CHARACTERIZATION OF LOCOMOTOR RESPONSE TO PSYCHOSTIMULANTS IN THE PARTHENOGENETIC MARBLED CRAYFISH (*Procambarus fallax* forma *virginalis*): A PROMISING MODEL FOR STUDYING THE EPIGENETICS OF ADDICTION

Cedric James Jackson

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2016

Committee:

Moira van Staaden, Advisor

Robert Huber

Vipaporn Phuntumart
CHAPTER I. ABSTRACT

Moira van Staaden, Advisor

Epigenetic regulation has been implicated as an important factor in understanding the molecular mechanisms of drug addiction. This is due to the life-long behavioral changes that commonly afflict addicted individuals long after drug exposure has been extinguished. Invertebrates, such as crayfish, offer excellent model systems to study these molecular mechanisms because they retain the ancestral neural reward circuit that is evolutionarily conserved across taxa, possess relatively few, large neurons, and have an accessible, modularly organized nervous system. The marbled crayfish (*Procambarus fallax* forma *virginalis*), in particular, has potential as a model for epigenetic studies because it is parthenogenetic, and thus all individuals are genetic clones. To provide the foundation of this model system for parsing the epigenetic mechanisms of drug addiction, here I characterize the locomotor response of juvenile *P. f. f. virginalis* exposed to the psychostimulant, d-amphetamine sulfate. Custom video-tracking software was used to record the movement patterns of juveniles exposed to water infused with varying concentrations of d-amphetamine sulfate. ANOVA demonstrated that crayfish locomotion was significantly impacted by drug concentration. These psychostimulant effects, along with the non-invasive mode of drug delivery, which avoids potential epigenetic changes resulting from the stress of direct injection into the hemolymph, set the stage for using *P. f. f. virginalis* as an animal model for epigenetic studies.
CHAPTER II. ABSTRACT

Moira van Staaden, Advisor

With the rising interest in using invertebrate models for behavioral epigenetic studies, and the tissue-specific nature of epigenetic changes, there is a need for effective methods of extracting genomic DNA from neural tissue. Invertebrates, such as crayfish, represent useful models in which to parse out the basic underlying epigenetic changes because of the relatively simplified anatomical structure of their nervous system. The parthenogenetic marbled crayfish (*Procambarus fallax* forma *virginalis*) offers a particularly elegant system because it reproduces asexually and produces large numbers of genetically identical offspring. However, the low DNA yield of fatty neural tissue combined with small sample size represent technical challenges in using invertebrate brains. The present study seeks to support the use of the marbled crayfish as a model for studying the mechanisms of behavioral epigenetics by providing an optimized method for genomic DNA extraction from invertebrate neural tissue.
Dedicated to Dr. T. Edward Weiss Jr. (1951-2013), a caring and supportive professor who always believed in and fostered the scientist within me. I owe a great deal of my success to the help and support Dr. Weiss gave as my Biology Advisor during the academically formative years of my undergraduate education at Christopher Newport University, without which I would not be the scientist I am today.
ACKNOWLEDGMENTS

I would like to express my appreciation for all of those who contributed to the success of my master’s project: to anyone who has taken the time to offer me advice, constructive criticism, technical help, manuscript review, or moral support.

I am deeply grateful to my advisor Moira van Staaden and my committee members Robert Huber and Vipa Phuntumart for all of their support, guidance, and constructive feedback at every stage of my thesis. I would like to thank Moira for allowing me the freedom to pursue a project that incorporated many of my areas of interest and for her excellent and numerous revisions of my manuscript. I am grateful for Robert’s technological contribution of the custom video tracking software he developed and the lab space he provided me to establish a breeding colony of my research animals. And I would like to thank Vipa for the opportunity to work in her molecular lab and for including me in her lab meetings while my advisors were away on sabbatical.

I also want to recognize the support and friendship I have been given from the members of the van Staaden Lab: Kami Stamey and Jessica LaHurd; the Huber Lab: Sayali Gore; and the Phuntumart Lab: Shannon Miller, Dilshan Beligala, Gayathri Beligala, Satyaki Ghosh, Alex Howard. All of whom have offered me much help on my thesis, through both technical and moral support. Thank you all.

I have received help and friendship from many of the graduate students and faculty of the Biology Department at BGSU, and, although too numerous to list individually, your efforts have been deeply appreciated.

I would like to offer my particular gratitude to Noah Ryan, who spent countless hours of his free time, after working eight to ten hour days as a computer scientist at NASA, to help me clean up my data, write my data analysis and statistical analysis programs, and countless hours
de-bugging said programs. In addition to offering technical support, he has offered continual moral support, motivation and friendship over the years and throughout the entire process of my thesis.

I would like to thank my family and friends for their unconditional support and their loving encouragement throughout the years.

Finally, my sincerest thanks to the marbled crayfish, without whom this research would not have been possible.
# TABLE OF CONTENTS

CHAPTER 1: LOCOMOTOR RESPONSE OF THE MARBLED CRAYFISH

*(Procambarus fallax forma virginalis)* TO D-AMPHE TAMINE SULFATE ADMINISTRATION ................................................................................................................ 1

INTRODUCTION ........................................................................................................ 1

METHODS ................................................................................................................... 5

Animal Care and Maintenance ............................................................................. 5

Behavioral Testing Apparatus ............................................................................. 6

Experimental Design and Procedure ................................................................. 7

Behavioral Analysis .......................................................................................... 9

Statistical Analysis ............................................................................................ 10

Euthanasia, Dissection and Brain Extraction .................................................... 10

RESULTS ..................................................................................................................... 11

Locomotion ....................................................................................................... 11

Space Use and Walking Pattern Behavioral Response ..................................... 14

DISCUSSION ............................................................................................................... 15

Crayfish Locomotion in Response to Novel Arena ........................................... 16

Effects of Initial Drug Exposure ....................................................................... 16

Effects of Continuous Chronic Exposure ......................................................... 20

Conclusion ........................................................................................................ 22

REFERENCES ............................................................................................................. 23
CHAPTER II: OPTIMIZATION OF GENOMIC DNA EXTRACTION FROM MARBLED CRAYFISH (Procambarus fallax forma virginalis) BRAIN TISSUE .......... 33

INTRODUCTION ................................................................. 33

METHODS .............................................................................. 35

Euthanasia, Dissection and Brian Extraction ....................... 35
Preparation of Crayfish Brian and Abdominal Tissue Samples ........ 35
Genomic DNA Extraction .................................................. 36
Methylation-sensitive Enzyme Digestion ................................ 37

RESULTS .................................................................................. 38

Genomic DNA Extracted from Different Starting Amounts of Abdominal Tissue ................................................................. 38
Genomic DNA Extracted from Assorted Number of Pooled Whole Brains ................................................................. 40
Methylation-Sensitive Enzyme Digests ..................................... 43

DISCUSSION ................................................................. 44

Genomic DNA Extraction .................................................. 45
Methylation-Sensitive Enzyme Digests ..................................... 46

REFERENCES ........................................................................... 47
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Total genomic DNA extracted from various amounts of <em>P. f. f. virginalis</em> abdominal tissue</td>
<td>39</td>
</tr>
<tr>
<td>2.2</td>
<td>Total genomic DNA extracted from assorted number of pooled <em>P. f. f. virginalis</em> brains</td>
<td>41</td>
</tr>
<tr>
<td>2.3</td>
<td>Total genomic DNA extracted from pooled <em>P. f. f. virginalis</em> brains after optimization</td>
<td>43</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Juvenile crayfish housing unit</td>
</tr>
<tr>
<td>1.2</td>
<td>Example of behavioral testing arena</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic outlining the experimental design</td>
</tr>
<tr>
<td>1.4</td>
<td>Mean distance travelled in the first 30 minutes of each recording period for d-amphetamine sulfate concentrations from 0.0 to 50.0 µM</td>
</tr>
<tr>
<td>1.5</td>
<td>Psychostimulant effects of d-amphetamine sulfate concentrations from 0.0 to 50.0 µM</td>
</tr>
<tr>
<td>1.6</td>
<td>Example of crayfish movement patterns over a one-hour period in drug-free water</td>
</tr>
<tr>
<td>2.1</td>
<td>Genomic DNA extracted from varying amounts of <em>P. f. virginalis</em> abdominal tissue</td>
</tr>
<tr>
<td>2.3</td>
<td>Genomic DNA extracted from assorted number of pooled <em>P. f. virginalis</em> brains during the early stage of DNA extraction optimization</td>
</tr>
<tr>
<td>2.4</td>
<td>Methylation sensitive enzyme (Msp I and Hpa II) digests of low and high concentrations of <em>P. f. virginalis</em> gDNA</td>
</tr>
</tbody>
</table>
CHAPTER I: LOCOMOTOR RESPONSE OF THE MARBLED CRAYFISH

*Procambarus fallax forma virginalis* TO D-AMPHETAMINE SULFATE ADMINISTRATION

INTRODUCTION

Drug addiction is a syndrome characterized by maladaptive plasticity of the neural reward circuit (reviewed in Hyman et al., 2006). Under normal circumstances, the neural reward circuit is triggered by natural rewards (food, sex, social interaction, etc.) to release dopamine signals from the ventral tegmental area to projections in the nucleus accumbens and the prefrontal cortex and from the substantia nigra to projections in the dorsal striatum (reviewed in; Hyman et al., 2006; Spanagel and Weiss, 1999; Wise, 1996). Therefore, it is unsurprising that the monoamine dopamine is required for motivated and appetitive behaviors and for reward-related learning (reviewed in Alcaro et al., 2007). However, drugs of abuse have the ability to falsely trigger and overstimulate, or “hijack,” the natural reward circuit by increasing the magnitude and the duration of these dopamine signals (reviewed in Kelley and Berridge, 2002; but see Hagen et al., 2013). In this way drugs of abuse act as strong reinforcers and can, with repeated exposure in susceptible individuals, develop into the syndrome of addiction. The addictive cycle is developed as drugs reinforce and strengthen drug-taking behavior and consumption, until the associated drug-seeking behaviors become compulsive despite negative repercussions (reviewed in Wise, 1998). Such behavioral abnormalities can persist even after long periods of abstinence, sometimes indefinitely, and are accompanied with a high susceptibility of relapse (O’Brien et al., 1998; Wise and Bozarth, 1987; Wise, 2000). These
persisting lifelong symptoms present particularly recalcitrant clinical challenges in developing effective treatments for addiction.

The long-term behavioral abnormalities that characterize the addiction syndrome are attributed to changes in neural gene expression. Epigenetics is the interaction of biochemical mechanisms that result in a tissue-specific change in gene expression, while the underlying DNA sequence remains unaltered (Jaenisch and Bird, 2003). These biochemical changes can be induced by environmental conditions experienced during an individual’s lifetime. In this way, epigenetics can be conceptualized as a means through which the environment interacts with an organism’s genome. As such, a subset of stable epigenetic changes have been identified as important molecular mechanisms underlying the neural plasticity associated with the addiction syndrome (reviewed in Nestler, 2014). Drugs of abuse affect the molecular environment within the brain’s reward regions, which can induce three major modes of epigenetic regulation: histone modifications (acetylation and methylation), DNA methylation, and non-coding RNA (reviewed in Nestler, 2014). DNA methylation is one of the most studied epigenetic mechanisms because it is considered important in the mediation of stable, and possibly heritable, changes in transcription (reviewed in Bestor, 2000).

Molecular and neural mechanisms underlying drug-induced reward have primarily been studied with the use of mammalian models (Chao and Nestler, 2004). Research in mammalian systems has successfully identified molecular targets, neurochemical systems and brain regions that mediate drug-induced reward (reviewed in Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Kalivas and McFarland, 2003; Kalivas and Volkow, 2005). Although, drugs of abuse target homologous brain regions in humans and other mammalian models (reviewed in Butler and Hodos, 2005; and Koob and Volkow, 2010), a similarity in the molecular neural
mechanisms for drug effects has been evolutionarily conserved across taxa (McClung and Hirsh, 1998; Andretic et al., 1999; Kaun et al., 2011).

Invertebrates possess a relatively simple, modularly organized nervous system. Unlike in mammalian models, the role of individual neurons and specific circuits can be determined in the invertebrate brain (Kaun et al., 2012). By taking advantage of these methods, invertebrate models have already greatly benefited our understanding of the basis of learning and memory, and of the mechanisms involved in molecular and neurochemical signals (Carew and Sahley, 1986). Exploration into how drugs alter neural function and plasticity across many different invertebrate taxa can help reveal the basic functional properties involved in the reward system.

Among invertebrate models of drug addiction, decapod Crustacea, such as crayfish, offer a particularly useful set of advantages. Crayfish possess relatively few, often large, easily identifiable, and modularly organized neurons (reviewed in Clarac and Pearlstein, 2007). Combined with a complex behavioral repertoire and relatively large size, this renders the crayfish particularly accessible to pharma-behavioral manipulations (Panksepp and Huber, 2002; Huber and Delago, 1998). Crayfish have been a longstanding neuroscience animal model and, consequently, a great deal is known about their central nervous system structure and function: both crayfish and lobster brains have been mapped (Beltz, 1999; Polanska et al., 2007; Sullivan and Beltz, 2001; Tierney et al., 2003), the neurochemistry of crayfish behavior is well-understood (reviewed in Kravitz, 2000; Edwards et al., 2003), and the lobster transcriptome has recently been characterized (McGrath et al., 2016). This foundation of neuroethological research sets the stage for delving into the basic mechanisms of drug-induced neural plasticity and reward-related learning.
Psychostimulant behavioral effects have been demonstrated in the rusty crayfish (Orconectus rusticus) in response to amphetamine (Alcaro et al., 2011), cocaine (Nathaniel et al., 2012), and morphine (Nathaniel et al., 2010). This includes sensitization (Dziopa et al., 2011; Nathaniel et al., 2010; Nathaniel et al., 2012), withdrawal (Huber et al., 2011; Nathaniel et al., 2009), and reinstatement with a single priming dose (Nathaniel et al., 2009). Drug-induced reward in O. rusticus has been demonstrated through the use of conditioned place preference (Panksepp and Huber, 2004; Nathaniel et al., 2009) and self-administration paradigms (Datta, 2015). Moreover, studies of drug-induced gene expression changes in the crayfish brain have shown changes of c-Fos mRNA expression in the accessory lobe (Nathaniel et al., 2012), which is hypothesized to be involved in high-order processing of environmental stimuli and an important region in the neural reward circuitry in crayfish (Nathaniel et al., 2012; Sullivan and Beltz, 2005).

In the current study I examine the locomotor response of P. f. f. virginalis to the psychostimulant d-amphetamine sulfate. Using a non-invasive mode of drug administration over 24-hour exposure periods, I explored differential behavioral effects and characterized the locomotor responses of juvenile P. f. f. virginalis (1) during initial exposure, de-coupled from a novelty-induced behavioral response, and (2) after continuous chronic exposure. The results of this work provide the foundation for P. f. f. virginalis as a potential model system for studying the epigenetic mechanisms underlying the maladaptive plasticity associated with drug addiction.
METHODS

Animal Care and Maintenance

Adult *P. f. f. virginalis* obtained from a commercial supplier were housed individually in the lab in clear plastic containers (Snapware Total Solution, 232.4 mm L x 186.7 mm W x 76.2 mm H). These were modified so as to allow water flow-through (4 mm holes drilled into container: 34 on lid, 7 along each length, and 5 along each width) and were kept within a single circulating-water tank system, at ambient room temperature (~19-22°C) and a 12:12 hour light/dark cycle. Adults were fed rabbit pellets supplemented with frozen bloodworms and brine shrimp twice a week. Each crayfish had access to a shelter and were handled as little as possible to minimize stress. First generation offspring (n = 48) of these females were used in all subsequent experiments. Once they had detached from their mother’s tail, offspring were moved to individual compartments within multi-partitioned containers, which simultaneously ensured social isolation and macro-environmental standardization (Figure 1.1). Isolated offspring were exclusively fed a diet of two parts frozen brine shrimp and one part frozen emerald entrée (San Francisco Bay Brand) until used in behavioral assays at 7-10 months of age (14-29 mm in length).
Figure 1.1: Juvenile crayfish housing unit (348 mm L x 218 mm W x 35 mm H). Holes (2.5 mm) were drilled in the top bottom of each compartment (52 mm L x 42 mm W x 31 mm H) to allow for water circulation throughout the unit. Each unit housed 30 individuals in separately-labeled compartments, was labeled with the mother’s identification number, and contained only juveniles from the same clutch.

Behavioral Testing Apparatus

A frosted plastic container (Figure 1.2) was made by bisecting a Whitmor stacking organizer box (492 mm L x 330 mm W x 91 mm H) to create six compartments, each measuring 91 mm L x 103 mm W x 61 mm H. A 64 mm diameter hole was drilled into the bottom center of each compartment and covered with a white plastic canvas mesh (Darice), which was adhered with VDR labeling tape. These compartmentalized containers were placed within white Sterilite ID boxes (371 mm L x 279 mm W x 130 mm H; 9.7 Liters) and wedged into place with cut pieces of PVC pipe (60 mm D x 47 mm L) within 2.8 L of water. This apparatus allowed for individual crayfish, placed within each compartment, to be transferred between containers of
drug-free water and drug-infused water with minimal disturbance, and ensured simultaneous and identical exposure time for all animals.

**Figure 1.2:** Example of behavioral testing arena

**Experimental Design and Procedure**

Juvenile crayfish were randomly assigned to one of four treatment groups: 0.0 μM, 0.5 μM, 5.0 μM, or 50.0 μM d-amphetamine sulfate (FW: 368.5; Sigma, St. Louis: A 5880). Two trials of six crayfish were run for each treatment group (n = 12 per group). In order to minimize confounding variables, all water used during experimental procedures was taken from the circulating tank in which crayfish were housed on the day of testing. For each trial, six crayfish were placed in individual compartments within the compartmentalized testing arena. This compartmentalized arena was then placed in a container of drug-free water for an acclimation
period of one hour. Following acclimation, the compartmentalized container was lifted and transferred to a second container containing either drug-free water (d-amphetamine sulfate 0.0 μM) for control trials, or one of three concentrations of d-amphetamine sulfate (0.5 μM, 5.0 μM, or 50.0 μM) for test trials, for an exposure period of 24 hours (experimental design outlined in Figure 1.3).

Figure 1.3: Schematic outlining the experimental design

During the one-hour acclimation period, the first two hours (early exposure) and last two hours (late exposure) of the 24-hour test period, crayfish movements were recorded at a rate of 4 frames s⁻¹ with a Microsoft Lifecam Studio 1080p HD camera (1920 x 1080 pixel resolution) mounted above the center of the testing arena. Custom video-tracking software (available at <http://iEthology.com>) recorded into a text file for subsequent behavioral analysis, the time stamps and x, y Cartesian coordinates of each individual’s position in real time (visualization of coordinate tracking shown in Figure 1.6). The experimental apparatus had diffuse light entering from above and was cordoned off with a black curtain to minimize light and other visual
disturbances to both the crayfish and the video tracker. After the exposure period, crayfish were immediately removed from the experimental arena and anesthetized on ice before further processing for molecular analysis. Between experimental trials, arenas and containers were rinsed with alcohol, water, and then air-dried to remove all traces of drug and chemical cues.

**Behavioral Analysis**

Juvenile *P. f. f. virginalis* are relatively small and largely translucent, with the darkest area generally being where consumed food in the stomach can be seen through the carapace. Consequently, tracking software would occasionally have difficulty discerning the crayfish from background shadows in the arena, which resulted in occasional dropped data frames. Because juvenile crayfish have a relatively slow walking speed, these dropped data frames had minimal impact on the final calculation of total distance travelled. Video tracking data was accumulated at an average rate of 254 frames per minute; minutes with no data totaled only 0.6%. The x, y Cartesian coordinates recorded by the tracking software were converted from pixel units into real distances (mm). Euclidian distance between coordinates was calculated to obtain the total distance travelled for each individual. Coordinate data were plotted and visually checked for errors: large jumps in position were filtered from data before further analysis was performed. To remove flickering in recorded position of crayfish, a threshold of 1 mm was considered no movement and a moving average filter of 5 frames was applied to movement calculations. Individuals that molted during testing, or for which more than 10% data was lost in any one recording session, were omitted from the data set entirely for statistical analysis (n = 5). The final analysis sample size after data clean-up for each treatment group was as follows: 0.0 µM (n=11), 0.5 µM (n=10), 5.0 µM (n=11), and 50.0 µM (n=11). Behavioral analysis was conducted on the
first 30 minutes of data from each recording session; due to technical error, data for some recording sessions was not recorded beyond this time frame.

**Statistical Analysis**

Custom scripts written in Python Version 3.4.2 (Python Software Foundation; <http://www.python.org>) were used to generate data sets used for descriptive and inferential statistics. Supporting packages/ functions installed were numpy, matplotlib, and pyplot. Descriptive statistics tabulated total distance travelled over the first 30 minutes of each recording session. Statistical analyses were conducted with R (version 3.2.3, <http://www.R-project.org>) with supporting installed packages R.utils, multcomp, AOV, boot, and car. In some datasets, treatment groups showed homoscedasticity, or equality of variance, (Levene’s Test) in the acclimation period, but there was large individual variation in response to drug exposure and data in the early and late exposure periods were not homoscedastic. Therefore, an ANOVA was run through bootstrap resampling (n=10,000) with a confidence interval of 95%; pairwise contrasts were performed to discern differences between control and treatment groups. In datasets that showed homoscedasticity, significance was tested using a one-way ANOVA (R package AOV), with a Bonferroni correction applied, and the post-hoc Dunnett’s test was used to determine the differences between treatment groups to the control group with pairwise comparisons.

**Euthanasia, Dissection and Brain Extraction**

Experimental crayfish were euthanized and dissected immediately following the 24-hour exposure period. After being deeply anaesthetized in an ice-slurry for at least 20 minutes, the crayfish was decapitated, the antennae were removed, and the head was mounted and pinned onto a Sylgard dish anterior side down and covered in 125 mM saline solution. Under a
microscope, what remained of the stomach, antennal glands, and other tissue was removed to expose the brain. The lateral brain connections and the eyestalks were dissected and the brain was removed from the head and placed on a wet slide where any non-neural tissue was removed. The crayfish body and the brain were placed in separate, correspondingly labeled containers and stored at -80°C for subsequent genetic analysis.

RESULTS

Locomotion

Crayfish of all treatment groups travelled the furthest distance during the acclimation period when they were first introduced to, and were exploring, the novel environment of the test arena: 0.0 µM (4584.7 mm; SE ± 266.5), 0.5 µM (4895.2 mm; SE ± 276.3), 5.0 µM (3566.4 mm; SE ± 308.7), and 50.0 µM (4111.3 mm; SE ± 362.1) (Figure 1.4). There was no significant difference in the distance travelled between treatment groups (p = 0.133). Over time, crayfish of all treatment groups slowly reduced their locomotion, covering smaller distances in each successive recording period.

During early exposure, when treatment groups were first exposed to varying concentrations of d-amphetamine sulfate, there was a significant effect due to treatment (p = 0.006). The 50 µM treatment group travelled a significantly greater distance, averaging 2828.4 mm (SE ± 306.5; Pairwise contrast p < 0.001), compared to the control group which travelled an average of 1514.4 mm (SE ± 205.6). There was no significant difference between the control group and the 0.5 µM treatment group which travelled an average of 2171.6 mm (SE ± 343.7; Pairwise contrast p = 0.201), nor with the 5.0 µM treatment group which travelled an average of 1083.3 mm (SE ± 343.7; Pairwise contrast p = 0.508).
During late exposure, after animals had remained in exposure tank overnight, there was a significant difference observed in distance travelled due to treatment ($p = 0.028$), with the control animals travelling further (1637.6 mm; SE ± 367.8) than any of the treatment groups: 0.5 µM (776.8 mm; SE ± 194.2), 5.0 µM (567.4 mm; SE ± 148.6), or 50.0 µM (813.9 mm; SE ± 200.3) (Figure 1.4).

A great deal of variability was observed amongst individual responses to first drug exposure. In order to better control for this large individual variation, the difference travelled between the early exposure and the acclimation period was calculated for each individual, then averaged together within each treatment group (Figure 1.5). This revealed a relationship resembling a dose-response curve in which the 0.0 µM control group exhibited the smallest reduction in distance travelled (2981.3 mm; SE ± 301.9), followed by 0.5 µM (2636.9 mm; SE ± 402.1), 5.0 µM (2369.3 mm; SE ± 302.3), and 50.0 µM (1216.6 mm; 320.6). A significant treatment effect was detected ($F(3,39) = 4.924$, $p = 0.005$). The Dunnett’s test revealed a significant difference ($p = 0.002$) between the 50.0 µM and control groups, but no significant difference between the control and either the 0.5 µM group ($p = 0.831$) or the 5.0 µM group ($p = 0.458$).
Figure 1.4: Mean distance travelled in the first 30 minutes of each recording period for d-amphetamine sulfate concentrations from 0.0 to 50.0 µM. As data does not show a normal distribution and does not pass the Levene’s test for homoscedasticity, an ANOVA was conducted with bootstrap resampling (n=10,000). No significant difference between treatment groups was observed in the acclimation period ($p = 0.133$). However, a significant difference was observed between treatment groups after crayfish were exposed to amphetamine (early exposure $p = 0.006$; late exposure $p = 0.028$). Bootstrapping pairwise contrasts revealed that, when compared to the 0.0 µM control group, the 50.0 µM treatment group was significantly different in the early exposure ($p < 0.001$) and all treatment groups showed a significant difference in the late exposure (0.5 µM, $p < 0.001$; 5.0 µM, $p < 0.001$; 50.0 µM, $p < 0.001$).
Figure 1.5: Psychostimulant effects of d-amphetamine sulfate concentrations from 0.0 to 50.0 µM: difference between mean distance travelled by individuals in first 30 minutes of early exposure and acclimation period. There was a significant difference due to treatment ($p = 0.005$), with Dunnett’s test differentiating the 50 µM treatment group from the control ($p = 0.002$).

Space Use and Walking Pattern Behavioral Responses

As crayfish generally show an edge-preference (Figure 1.6), each arena compartment was divided into space divisions of “edge” (inner 15 mm around compartment perimeter) and “middle” (the remaining inner portion of arena). Percent time spent and distance travelled in each area was quantified for all treatment groups for each recording session. Crayfish of all treatment groups displayed a clear preference for the edge and there was no significant difference between treatment groups (data not shown).
Figure 1.6: Example of crayfish movement patterns over a one-hour period in drug-free water. Tracking coordinates overlaid on picture of experimental arena.

Crayfish move with an intermittent walking pattern in which they take frequent pauses to sample with their antenna before continuing. In order to characterize whether amphetamine exposure affected this behavioral pattern, pause duration, number of pauses, and duration of walking between pauses were quantified, both for the entire arena and for each arena space (edge and middle) separately. No significant differences were found between treatment groups for any of these parameters (data not shown).

DISCUSSION

An ideal experimental system for studying the epigenetics of addiction would combine an animal model with little or no genetic variation, with a non-invasive mode of drug delivery. The
results of this study demonstrate that the parthenogenetic marbled crayfish, *P. f. f. virginalis*, is just such a system, displaying a significant, dose-dependent, psychostimulant-induced locomotor response when immersed in water containing d-amphetamine sulfate.

**Crayfish Locomotion in Response to Novel Arena**

The uniformly high levels of locomotion observed on initial introduction of crayfish into the drug-free environment are typical of animals exposed to novel stimuli. Under natural conditions, such behaviors facilitate exploration, the acquisition of information, and the procurement of other resources essential for survival (Di Chiara and Bassareo, 2007; Marinelli, 2005). Similarly, the reduction of locomotion as crayfish became accustomed to the test arena is concordant with prior observations in *O. rusticus*, where animals with initial high levels of exploration, gradually decrease movement over time as they habituate, until they eventually settle into one location along the edge of the arena (Panksepp and Huber, 2004; Alcaro et al., 2011).

**Effects of Initial Drug Exposure**

On initial exposure to d-amphetamine, the 50.0 µM treatment group of *P. f. f. virginalis* showed a significant increase in locomotion compared to that of control animals during the first 30 minutes of exposure. This result is congruent with the typical psychostimulant-induced effects noted in both a range of mammals (Fog, 1969; Schiorring, 1971; Segal and Mandell, 1974; Hoebel et al., 1983), as well as in other invertebrate systems (reviewed in Wolf and Heberlein, 2003). In *O. rusticus*, d-amphetamine exposure stimulates enhanced locomotion, along with other exploratory behaviors, including rearing and antennal sampling (Alcaro et al., 2011).

The behavioral locomotor variability between individuals appears to be reduced in *P. f. f. virginalis* compared to that observed in *O. rusticus*. The degree of variation in locomotion
observed in the acclimation period in *P. f. f. virginalis* was about half that observed in *O. rusticus* (Datta, 2015) and one sixth of what was observed in Bhimani and Huber (2015). The greater locomotor variability observed in *O. rusticus* may be attributed to the fact that these were wild-caught individuals of a sexually-reproducing species, with a (presumably) higher degree of genetic and developmental variation compared to *P. f. f. virginalis*, which were lab-reared, asexually reproducing genetic clones. It is notable that individual behavioral and morphological variation was observed in *P. f. f. virginalis* regardless of genetic identity, likely as a consequence of uncontrolled microenvironmental influences on development.

Evidence suggests that exploratory behaviors are specifically mediated by the dopaminergic system in both mammals and invertebrates (Hills et al., 2004; reviewed in Alcaro, et al., 2007). This dopaminergic system is thought to be a key component in the control of the complex appetitive-motivational neural circuit (Alcaro et al., 2007), as its primary function is to sustain exploratory behaviors and reinforce adaptive behaviors that increase the acquisition of rewards (food, shelter, sex, etc.). Therefore, activation of these neural systems by human drugs of abuse is perceived as rewarding (Di Chiara and Bassareo, 2007), and may result in an increase of exploratory behaviors in crayfish.

The appetitive-motivational neural circuit also appears to be stimulated by, in addition to more direct rewards, novel stimuli. In *O. rusticus*, the simultaneous presentation of a novel environment with the onset of drug administration facilitated an increased psychostimulant behavioral response to d-amphetamine (Alcaro et al., 2011). In the current study, *P. f. f. virginalis* were acclimatized for one hour in the test arena prior to drug exposure; an experimental design which effectively decoupled the d-amphetamine-induced locomotor response from the novel stimulus-induced locomotor response. This demonstrated that d-
amphetamine independently induces a psychostimulant response in *P. f. f. virginalis*. However, this may also be one reason why a significant dose-response curve was not demonstrated. Had the test subjects been exposed to the novel arena and d-amphetamine simultaneously, individuals exposed to lower concentrations (0.5 and 5.0 µM) may have demonstrated a greater locomotor response, which may have exaggerated the data trend observed in the data that was controlled for individual-variation (Figure 1.5). In order to determine the effect that novel stimuli have on *P. f. f. virginalis* response to psychostimulants, future research could characterize the locomotor response elicited by the presentation of different concentrations of d-amphetamine in a novel environment.

The observed psychomotor effects establish immersion as an effective method of drug delivery in juvenile crayfish, although knowledge of drug absorption and metabolism rates is limited and requires more study. Possible pathways of drug intake include absorption through the respiratory system via the gills and through the gastrointestinal tract. The method of drug delivery will have a direct influence on crayfish locomotor response. According to the rate hypothesis of psychoactive drug action, the quicker a drug reaches the brain, the greater is both the perceived reward and the resulting reinforcing effect (Nelson et al., 2006; de Wit et al., 1992; Gorelick, 1998). Higher drug infusion rates generate greater subjective responses (Abreu et al., 2001; Nelson et al., 2006), and when given a choice, rats have been shown to prefer higher infusion rates of the same drug dose (Schindler et al., 2009). Furthermore, a rapid delivery time frame results in an intensified motivation for obtaining the drug (Minogianis et al., 2013). Consequently, the route of administration affects how reinforcing the drug is perceived to be, due to varying pharmacokinetic effects in when the drug reaches the brain (Samaha and Robinson, 2005; Volkow et al., 2000). In crayfish, Datta (2015) demonstrated with *O. rusticus* that the
reinforcing properties of drug-reward were much more robust when psychostimulants were injected directly into the brain, compared to injection into the general hemolymph circulation. By extension then, simple immersion of the animals into drug-infused water is almost certain to have slowed the time course of drug delivery to the active sites in the nervous system, attenuated the behavioral responses to the drug, and diminished any resultant reinforcing effect. However, because route of administration can influence drug effect, it is important to have methods of drug delivery in animal models that are comparable to the methods humans use to self-administer drugs (Sovik, 2013). The route of administration the crayfish are experiencing, by absorbing drug through the respiratory system and the digestive tract simultaneously, may be likened to the human methods of inhalation and insufflation. Therefore, this mode of drug delivery in crayfish may offer a nice complement for studying the differential effects route of drug administration have on the molecular processes and behavioral responses of human drugs of abuse, although research is needed to determine the validity of this comparison.

Drug exposure via immersion may also directly affect a crayfish’s behavioral response, in ways other than locomotion. Crayfish exploration relies heavily upon mechanoreception from antennal sampling (Koch et al. 2006; Basil and Sandeman, 2000), with the antennae and antennules conveying perceived sensory information to the olfactory lobe in the central nervous system (Mellon, 2000; Sullivan and Beltz, 2005). The olfactory lobe is mediated by dopamine and serotonin (Sandeman and Sandeman, 1987; Sandeman et al., 1995; Schmidt, 1997), and therefore, because amphetamine produces psychostimulant effects by hijacking dopamine-mediating cellular mechanisms, the olfactory lobe is likely a site of action for psychostimulants. As such, increased dopamine signals in the olfactory lobe may induce crayfish to engage in increased antennal sampling behavior. P. f. f. virginalis treated by direct immersion in d-
amphetamine were simultaneously being administered drug while also actively sampling a novel chemical in their environment. This may have induced crayfish to spend a greater proportion of their time in antennal sampling exploratory behaviors compared to *O. rusticus*, which were administered drug through direct injection to the brain and spent approximately equal time engaged in the exploratory behaviors of locomotion and antennal sampling (Alcaro et al., 2011; Panksepp and Huber, 2004). In preliminary experiments, it was observed that crayfish did increase antennal sampling rate when exposed to amphetamine, however this was not measured in the current study. Quantifying this exploratory response of crayfish to a drug-infused water route of exposure is a possible avenue for future study.

**Effects of Continuous Chronic Exposure**

Following extended immersion (i.e. ‘late exposure’), all d-amphetamine treatment groups displayed significantly reduced levels of locomotion compared to that observed in the control (no drug) group. At this point, test subjects had been exposed to d-amphetamine for a period of 22-24 hours, a duration which is considered continuous chronic drug exposure (Robinson and Becker, 1986). Amphetamine is relatively stable in urine samples with a pH of 6, demonstrating only a 1.3% degradation after three days at 37°C (Jimenez et al., 2006). Therefore, it is likely that the d-amphetamine to which *P. f. f. virginalis* was exposed, at a pH of 6.8 and a temperature of 21°C, was still biologically active throughout the entire 24-hour duration of the experiment. The ability to test continuous chronic d-amphetamine exposure was made possible by the mode of drug delivery employed in this study, by direct immersion in drug-infused water. When psychostimulant effects and drug-induced reward to the administration of cocaine and amphetamine were demonstrated in *O. rusticus*, drug delivery was achieved by direct injection through a cannula surgically implanted in the crayfish carapace (Datta, 2015; Nathaniel et al.,
Such invasive procedures have limited utility in epigenetic studies where the stress experienced by experimental animals can, itself, induce epigenetic changes in neural tissue (Hunter et al., 2009). As such, this non-invasive method lends itself nicely for epigenetic studies.

Continuous chronic amphetamine exposure in mammals (humans and rats) results in hyperactivity, and increased locomotion, shortly after the onset of administration (reviewed in Ellison and Eison, 1983). Rats are administered continuous chronic drug through the use of subcutaneously-implanted, slow-release pellets containing 50 mg amphetamine, which maintain brain drug levels for 10 days (Huberman et al., 1997). In rats however, a few hours after the onset of exposure, this hyperactivity and increased locomotion is gradually curtailed, over approximately an eight-hour period, until their behavior is consumed by motor stereotypies. These stereotyped behaviors last for approximately two days and are then followed by a lengthy period of inactivity (reviewed in Ellison and Eison, 1983). This is similar to the time course of locomotor activity that was generally observed in *P. f. f. virginalis*: the highest d-amphetamine treatment group (50.0 µM) displayed a significantly higher level of locomotion during the early exposure, then in the late exposure all treatment groups (0.5, 5.0, and 50.0 µM) displayed reduced locomotion compared with controls. However, the increased locomotor response demonstrated in the 50.0 µM treatment group had a much faster onset (within 30 minutes) compared to that seen in rats, which only started to show increased locomotion after a few hours (Ellison and Eison, 1983). Therefore, it is possible that, compared to rats, the behavioral response time course of chronically administered d-amphetamine in *P. f. f. Virginalis* may be accelerated.

In mammals, amphetamine exposure induces these behavioral changes by stimulating the release of dopamine and inhibiting the dopamine reuptake transporter (Hyman et al., 2006). This
results in prolonged and elevated levels of dopamine that can last for hours, disrupting typical patterns of dopamine release and motor activity (Knutson et al., 2004). In preliminary observations of exposure to a high d-amphetamine concentration (50.0 µM), crayfish started to display motor stereotypies, such as leg twitching, within an hour from onset of continuous administration. *O. rusticus* also display muscle tremors in walking legs when administered high levels of amphetamine (Panksepp et al., 2004). This behavioral response to amphetamine inevitably results in reduced locomotion. However, in mammals, the disruption of dopamine systems that result from chronic exposure also eventually leads to a decrease of stimulation-dependent dopamine release (Schmitz et al., 2001), which is associated with hypoactivity (Johnels, 1982; Monti et al., 1990) and results in amphetamine tolerance (Shoblock et al., 2003). This tolerance and hypoactivity results from a depletion of striatal dopamine and dopamine receptors, which may be due to dopamine terminal degeneration (a.k.a. amphetamine neurotoxicity) (reviewed in Robinson and Becker, 1986). Thus, by the late exposure period, treatment animals may be demonstrating continuous chronic amphetamine exposure-induced motor stereotypies or hypoactivity.

**Conclusion**

The present study has demonstrated (1) psychostimulant-induced locomotor effects in a parthenogenetic crayfish, *Procambarus fallax* forma *virginalis*, (2) locomotor response after continuous amphetamine administration, and (3) an effective non-invasive method of drug delivery for crustaceans. With initial onset of d-amphetamine administration, *P. f. f. virginalis* demonstrated behavioral and locomotor hyperactivity responses, expressed as an increase in exploratory behaviors, which are comparable to those that have been previously observed in *O. rusticus* (Alcaro et al., 2011; Panksepp and Huber, 2004). For the first time, however, the
locomotor response of a decapod crustacean has been characterized to continuous chronic d-amphetamine exposure. After continuous chronic d-amphetamine administration, all treatment crayfish demonstrated reduced locomotion, which offers a possible avenue for exploring the molecular mechanisms involved in drug-tolerance.

The non-invasive method of drug administration used in this study offers distinct advantages and opportunities for future research: it is advantageous for epigenetic studies because it minimizes potential stressors, it allows for the use of smaller animals with which direct injection is not feasible, and it may be a method for drug-delivery in animal models that may be comparable to the human method of inhalation or insufflation. Lastly, as a parthenogenetic invertebrate, *P. f. f. virginalis* possesses a genetically identical, modularly organized and relatively simple nervous system with a conserved neural reward circuit. Therefore, *P. f. f. virginalis* offers an elegant model system available for exploring many different avenues of research into the basic underlying behavioral, neural, and epigenetic mechanisms of drug-induced neural plasticity.

REFERENCES


*Behavioural Brain Research*, 197(2), 331-338.


CHAPTER II: OPTIMIZATION OF GENOMIC DNA EXTRATION FROM MARBLED CRAYFISH (Procambarus fallax forma virginalis) BRAIN TISSUE

INTRODUCTION

Epigenetic changes occurring in neural and brain tissue may affect an animal’s behavior via subsequent changes in gene expression levels (reviewed in Crews, 2010; Nestler, 2014). Several studies, in humans and mammalian animal models, suggest that DNA methylation is one of the critical epigenetic regulators of adult neural gene expression (Brooks et al., 1996; Endres et al., 2000; Endres et al., 2001; Fan et al., 2001). For instance, rapid DNA methylation regulation has been demonstrated in the hippocampus following fear conditioning (Miller and Sweatt, 2007). DNA methylation changes in the nucleus accumbens may also be an important mechanism underlying behavioral sensitization to repeated cocaine exposure (Anier et al., 2010). Although DNA methylation seems to play a vital role in neural gene expression, its processes are not well understood. Robust and effective methods for extracting genomic DNA (gDNA) from target neural tissues are thus vital to studying the underlying epigenetic mechanisms of behavior.

Although global DNA methylation has been implicated as a vital mechanism regulating neural gene expression and, by extension, behavior, the large genome and high (ca. 70%) methylation rates found in mammals represent difficulties in parsing out the particularities of the processes. Because invertebrates have smaller genomes (Adams, 2000; Weinstock et al., 2006), global DNA methylation studies are more feasible. Unfortunately, many invertebrate models, including C. elegans and Drosophila, do not have fully functional DNA methylation systems at all life stages and are therefore unsuitable for these studies (reviewed in Lyko and Maleszka, 2011).
The marbled crayfish (*P. f. f. virginalis*), has significant advantages for research into the epigenetic mechanisms of behavior. It demonstrates DNA methylation during all life stages (Vogt et al., 2008), and exhibits relatively low levels of global DNA methylation (between 1-2%) for the two tissues (abdominal musculature and hepatopancreas) that have been measured thus far (Vogt et al., 2008; Schiewek et al., 2007). Because small changes in global DNA methylation can have large downstream effects on phenotype (Hinendleder et al., 2006) this is advantageous as it is easier to detect small changes in methylation when overall methylation rate is low (reviewed in Lyko and Maleszka, 2011). Additionally, *P. f. f. virginalis* is the only decapod of over 10,000, that reproduces obligatorily through apomictic parthenogenesis: all individuals are genetically identical, asexually reproducing, females (Scholtz et al., 2003; Martin et al., 2007; Vogt et al., 2008). Minimizing genetic variability amongst experimental animals removes a significant confounding factor. However, behavioral epigenetic changes have yet to be studied in the crayfish.

Common problems in working with invertebrates derive from the related challenges inherent in lab rearing requirements and small body size, which limits tissue sample sizes. Consequently, tissue samples from multiple individuals often need to be pooled in order to obtain sufficient DNA for genetic analyses (Wang et al., 2006; Lockett et al., 2012). Moreover, epigenetic modifications are tissue-specific (reviewed in Jaenisch and Bird, 2003), hence behavioral epigenetic studies require DNA extracted from neural tissue, a fatty material which yields lower concentrations of DNA. In order to fully exploit the potential of invertebrates as model systems for studying the epigenetics of behavior, it is essential to have effective methods for DNA extraction from small sample sizes.
The present study seeks to provide the parthenogenetic *P. f. f. virginalis* as a model organism for studying the molecular mechanisms underlying behavioral epigenetics. Modifying the procedure of the Qiagen Puregene Blood Core Kit B (Qiagen, MD) for small tissue samples, I optimized the method for gDNA extraction from invertebrate brain tissue. The results of this work provide the methodological foundation for use of the parthenogenetic *P. f. f. virginalis* to study the epigenetic processes underlying behavior.

**METHODS**

**Euthanasia, Dissection and Brain Extraction**

Juvenile *P. f. f. virginalis* (15-19 mm; 86.2-136.0 mg) were dissected immediately after being deeply anaesthetized in an ice-slurry for at least 20 minutes. The crayfish was decapitated, the antennae were removed, and the head was mounted and pinned onto a Sylgard dish anterior side down and covered in 125 mM saline solution. Under a microscope, what remained of the stomach, antennal glands, and other tissue was removed to expose the brain. The lateral brain connections and eyestalks were dissected and the brain moved from the head to a wet slide where any remaining non-neural tissue was removed. The crayfish body and the brain were placed in separate, correspondingly labeled, screw-top 1.5 mL microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -20°C to await subsequent processing.

**Preparation of Crayfish Brain and Abdominal Tissue Samples**

Due to the small size of the juvenile crayfish brain, samples were prepared with an increasing number of brains pooled together in order to optimize the extraction of total amount of DNA necessary for subsequent genetic analysis, with minimal number of samples pooled.
For abdomen samples, frozen body samples (brains removed) were thawed on ice. The abdomen was dissected from the body and the carapace removed. Abdominal tissue was then dissected and weighed into desired quantities. Prepared samples were placed in separate containers for subsequent analysis.

**Genomic DNA Extraction**

Genomic DNA was isolated from frozen samples of brain or abdomen tissue with Qiagen Puregene Blood Core Kit B according to the ‘DNA purification from tissue’ protocol included in the supplier’s instruction manual (Qiagen, MD). In order to accommodate small sample volume and optimize DNA yield, the supplier’s standard protocol was modified as follows.

Procedure for cell lysis from small samples: Frozen samples (each in 1.5 mL centrifuge tube) were placed in ice. Tissue cells were lysed by adding 300 µL Cell Lysis Solution together with five 1 mm glass beads to each sample, then vortexed for 20 seconds. Proteinase K (30 mg) was added and then mixed by 25 gentle inversions. In order to gain maximum DNA yield, samples were incubated at 55ºC in an AccuBlock Digital Dry Bath (Labnet international, Inc.) overnight (~12 hr).

Procedure for gDNA extraction from small samples: Samples were removed from the dry bath and treated with RNase A solution (1.5 mg), gently inverted 25 times, and incubated at 37ºC for 60 minutes. Samples were cooled for 1 minute on ice. Proteins were then precipitated by vortexing at high speed for 20 seconds with 300 µL Protein Precipitation Solution, followed by 5 minutes’ centrifugation at 11,000 rpm. The supernatant was transferred to new centrifuge tubes containing 300 µL isopropanol, gently inverted 50 times, and then DNA was precipitated by centrifugation for 5 minutes at 11,000 rpm. The isopropanol was discarded. The DNA pellet was then washed with 300 µL of 70% ethanol and centrifuged at 11,000 rpm for 5 minutes. Ethanol
supernatant was removed and DNA pellet was allowed to air dry at 55°C for 5 minutes, until no trace of ethanol remained. DNA was then resuspended by vortexing at medium speed for 5 seconds with 20 µL distilled water. Samples were then incubated for 1 hour at 65°C to resuspend DNA, after which they were incubated at 21°C with gentle shaking (35 rpm) for 18 hours. The next day, samples were briefly centrifuged and analyzed for DNA purity and concentration using a Thermoscientific NanoDrop 2000 Spectrophotometer. Final gDNA preparations were either run on a gel as an additional quality check, or stored at -20°C for subsequent molecular analysis.

**Methylation-sensitive Enzyme Digestion**

Methylation sensitive enzyme digestion was performed with the procedure described in Singh (2014). Each gDNA sample was separately digested with Msp I and Hpa II (New England BioLabs Inc.) which both recognize the sequence (5’-CCGG-3’). The presence or absence of a methyl group attached to the internal cytosine determines whether either enzyme will cut or not. Although Msp I cuts this sequence regardless of methylation, Hpa II will only do so if the internal cytosine is unmethylated (McCelland et al., 1994). Samples were digested at 37°C overnight and then, to confirm digestion, run on a 1.5 % agarose gel.

An additional digestion of the samples was performed with the enzyme CpG Methyltransferase M.SssI and then with the enzyme McrBC (New England BioLabs Inc.). CpG Methyltransferase M.SssI adds a methyl group to the 5-carbon of CpG islands, recognizing the sequence (5’-CG-3’). McrBC cleaves these methylated CpG sites, but it does not recognize the methylation of the internal cytosine of (5’-CCGG-3’), which is the sequence recognized by Msp I and Hpa II. Genomic DNA samples digested with CpG Methyltransferase M.SssI were prepared with 1.4 µL dH₂O, 5 µL 10x NEB buffer 2, 5 µL of SAM (1600 µM), 36.1 µL gDNA (1000 ng), and 2.5 µL CpG Methyltransferase M.SssI (4 U/µL). Genomic DNA samples to be
digested with McrBC were prepared with 0.4 µL BSA (100x), 0.4 µL GTP (100x), 4 µL buffer (10x), 1 µL McrBC, and 14.2 µL gDNA (393 ng). Genomic DNA samples digested with CpG Methyltransferase M.SssI were digested at 37°C for one hour, and then 65°C for twenty minutes. Both gDNA samples that had and had not been digested with CpG Methyltransferase M.SssI were then digested with McrBC at 37°C for three hours and then at 65°C for twenty minutes.

RESULTS

Genomic DNA Extracted from Different Starting Amounts of Abdominal Tissue

When DNA extraction from *P. f. f. virginalis* abdominal tail tissue was conducted following the unmodified protocol for tissue samples from Qiagen Puregene Blood Core Kit B, the procedure resulted in no obtained product (data not shown). After procedure modification (detailed in methods above), each successively larger starting tissue sample yielded higher concentrations and total amounts of extracted gDNA (Table 2.1). The smallest starting tissue sample (1 mg) yielded a concentration of 8.5 ng/µL gDNA, and a total of 42.5 ng gDNA run on the gel is visible as a very faint band (Figure 2.1). Each larger starting tissue sample resulted in higher gDNA concentrations. Quality of DNA was visualized by agarose gel electrophoresis.
Table 2.1: Total genomic DNA extracted from various amounts of *P. f. f. virginalis* abdominal tissue.

<table>
<thead>
<tr>
<th>Starting tissue quantity (mg)</th>
<th>Extracted gDNA concentration (ng/µL)</th>
<th>Total gDNA yield (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.5</td>
<td>42.5</td>
</tr>
<tr>
<td>2</td>
<td>15.6</td>
<td>78.0</td>
</tr>
<tr>
<td>5</td>
<td>27.4</td>
<td>137.0</td>
</tr>
<tr>
<td>10</td>
<td>33.7</td>
<td>168.5</td>
</tr>
</tbody>
</table>

Figure 2.1: Genomic DNA extracted from varying amounts of *P. f. f. virginalis* abdominal tissue. Five µL of each extract was loaded on the gel. Amount of starting tissue and total DNA added to gel reported for each sample respectively: lane (1) 1 mg, 42.5 ng; (2) 2 mg, 78.0 ng; (3) 5 mg, 137.0 ng; (4) 10 mg, 168.5 ng. L = 1 kilobase pair DNA ladder (300 ng).
**Genomic DNA Extracted from Assorted Number of Pooled Whole Brains**

When first extracting DNA from crayfish brain tissue, all modified procedures detailed in methods for tissue samples from Qiagen Puregene Blood Core Kit B were followed apart from the cell lysis procedure, which was achieved by physical homogenization in liquid nitrogen. These early DNA extractions demonstrated that samples comprising a larger number of crayfish brains generally yielded higher concentrations of extracted gDNA (Table 2.2; Figure 2.2). The tissue samples shown in lanes 3 (homogenized) and 4 (not homogenized, Figure 2.2) were differentially treated in order to examine the effect of physical lysis on very small tissue samples. gDNA yields were comparable in both treatments. Most of the tissue from the sample initially containing 3 brains (lane 5, Figure 2.2) was lost during the cell lysis procedure due to human error, and therefore resulted in lower concentration of extracted gDNA. Overall, samples containing more than 1 brain demonstrated visible bands when run on agarose gel electrophoresis (with the exception of that in lane 5 which was lost due to a processing error).
Table 2.2: Total genomic DNA extracted from assorted number of pooled *P. f. f. virginalis* brains during the early stage of DNA extraction optimization. Much tissue from sample containing starting tissue quantity of three brains was lost due to an error in the cell lysis procedure, resulting in low total DNA extracted.

<table>
<thead>
<tr>
<th>Starting quantity of brains</th>
<th>Extracted gDNA concentration (ng/µL)</th>
<th>Total gDNA yield (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>35.0</td>
</tr>
<tr>
<td>1</td>
<td>4.7</td>
<td>23.5</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>28.0</td>
</tr>
<tr>
<td>2</td>
<td>5.9</td>
<td>29.5</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>22.5</td>
</tr>
<tr>
<td>6</td>
<td>25.3</td>
<td>126.5</td>
</tr>
</tbody>
</table>
Figure 2.2: Genomic DNA extracted from assorted number of pooled *P. f. f. virginalis* brains during the early stage of DNA extraction optimization. Number of brains per sample in lanes 1 through 6 are 1, 1, 2, 2, 3, and 6 respectively. Five µL of each extract was loaded on the gel. Total gDNA added to gel reported for each sample respectively: (1) 35 ng; (2) 23.5 ng; (3) 28.0 ng; (4) 29.5 ng; (5) 22.5 ng; (6) 126.5 ng. Much tissue from sample 5 was lost due to an error in the cell lysis procedure, resulting in low total DNA extracted. L = 1 kilobase pair DNA ladder (300 ng).
All subsequent gDNA extractions were conducted with the fully optimized method detailed in methods, with cell lysis conducted through physical homogenization with glass beads. Pools of three brains each were utilized and resulted in a mean concentration of 19.06 ng/µL ± 24.49 SD; mean purity = 1.83 ± 0.09, n = 10 (Table 3).

Table 2.3: Total genomic DNA extracted from pooled *P. f. f. virginalis* brains after optimization of the DNA extraction method.

<table>
<thead>
<tr>
<th>Starting number of brains</th>
<th>Purity (260/280)</th>
<th>Extracted gDNA concentration (ng/µL)</th>
<th>Total gDNA yield (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.75</td>
<td>8.7</td>
<td>174</td>
</tr>
<tr>
<td>3</td>
<td>1.82</td>
<td>9.8</td>
<td>196</td>
</tr>
<tr>
<td>3</td>
<td>1.80</td>
<td>90.9</td>
<td>1,818</td>
</tr>
<tr>
<td>3</td>
<td>1.82</td>
<td>11.6</td>
<td>232</td>
</tr>
<tr>
<td>3</td>
<td>1.80</td>
<td>9.3</td>
<td>186</td>
</tr>
<tr>
<td>3</td>
<td>2.06</td>
<td>4.6</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>1.77</td>
<td>6.8</td>
<td>136</td>
</tr>
<tr>
<td>3</td>
<td>1.95</td>
<td>8.8</td>
<td>176</td>
</tr>
<tr>
<td>3</td>
<td>1.75</td>
<td>23.8</td>
<td>476</td>
</tr>
<tr>
<td>3</td>
<td>1.77</td>
<td>16.3</td>
<td>326</td>
</tr>
</tbody>
</table>

**Methylation-Sensitive Enzyme Digests**

Controlled enzymatic digestion of both low (8.4 ng/µL) and high (21.2 ng/µL) concentrations of gDNA with Msp I and Hpa II separately resulted in smearing patterns (Figure 2.3). Both enzymes produced distinctly stronger gel banding patterns in the higher concentration
digests. Results from CpG Methyltransferase M.SssI and McrBC digests were unable to be interpreted due to technical error (data not shown).

**Figure 2.3:** Methylation sensitive enzyme (Msp I and Hpa II) digests of low and high concentrations of *P. f. f. virginalis* gDNA. Samples were as follows: (1) low concentration undigested gDNA (84.0 ng); (2) low concentration (84.0 ng) gDNA digested with Msp I; (3) low concentration (84.0 ng) gDNA digested with Hpa II; (4) high concentration undigested gDNA (212.0 ng); (5) high concentration (212.0 ng) gDNA digested with Msp I; (6) high concentration (212.0 ng) gDNA digested with Hpa II. L = 1 kilobase pair DNA ladder (300 ng).

**DISCUSSION**

The tissue-specific nature of epigenetic changes (reviewed in Jaenisch and Bird, 2003) necessitates the use of DNA extracted from neural tissue if one is interested in studying
epigenetic mechanisms that influence behavior. Using invertebrate brains represents technical challenges in small sample sizes and fatty tissues with low DNA yield. Developing an effective gDNA extraction method for such tissues will enable much research to be conducted using potentially useful model organisms, such as the parthenogenetic marbled crayfish, to parse out the underlying mechanisms involved in epigenetically-mediated behaviors.

**Genomic DNA Extraction**

Using samples of abdominal muscle tissue (ranging from 1 mg to 10 mg) from juvenile *P. f. f. virginalis*, first I confirmed that the Qiagen Puregene Blood Core Kit B (which is produced for the extraction of DNA from mammalian blood cells), with some modifications, could be used to extract high-quality and high quantities of gDNA from invertebrate crayfish neural tissue (mean purity = 1.83 ± 0.09, n = 10). As expected, larger quantities of starting material provided concomitantly larger gDNA yields.

Results of early attempts showed similarly effective isolation of gDNA from neural brain tissue (Figure 2.2) with a concentration of 5.85 ± 1.15 ng/µL and a mean purity of 2.17 ± 0.26 from starting tissue of single brain. Following a series of optimization procedures (detailed below), later attempts provided much higher yields of gDNA, on the order of 19.06 ng/µL ± 24.49 SD from samples of three pooled brains. Different amounts of total gDNA extracted from starting tissue of three pooled brains may be due to different donor specimen size and/or human error. These three pooled brain samples were preserved for subsequent genetic analysis and were hence not run through gel electrophoresis nor illustrated here.

Similar to initial tests using muscle tissue, larger amounts of starting brain tissue generally yielded higher gDNA concentrations and total amounts. Tests in which two equal samples of starting neural tissue were subjected to different physical treatments yielded
interesting results. Whether samples underwent additional physical disruption during the cell
lysis procedure or not, both treatments yielded approximately equivalent amounts of high quality
gDNA. This is comparable to what was found by Wang et al. (2006), who reported that delicate
tissues, such as the honey bee brain, dissolve sufficiently well in NTE buffer and Proteinase K as
to render physical disruption superfluous. This suggests that the physical homogenization step
may be omitted in the case of crayfish brain tissue, although it may be of benefit in processing
larger volume samples. Physical homogenization of small invertebrate neural tissue samples in
liquid nitrogen represented significant difficulties. When frozen in liquid nitrogen, the neural
tissue hardened and would often stick to the pestle, preventing it from being broken up or
pulverized and would result in much of the tissue sample being lost. Homogenization via glass
beads was a much more effective cell lysis method and resulted in fewer complications.

**Methylation-Sensitive Enzyme Digests**

Methylation-sensitive enzymes Msp I and Hpa II, both resulted in smears on digesting *P.
f. f. virginalis* gDNA. Since both of these enzymes recognize the identical sequence (5’-CCGG-
3’: Msp I cuts regardless of methylation and Hpa II cuts only when the sequence is
unmethylated), this demonstrates that *P. f. f. virginalis* possesses a substantial number of this
motif in its genome. Previous studies have demonstrated that the *P. f. f. virginalis*
hepatopancreas and abdominal musculature exhibits low levels of methylation (Vogt et al., 2008;
Schiewek et al., 2007). Quantification of methylation levels in the brain of *P. f. f. virginalis* has
not previously been reported. In addition to using Msp I and Hpa II, the use of the enzymes CpG
Methyltransferase M.SssI, which adds methyl groups to the 5-carbon of cytosines located in CpG
islands in the sequence (5’-CG-3’), and McrBC, which cleaves at said methylated locations, may
provide a useful accompaniment in quantifying methylation levels in the *P. f. f. virginalis* neural
genome. Although I was unable to reach this endpoint, the present study does provide the basis on which such measures may be determined in the future.

This optimized method for extracting genomic DNA from crayfish brain tissue will significantly enhance the utility of the parthenogenetic marbled crayfish as an animal model for use in research studying the molecular epigenetic processes underlying behavior.

REFERENCES


