color Depth:

        -Decompression format: “Autoselect.”

        -Output format to compression display: “Luminance only (Y8).”

5. File: “Save as AVI.”
6. Close Virtual Dub and reopen, repeat steps 1-4.

-When opening video from file menu, select “VDUB” version,

i.e. “BMAA_0909_CAM19D1_VDUB.avi”

7. File: “Save as old formated AVI.”

    -Save move as “VDUB2” i.e. “BMAA_0909_CAM19D1_VDUB2.avi”

APPENDIX B: CTRAX

C-trax Instructions:

1. C-traxs will prompt you to open movie:

-Open “BMAA_0909_CAM19D1_VDUB2.avi.”

2. C-traxs will prompt you to save annotation file:

-Save As “ANN_0909_CAM19D1.ann”

3. On top menu find “Setting:”

-Select “Background Model” and enter full amount of frame numbers, i.e. 6400, and hit “Compute.”

-Select “Background Subtraction,” make sure settings is set on “Light flies on Dark Background.” Set high and low threshold until all flies are in field of view, (Look at **Appendix II** for threshold settings).

-If your arena is not circular, you need to deselect circular arena, **Set Circular Arena Region of Interest.** Now select **Set Regions of Interest**, and set boundaries by drawing regions of interest and hit **Save** then **Quit.**

-Select “Track Setting:”

-If all flies are detected for after “Background Subtraction,” select “Auto Compute Bond,” compute bond.

-If not all flies are detectable after “Background Subtraction,” select “Manual Compute Bond,” (must enter all parameters manually).

4. On top menu find “Track:”

- Select “Create compressed SBFMF file with tracks,” Save AS i.e. SBFMF_BMAA_0909_CAM19D1.sbfmf.

-Select “Start Tracking.”

5. When tracking is done, find “File” on top menu.

-Select “Export as MAT file” i.e. MAT_BMAA_0909_CAM19D1.mat.

-(Note) If you exit the program without exporting a MAT file, you can just reopen the tracking file with C-trax and open the ANN file to export the MAT file.

(50 mM Injected)

Background Subtraction	
Background Type	Light flies on dark background
Normalize by	Standard Deviation
Std Range	1.00-10.00
Minimum Non-Foreground Intensity	256.00
Maximum Non-Foreground Intensity	-1.00
High Thresh	15.0
Low Thresh	3.2

Tracking Settings			
	Minimum	Mean	Maximum
Area of Target	36.62	79.16	121.7
Major axis length	3.05	4.52	5.99
Minor axis length	1.01	1.39	1.77
Eccentricity	0.16	0.32	0.49

(Saline Control)

Background Subtraction	
Background Type	Light flies on dark background
Normalize by	Standard Deviation
Std Range	1.00-10.00
Minimum Non-Foreground Intensity	256.00
Maximum Non-Foreground Intensity	-1.00
High Thresh	15.0
Low Thresh	3.2

Tracking Settings			
	Minimum	Mean	Maximum
Area of Target	43.45	76.03	108.61
Major axis length	2.95	4.68	6.42
Minor axis length	0.89	1.29	1.67
Eccentricity	0.13	0.28	0.43

APPENDIX C: FIXERRORS

MATLAB Fixerrors Instructions:

1. MATLAB version 7.0.6 (R2008a) and Fixerrors version 0.1.04 used.
2. Open MATLAB and change directory to otto/useful/fixerrors/fixerrors.

-Select fixerror.m from "Current Directory" and drag into "Command Window."

3. Prompt to open "Movie," select SBFMF formatted movie, i.e. SBFMF_L-BMAA_0909_CAM19D1.sbfmf.
4. Prompt to open "MAT File," select Copy of MAT, i.e. Copy of MAT_L-BMAA_0909_CAM19D1.mat.
5. Prompt to open "ANN File," select Copy of ANN, i.e. Copy of ANN_L-BMAA_0909_CAM19D1.ann.
6. Prompt to enter "Frames per second," enter **10.**
7. Prompt to compute pixel to mm conversion, select "Compute:"

-Draw a straight line in one dish to indicate diameter on dish, make sure there in no brake in the line.

-Double click the line and enter 76 to indicate diameter of dish.

8. Prompt to "Save results for input," i.e. trx_L-BMAA_0909_CAM19D1_MAT.mat.
9. Prompt to enter "Suspiciousness Parameters:"

-***Minimum suspicious prediction-detection error (mm)*** **15.**

-***Minimum suspicious orientation change (deg)*** **180.**

-***Minimum suspiciously large major axis (mm)*** **6.7224.**

-***Minimum suspicious orientation-velocity direction mismatch (deg)*** **180.**

-***Minimum walking speed (mm/frames)*** **0.25.**

-***Minimum ambiguous error (mm^2)*** **0.625.**

10. After fix, Save AS fixed_L-BMAA_0909_CAM19D1.mat.

11. Change directory to **behaviormicroarray**, i.e. StorageD/Otto/Useful/behaviormicroarrya.

-Scroll down and locate **showtrx.m,** allow you to determine which fly is which by numbering each fly with a separate value. Just select and fly and it will give you the identity, record identities in an excel sheet, i.e. CAM19FLYID.xls

APPENDIX D: PERFRAMESTATS

Perframestats Text file:

1. Open MATLAB Change Directory to **behaviormicroarray**, i.e. StorageD/Otto/Useful/behaviormicroarry.
2. Scroll down and locate **fix_length_mismatch.m** MAT file and drag into command window. Prompt to select MAT file, select fixed_L-BMAA_0909_CAM19D1.mat.

-Scrip "Saves File As" fixed_L-BMAA_0909_CAM19D1.mat_coverted.

3. In same directory, locate **compute_perframe_stats.m** and drag into command window. Prompt to select MAT file, select fixed_L-BMAA_0909_CAM19D1.mat_coverted.

-Prompt to "Do you want to compute distance, angle to arena wall statistics?" select **NO.**

-Prompt to "Do you want to compute statistics relating to closest fly?" select **YES.**

-Prompt to "Save File " i.e. perframestats_fixed_L-BMAA_0909_CAM19D1.mat.

-Prompt to enter "Field of View of fly in degrees" enter **180.**

-Save results as perframestats_fixed_L-BMAA_0909_CAM19D1.mat_coverted.

4. In same directory, locate **Sameet Code** and open folder. In folder, there should be another folder labeled **MikeMat** open folder and drag into command window **executeFruitFlyStatsEngine.m**

-Prompt to "Pick an MAT-File," open **perframestats_fixed_L-BMAA_0909_CAM19D1.mat.**

****Global Settings

Start Fly:

1
Stop Fly:
1
Show Exceptions:
0
Notepad Output
1

****Setting for Stats

Enter StDev Threshold:
4.5
Range Size:
25
Show Exceptions:
0
Do not ask again
1

****Settings for Boundaries

Speed Threshold:
2
Range Size:
25
Min Frames per Bout:
5
Show Exceptions:
0
Do not ask again
1

California State University, Fresno

Non-Exclusive Distribution License

(to make your thesis/dissertation available electronically via the library's eCollections database)

By submitting this license, you (the author or copyright holder) grant to CSU, Fresno Digital Scholar the non-exclusive right to reproduce, translate (as defined in the next paragraph), and/or distribute your submission (including the abstract) worldwide in print and electronic format and in any medium, including but not limited to audio or video.

You agree that CSU, Fresno may, without changing the content, translate the submission to any medium or format for the purpose of preservation.

You also agree that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright.

If the submission reproduces material for which you do not hold copyright and that would not be considered fair use outside the copyright law, you represent that you have obtained the unrestricted permission of the copyright owner to grant CSU, Fresno the rights required by this license, and that such third-party material is clearly identified and acknowledged within the text or content of the submission.

If the submission is based upon work that has been sponsored or supported by an agency or organization other than California State University, Fresno, you represent that you have fulfilled any right of review or other obligations required by such contract or agreement.

California State University, Fresno will clearly identify your name as the author or owner of the submission and will not make any alteration, other than as allowed by this license, to your submission. **By typing your name and date in the fields below, you indicate your agreement to the terms of this distribution license.**

Athena Goodarzi

Type full name as it appears on submission

July 8, 2012

Date