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DEPARTMENT OF BIOBEHAVIORAL HEALTH

EFFECTS OF ADOLESCENT LABORED BREATHING ON ANXIETY-LIKE BEHAVIORS AND NEUROENDOCRINE FUNCTION IN ADULT MICE

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ABSTRACT

BACKGROUND: In humans, individuals who develop asthma in adolescence have been found to have a doubled risk for developing internalizing disorders such as adult anxiety and depression. The goal of this study is help elucidate the physiological and behavioral mechanisms underlying this co-morbidity and potential bidirectional relationship using a rodent model of asthma. Specifically, this study focuses on experimentally determining how induced severe and frequent labored breathing in periadolescent mice affects adult anxiety-like behaviors and both glucocorticoid and serotonin regulation. The goals of the study are clinically relevant in evaluating possible interventions for minimizing the risk of anxiety in asthmatic adolescents.

METHODS: To induce labored breathing, aerosolized methacholine was administered to mice. Mice were divided into one of five treatment groups: **(1)** non-handled control (n=9), **(2)** vehicle control (n=10), **(3)** low methacholine exposure (n=12), **(4)** medium methacholine exposure (n=12), and **(5)** high methacholine exposure (n=13). To measure baseline anxiety-related temperament, neonatal isolation-induced ultrasonic vocalizations (USV) were used to characterize high- and low- aversive affective (i.e. anxiety-prone) pups prior to methacholine treatment. On PND 22-57, experimental groups (N = 3-5) were treated with the bronchoconstrictor methacholine while procedural controls received PBS saline using similar methods and non-handle controls received no treatment. Labored breathing was measured as enhanced pause (Penh) via whole-body plethysmography. In adulthood, anxiety and depression-like behaviors (PND 60, 63) relative to baseline individual differences in anxiety were measured by standard tests such as exploratory activity in the elevated plus maze (EPM) and anhedonic measures in the sucrose preference test (SPT). To measure glucocorticoid regulation, fecal

samples were collected (i.e. for analysis of fecal corticosterone metabolites) for a two-day period (PND 58-59). To measure serotonin regulation, brainstem sections were preserved for analysis of serotonin transporter gene expression (*Sert*), and serum samples were collected at necropsy (PND 80) and analyzed for corticosterone levels.

RESULTS: Mice with greater labored breathing (Penh) during adolescence were found to have elevated adult anxiety-like behavior (EPM) compared to controls, regardless of basal affective behavior (USV). Increased labored breathing was found to have a significant linear relationship with adult anxiety behavior, explaining 19.0% of the variance in anxiety-related behavior after accounting for cohort effects. Penh and EPM were not significantly associated with *Sert* gene expression, CORT levels, or sucrose preference. Interestingly, body weight had a significant effect on Penh values, presumably because labored breathing was more difficult to detect in small (i.e. <15 g) periadolescent mice.

CONCLUSION: Labored breathing had a significant linear relationship with anxiety-related behavior in adulthood. Going forward, the findings from this study may help researchers develop more accurate mouse models for studying the relationship between adolescent asthma and adult anxiety.

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Chapter 1

Introduction

Asthma is a chronic inflammatory disorder affecting over 25 million Americans each year (NIH, 2014). With one in ten children experiencing asthma and associated symptoms of wheezing, chest tightness, and labored breathing, it is the most common respiratory illness affecting youth worldwide, especially adolescents (ISAAC, 1998).

Several studies to date have demonstrated a strong co-morbidity between asthma in adolescence and the development of internalizing disorders later in life. A study conducted by researchers at the University of Washington in 2001 found that 54% of adult asthmatic patients suffered from disorders such as major depression and generalized anxiety disorder, suggesting that predispositions to certain psychiatric conditions are common among asthmatic patients (Afari, 2001). Studies have also shown increased anxiety- and depression-related behaviors and temperament among asthmatic children and adolescents compared to age and sex-matched controls (Rajesh, 2008). More recent studies have also confirmed this finding, with adolescent asthmatics having a doubled risk for developing anxiety and depression that continued into adulthood (Goodwin, 2003; Ross, 2007; Bruzzese, 2009). In addition, asthma has been shown to accentuate anxiety and depression while anxiety and depression have similarly been linked to accentuated asthmatic symptoms, indicating a potential bidirectional relationship (Richardson, 2006; Chida, 2008).

Despite the co-morbidity between adolescent asthma and adult internalizing behavior, the underlying physiological and behavioral mechanisms between adolescent asthma and adult

anxiety have yet to be established. Previous human and animal studies have suggested the implication of long-term dysregulation of stress hormone levels – a well-known correlate of internalizing disorders – in asthmatic individuals as a possible link between adolescent asthma and adult anxiety (Heim, 2001; Feder, 2004). Notably, chronic psychosocial stress has been found to exacerbate asthmatic symptoms in humans (Chida, 2008). Meanwhile, mouse studies have indicated that social stress induces corticosterone insensitivity that may be attributable to impaired glucocorticoid regulation (Haczku, 2010). In addition, humans suffering from anxiety and depression have also been found to exhibit increased sensitivity to the mood-associated neurotransmitter, serotonin, while rodent models with decreased serotonin transporter function have demonstrated increased internalizing behavior (Jørgensen, 2007; Holmes, 2003; Murphy, 2004; Kalueff, 2010).

1.1. Benefits of Animal Models in Studying Asthma and Anxiety

Determining causal pathways in human studies is difficult due to the inability to directly manipulate study conditions. However, the use of rodent models of asthma can complement findings from human research and strengthen arguments for potential mechanisms linking asthma and anxiety. Animal models can test for causality by manipulating early life conditions and allowing for longitudinal monitoring and documentation of behaviors and physiology later in life in an environmentally controlled setting. By using genetically inbred mice strains and distributing animals within the same litter among different treatment conditions, researchers can control for potential genetic predispositions and maternal environment effects that may otherwise confound findings.

1.2. The Role of HPA Axis Hormones in Anxiety

CORT and Internalizing Behaviors

The hypothalamic-pituitary-adrenal (HPA) axis is a well-known modulator of stress regulation in mammals, with circulating glucocorticoids such as cortisol in humans, involved in the stress response (McEwen, 2000; Sapolsky, 2003; de Graaf-Peters, 2006; Ruttle, 2011). In addition to its role in stress, cortisol is found at basal levels in the body and fluctuates throughout the day with a circadian rhythm (Klimes-Dougan, 2001).

While cortisol secretion is adaptive in coping with acute stress, prolonged stress exposure has been associated with the development of various internalizing disorders (Monroe 2002; van Praag, 2004; Juruena, 2014). Studies have shown that stress may dysregulate the cortisol circadian rhythm, resulting in extremely low morning and high evening cortisol (Ruttle, 2011) and early-life stress exposure may influence HPA axis responsivity later in life (Jurena, 2014). In human studies, anxiety and depression have been associated with a flattened circadian rhythm, indicating that dysregulated cortisol production may be a risk factor for internalizing disorders (Feder, 2004; Van den Bergh, 2009; Hsiao, 2010). Animal models have confirmed this finding, with mice exposed to chronic social stress during periadolescence (~PND 30-59 days) having a flattened corticosterone circadian rhythm and increased adult anxiety- and depression-related behavior (Sullivan, 2006; McCormick, 2008; Sterlemann, 2008). Together, these findings indicate that chronic adolescent stressors may have long-term influences on glucocorticoid regulation and the development of anxiety and depression.

Asthma and CORT

Research has indicated that chronic inflammatory disorders, such as asthma, may be linked to a hyporesponsive HPA axis (Priftis, 2007). Specifically, children and adolescents with asthma tend to have lower levels of circulating cortisol than non-asthmatic individuals, regardless of whether they use cortisol-altering anti-inflammatory medications, indicating attenuated HPA axis responsivity in asthmatic children (Landstra, 2002; Priftis, 2007).

1.3. The Role of Serotonin and Serotonin Transporter Gene Expression in Anxiety

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter derived from the essential amino acid, tryptophan, and secreted in several regions of the body, including the gastrointestinal tract and central nervous system (Sangkuhl, 2009; Borue, 2010). In the brain, 5-HT is primarily released into synapses by neurons of the Raphe nuclei of the brainstem (Borue, 2010). As shown in **Fig. 1**, 5-HT diffuses across brain synapses, or intracellular spaces between neurons, to affect neighboring cells.

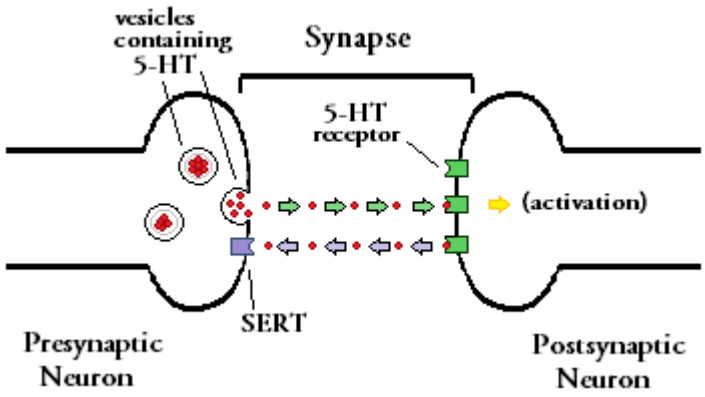


Figure 1: Serotonin at the brain synapse. Vesicles containing 5-HT in the presynaptic neuron fuse with the cell membrane, resulting in the release of the neurotransmitter via exocytosis. 5-HT diffuses across the synapse to bind and activate 5-HT receptors on the postsynaptic neuron. SERT re-uptakes 5-HT from the synapse.

Mechanistically, 5-HT binding activates 5-HT receptors on dendrites of the postsynaptic neuron. Another membrane-bound protein, the serotonin transporter (SERT in humans; Sert in mice) is involved in "resetting" the synapse by re-uptake of the 5-HT neurotransmitter molecules back into the presynaptic neurons to reform vesicles (Murphy, 2004; Borue, 2010). Thus, 5-HT availability is derived from two main sources: (1) newly synthesized molecules from tryptophan and (2) 5-HT recycled from the synapse by SERT (Borue, 2010).

1.4. Serotonergic Function

Since 5-HT influences multiple cognitive processes, including those involved with emotion and mood, several studies have implicated abnormalities in serotoninergic function with the pathogenesis of affective disorders (Heisler, 2007; Sangkuhl, 2009). Among other possible factors, a shortage in tryptophan (the 5-HT precursor), reduced conversion of tryptophan to 5-HT, low circulating 5-HT levels, and 5-HT receptor insensitivity have been proposed as putative underlying mechanisms (Borue, 2010). Notably, drugs such as selective serotonin reuptake inhibitors (SSRIs) have been clinically effective in treating certain states of anxiety and depression by blocking 5-HT transporters and increasing synaptic serotonin levels (Baldwin, 1998; Sangkuhl, 2009). In contrast, other studies have found that reduced *Sert* gene expression, which may correspond with elevated 5-HT in the synapse due to reduced 5-HT re-uptake, are associated with affective disorders (Holmes, 2003; Borue, 2010).

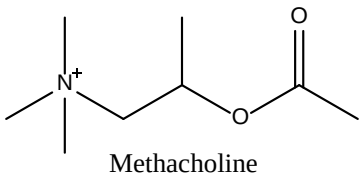
Because 5-HT is required for the activation of postsynaptic neurons in brain regions associated with emotion and stress responses, an imbalance of 5-HT release and re-uptake may contribute to anxiety and depression. Specifically, it is thought that SERT may play a role in the

modulation of serotonergic responses to stress since *Sert* knock-out mice have previously shown increased fearful, anxiety-like behavior and elevated stress hormone responses to stress (Caspi, 2003) and increased extracellular concentrations of serotonin (Borue, 2010).

1.5. CORT, Serotonin, and Internalizing Disorders

The influence of stress in internalizing disorders likely involves both altered glucocorticoid and serotonin regulation. Several studies have indicated that coping responses to chronic stress may be regulated by serotonin (Deakin, 1998; Zhang, 2012; Chilmonczyk, 2015). Studies of mice exposed to early life maternal separation stress demonstrated increased plasma CORT levels, reduced *Sert* gene expression in the amygdala, and increased anxiety-like behavior (Sachs, 2013). However, in a separate study in rats, chronic stress was shown to upregulate *Sert* expression in the dorsal raphe nucleus (Zhang, 2012). In addition, ingested CORT increased *Sert* expression, indicating that chronic stress may increase *Sert* gene expression in a CORT-dependent manner (Zhang, 2012). Since adolescence is a critical period for the development and maturation of neuronal systems involved in emotion regulation (Henry, 2015), severe stress precipitated by respiratory asthmatic symptoms may have long-term influences on the regulation of stress hormones and heightened serotonin activity. The current research project addresses whether the highly stressful experiences of chronic labored breathing in adolescent asthma is a contributing factor that increases susceptibility to adult anxiety-related behavior and physiology.

1.5. Methacholine as a Bronchoconstrictor



Methacholine

Figure 2: Structure of Methacholine. Drawn in ChemDraw® software

Methacholine (**Fig. 2**) is a synthetic ester and verified bronchoconstrictor that induces asthma-like symptoms of labored breathing in mice by acting on G-protein-linked M_3 muscarinic receptors in the lungs and smooth muscle lining bronchial passageways (Brusasco, 2001; Brannan, 2012; PubChem, 2015). Specifically, activation of G-protein-coupled M_3 receptors activates a downstream IP3 pathway increasing cytosolic Ca^{2+} concentrations, resulting in smooth muscle contraction (Brusasco, 2001; see **Fig. 3**).

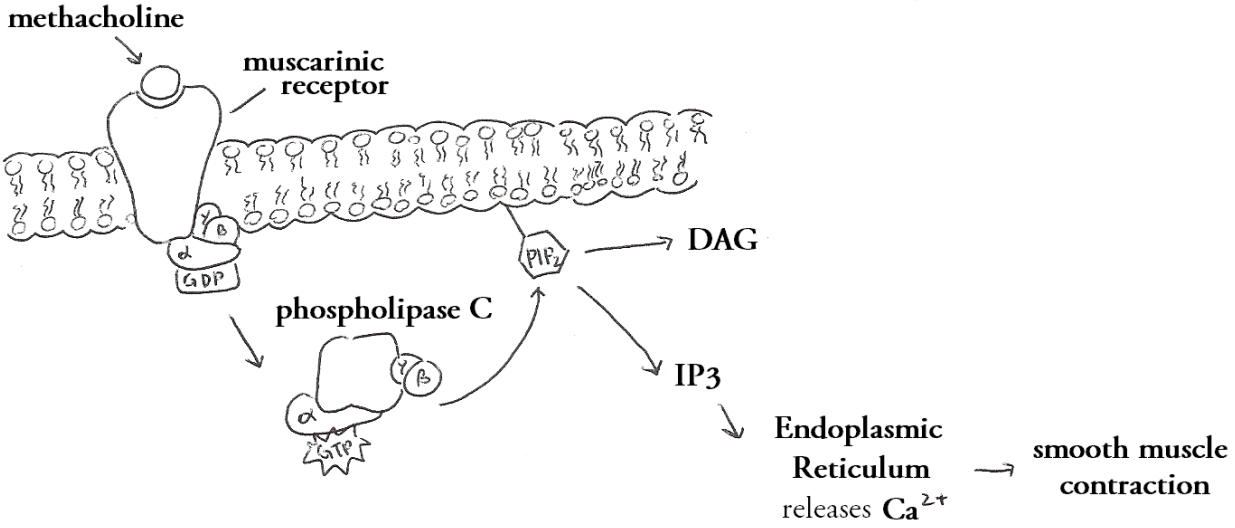


Figure 3: Methacholine mode of action. Activation of a muscarinic receptor activates the coupled G-protein, which activates phospholipase C to cleave PIP2 into DAG and IP3. IP3 triggers the endoplasmic reticulum to release Ca^{2+}, which causes smooth muscle contraction. Adapted from: Cooper G. M. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Pathways of Intracellular Signal Transduction. Available from: http://www.ncbi.nlm.nih.gov/books/NBK9870/

Aerosolized methacholine is also used to diagnose and characterize the severity of asthma in humans via a bronchial challenge test in which a patient inhales varying doses of methacholine and is monitored for airway hyper-reactivity (Birnbaum 2007; Brannan, 2012; PubChem, 2015). Because of its charged quaternary amine group, methacholine is pharmacologically insoluble to lipid cell membranes and thus unable to cross the blood-brain barrier. Thus, any changes in long-term glucocorticoid dysregulation or serotonergic function after methacholine administration is likely not due to direct action of methacholine at brain synapses, but instead due to the effects of chronic psychological stress associated with the induced asthma-like symptom of severe, potentially life-threatening labored breathing.

1.6. Purpose & Hypotheses

The development of verified animal models mimicking asthma labored breathing is a key first step in designing longitudinal studies to model the bidirectional relationship between asthma and internalizing disorders. Distinguishing the role of asthmatic stress in the development of anxiety and depression would provide clinically relevant information for evaluating asthma management in adolescence to minimize the risk of developing internalizing disorders later in life.

The purpose of this study was therefore to gain insight into the possible mechanisms underlying the co-morbidity between adolescent asthma and adult anxiety. Prior data in the lab indicate that methacholine treatment leads to anxiety-related behavior and *Sert* expression in mice. Based on this background research, the goal of the current study was to test whether there is a dose-dependent relationship between labored breathing and adult anxiety-related behavior

and physiology. It was originally hypothesized that greater methacholine-induced periadolescent labored breathing would result in greater glucocorticoid dysregulation and corresponding higher anxiety- and depressive-like symptoms in adulthood in a dose-dependent fashion. In addition, increased susceptibility to internalizing disorders resulting from greater labored breathing was expected to depend on individual basal differences in postnatal affective behavior (i.e. ultrasonic vocalizations). Specifically, we expected high adolescent affective behavior to modulate the above relationship with high pre-asthma fear behavior predicting increased susceptibility to asthma-induced anxiety-related behavior and physiology. Overall, the findings from this study aim to complement current epidemiological and observational research to further our understanding of the relationship between adolescent asthmatic experiences and adult internalizing disorders in humans.

Chapter 2

Methods

The present study used a rodent model to experimentally test for a dose-dependent influence of labored breathing on glucocorticoid regulation and *Sert* gene expression as possible mediators of adult anxiety-related behavior (see overall study timeline, **Fig. 4**). To simulate labored breathing of asthma, mice were exposed to the verified bronchoconstrictor methacholine weekly during adolescence on postnatal days (PND) 22-57. Fecal extracted corticosterone metabolites and anxiety- and depression-related behavior was measured in early adulthood (PND 59-66) and animals were sacrificed at PND 80 to measure brain parameters. Prior to methacholine manipulations, mice ultrasonic vocalizations (USV, PND 4-6) were measured for each cohort as an estimate of pre-adolescent fear-related behavior.

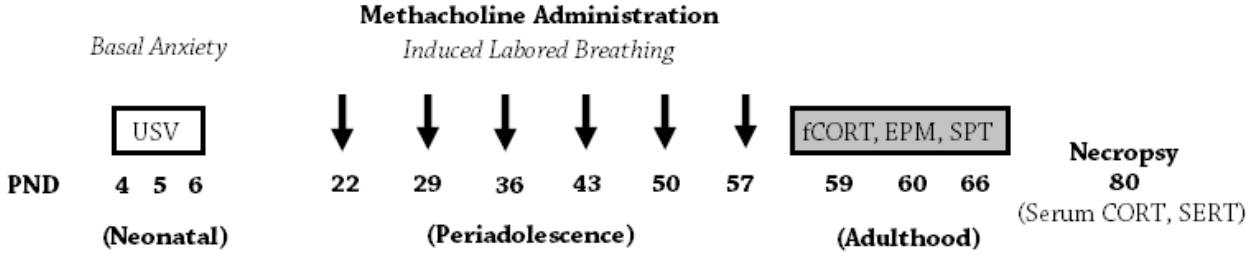


Figure 4: Overall study timeline. The white block indicates neonatal USV measures of anxiety-related behavior on PND 4-6. Arrows indicate methacholine exposure to induce labored breathing for experimental animals once a week on PND 22-57. The gray block indicates adult glucocorticoid analysis and behavioral testing: fCORT (PND 58-59), EPM (PND 60), and SPT (PND 66), followed by necropsy on PND 80 for serum CORT and *Sert* gene expression analysis.

2.1. Animals

Due to their significant airway reactivity to methacholine in previous studies (Gueders, 2009), BALB/cJ strain mice were used as rodent models that mimic asthma. Adult mice were purchased from Jackson Laboratories and bred to produce 12 litters across three cohorts (n=21, n=18, and n=17; respectively Cohorts 1, 2, and 3). A total of 56 pups (27 females, 29 males) were born (with day of birth noted as postnatal day 0, or PND 0). Each mouse was assigned a unique "Mouse ID" determined by litter (mother ID, father ID) and number. (Example: for the mouse DEDF 1, the mother's ID was DE and the father's ID was DF. The assigned number was used to determine an ear-notch pattern to distinguish between littermates.) Mice were weaned on PND 22 and housed with 2-3 same-sex littermates in 19 × 29 × 13 cm cages with food and water *at libitum*. The colony room was ventilated and controlled at 20-22° C with 40% relative humidity under a reverse 12L:12D light cycle (lights off at 8:00 and on at 20:00). Mice were weighed periodically to accustom them to handling. On PND 50, mice were singly-housed on wire bottom cages. All methods detailed below were approved by The Pennsylvania State University Institute for Animal Care and Use Committee IAUC #43671.

2.2. Ultrasonic Vocalization and Basal Anxiety

On PND 4-6, ultrasonic vocalizations (USV) were measured to estimate baseline anxiety. For each measure, pups were individually separated from their mother and placed into an isolated cage for 2 min, and the number of times each pup called for its mother was measured with a bat detector set to a frequency of 65 kHz. This frequency was chosen since earlier studies showed that isolation-induced USV calls by periadolescent mice generally ranged between 50-80

kHz, with 65 Hz as the midpoint frequency (Hofer et al, 2002). Since other studies have indicated that mice bred for high anxiety-related behavior on the EPM tended to have "aversive affective" states with higher USV call rates than lower anxiety mice (Krömer et al., 2005; Kessler et al., 2011), pups in this study were characterized as having either high- or low- anxiety tendencies based on whether their call rate was above or below the litter median at each test (PND 4, 5, and 6). In the present study, an animal was considered "fearful" with a high anxiety-prone aversive affective state if its USV call rate was higher than the litter's median on at least 2 of the 3 test days ("high-USV"), while an animal was considered low anxiety if its USV call rate was lower than the litter median for at least 2 of the 3 days ("low-USV"). When assigning animals to experimental groups, high- and low- USV pups were randomized and distributed evenly to control for individual differences in baseline fear-related behavior.

2.3. Methacholine and Induced Labored Breathing

All animals were divided into one of five treatment groups: **(1)** non-handled control animals (n=9), **(2)** a vehicle control group (n=10) receiving the same procedures as experimental animals except without methacholine exposure, **(3)** low methacholine exposure (n=12), **(4)** medium methacholine exposure (n=12), and **(5)** high methacholine exposure (n=13). The bronchoconstrictor, methacholine, was purchased from the supplier, Sigma-Aldrich (St. Louis, MO). From PND 21 to PND 57, methacholine-treatment pups were exposed once a week to 4 increasing doses of aerosolized methacholine via a whole-body plethysmograph (Buxco, model PLY3211 coupled to a MAX1320 modular accessory unit and PLY1040 bias flow regulator with BioSystemXA software, Wilmington, NC). In the low methacholine condition, pups received

1.9, 3.75, 7.5, and 15 mg/mL doses, medium methacholine pups receiving 3.75, 7.5, 15, and 30 mg/mL doses, and high methacholine pups receiving 7.5, 15, 30, and 60 mg/mL doses during each exposure. See **Table 1** below for a summary of doses administered during each exposure for each experimental condition.

Table 1. Doses Administered During Each Exposure (mg/mL) for Each Experimental Group

	D1	D2	D3	D4
Low Meth	1.9	3.75	7.5	15
Med Meth	3.75	7.5	15	30
High Meth	7.5	15	30	60
PBS	--	--	--	--

Each dose was administered for 2.5 min with a 30-sec resting period between each dose. Vehicle controls received phosphate-buffered saline (PBS) instead of methacholine for the same time duration in the plethysmograph while non-handled control mice were never placed in the plethysmograph.

Penh as a Measure of Labored Breathing

Enhanced pause (Penh) was used as a non-invasive measure of labored breathing in the study animals. This measure is also used to quantify airway reactivity and lung function in humans (Houghton, 2004). Aerosolized methacholine was administered by placing each animal in a Buxco® whole body plethysmograph chamber (**Fig. 5**).

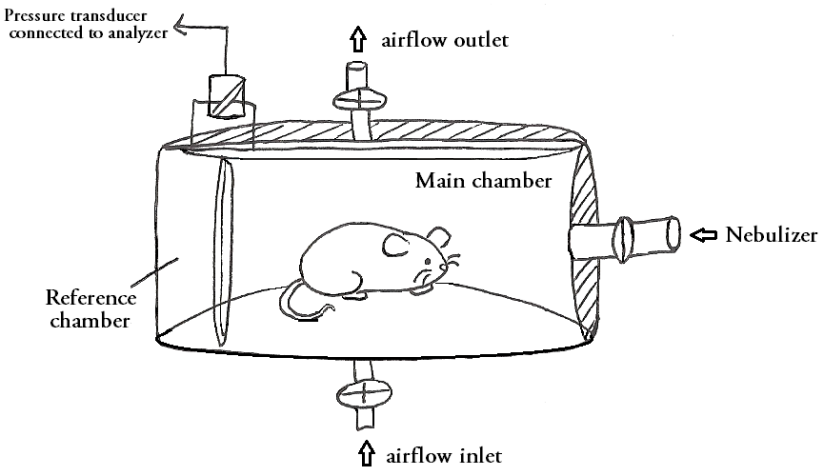


Figure 5: Schematic Diagram of Whole-Body Plethysmography Set-Up. Redrawn from Hamelmann et. al, 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156, 767.

By measuring changes in air flow volume and pressure as the animal inhales and exhales in the enclosed chamber relative to the reference chamber, Penh measurements of labored breathing (i.e. increased delay times in between breaths) can be detected for each animal (Hamelmann, 1997; Irvin, 2003; Glaab, 2007; Buxco, 2015). Penh is indicative of induced asthma-like labored breathing as the mouse attempts to hold onto each breath longer to absorb enough oxygen (Hamelmann, 1997).

More recent studies have indicated flaws with relying solely on Penh as a repetitive measure (Lundblad, 2007; Lundblad, 2012). However, for this study, Penh was utilized as a noninvasive, unrestrained measure for mice to allow for developmental research and to mimic natural environments as much as possible and minimize the potential confound of restraint stress.

All Penh readings were saved as a Microsoft Database file by mouse ID and test date before exporting to Excel. Mean Penh values were then calculated for each animal at each dose (baseline, PBS, and methacholine doses 1-4) and for administrative age (PND 21, 28, 35, 42, 49, and 56; or weeks designated as T1-T6).

2.4. Fecal CORT extraction and RIA analysis

On PND 58-59, corticosterone (CORT) metabolites found in rodent feces were analyzed as a non-invasive method to estimate serum glucocorticoid regulation. To control for circadian rhythm effects on CORT production, fecal samples from each animal were collected at 20:00 and 24:00 on PND 58 and 8:00 and 12:00 on PND 59. Samples were then stored at -80°C until processed for extraction, in which samples were thawed and weighed before drying in a centrifuge evaporator. Crushed feces (0.05 g) were placed in a 15 mL polypropylene centrifuge tube with 10 mL of HPLC reagent alcohol (Sigma-Aldrich, St. Louis, MO) and boiled for 20 min before centrifugation at 2000 *g* for 15 min at room temperature. The supernatant was decanted into a 16 mm × 125 mm glass tube, and 5 mL of HPLC reagent alcohol was added to the pellet. The sample was subsequently vortexed for 1 min and centrifuged at 2000 *g* for 15 min at RT before decanting the resulting supernatant liquid into the same 16 mm × 125 mm tube. The supernatant was dried thoroughly and re-suspended in 1 mL of methanol before analysis with a commercial ImmuChem™ Corticosterone radioimmunoassay (RIA) kit, purchased from MP Biomedicals (cat. #07-120103; Solon, OH). Extracts were diluted 1:5 and reaction volumes were halved from what the protocol called for. Radioactivity after each reaction was determined with a gamma counter.

2.5. Elevated Plus Maze

EPM and Anxiety-Related Behavior

The elevated plus maze (EPM) is a commonly used test of anxiety-related behavior in rodents. Since rodents tend to fear and avoid open areas while favoring dark, enclosed spaces, an increase in time spent in the open typically indicates reduced anxiety-like behavior (Walf, 2007). Several studies have demonstrated that anxiolytic, or anxiety-reducing, drugs increase time spent on open arms while anxiogenic, or anxiety-causing, drugs decrease time spent on open arms, verifying the construct validity of the EPM model as a rodent measure of anxiety (Pellow, 1985; Mechiel Korte, 2003).

Currently, ideal animal models will have face validity, predictive validity, and construct validity (Bourin, 2007). It is difficult to discriminate between reduced anxiety-related avoidance and increased novelty-seeking in both the EPM and other behavioral tests of anxiety including the light-dark box test. The EPM specifically tests the unconditioned, spontaneous behavioral conflict model and exploration and approach-avoidance conflict (Pellow, 1985).

To quantify adult anxiety-like behavior, exploratory activity in the elevated plus maze (EPM) test was measured for each animal on PND 60. The EPM was designed as a cross-shaped maze elevated 42 cm from the ground with two “closed” arms (30 cm × 5 cm) with walls 14.5 cm high, and two unsheltered “open” arms (30 cm × 5 cm) with a 5 mm high lip to prevent mice from falling off the maze. Each trial began by placing the mouse at the center facing an “open” arm and allowing the mouse to explore freely for 5 min. The test was conducted under red light with a video camera mounted above to record behaviors for coding. Measures such as the amount of time the animal spent on closed vs. open arms and frequency of arm entries

(determined by 4-limbs crossing over each section) were recorded to quantify exploratory activity and anxiety-related behavior. At the end of each test, the maze was fully cleaned with 30% EtOH and the animal was returned to the home cage.

2.6. Sucrose preference test

To measure adult anhedonic, depressive-like behavior, the sucrose preference test was given to each animal on PND 66 (Willner, 1987). The test was administered for a 24-hr period. Mice were allowed to consume water from either a regular tap water control or a 1% sucrose solution bottle. To control for possible side preferences, the bottle locations were switched halfway through the test. Results were analyzed as % sucrose solution consumption relative to total liquid consumption.

2.7. Serum CORT at Necropsy

To measure basal circulating CORT levels, serum samples were collected 3-7 min (avg. 4.33 min) after the animal was moved from the colony room at necropsy on PND 80. Blood was collected via cardiac puncture following euthanasia by CO_2 inhalation. Blood was centrifuged to separate serum and 5µl aliquots were stored at -80°C until processed. Samples were run on the same commercial kit as the fecal CORT extracts but at a dilution of 1:200. Reaction volumes were similarly halved from the commercial protocol. All serum CORT samples were run on the same assay.

2.8. Quantitative RT-PCR Analysis of *Sert* Gene Expression

Brainstem sections were preserved at necropsy for analysis of serotonin transporter mRNA expression (*Sert*). TRIzol reagent (purchased from Invitrogen; Carlsbad, CA) and Qiagen "RNeasy" columns (Qiagen; Germantown, MD) were used to extract cellular RNA. To obtain complementary DNA (cDNA) from the RNA extracts, reverse transcription was completed via a commercial High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Wilmington, DE). cDNA samples containing *Sert* were subsequently analyzed via quantitative real time PCR (qRT-PCR) using validated TaqMan probes. qRT-PCR reactions were conducted using cycles of 50^{o}C for 2 min, 95^{o}C for 10 min, 40 cycles of 95^{o}C for 15 sec, and a test at 60^{o}C for 1 min, and all data were collected on the StepOnePlus Real-Time PCR System (Applied Biosystems). The relative abundance, and thus approximate gene expression level, of *Sert* in each sample was determined by quantitative comparisons to the constantly-expressed gene beta actin (*Actb*). Specifically, *Sert* expression was normalized to *Actb* expression by subtracting *Sert* cycle threshold (CT) values from *Actb* CT values to obtain ΔCT values. The relative abundance of *Sert* in each sample was then calculated using the formula: $2^{-\Delta\Delta CT}$.

2.9. Statistical Analyses

All statistical tests were run on IBM SPSS 22.0 software.

Statistical ANOVA tests were used to analyze the effect of periadolescent methacholine-induced labored breathing on adult internalizing behavior, as measured by EPM open arm time and percent sucrose consumption in the SPT, as well as physiological measures: fecal CORT,

serum CORT, and *Sert* gene expression. Prior to parametric testing, each outcome variable was examined to evaluate normal distribution by visual inspection (i.e. histograms and boxplots) as well as by the Shapiro-Wilk's normality test ($P>0.05$) and skewness and kurtosis z-values (between -1.96 and +1.96 for approximate "normal distribution"). Skewed and kurtotic distributions were natural log-transformed, and if necessary, outliers greater than 2.5 standard deviations away from the mean were excluded to obtain more normally-distributed data and achieve assumptions for parametric statistical tests.

Univariate ANOVA analyses were run on normally-distributed outcome variables with experimental treatment group as a *fixed factor* (i.e. the factor intentionally being manipulated) and any other possible confounds controlled for as a random factor or covariate. To see if there were any confounding factors interacting with the experimental group effect and influencing results, univariate ANOVA analyses were run separately for each possible confound including cohort, sex, and USV. If no interaction was found, the confounding variable criterion was removed to maximize the test's power. Since cohort effects were present, the cohort mean for the outcome variable was included as a *covariate* during the ANOVA analysis.

Hierarchical multiple linear regression analyses were completed to determine whether there was a statistically significant linear relationship ($P<0.05$) between Mean Penh and EPM Open Arm Time, sucrose preference, *Sert* gene expression, and CORT measures. Scatterplots of Mean Penh vs. each outcome variable were generated to observe overall trends.

Reported F-statistics and p-values were derived from natural log-transformed data to meet normal distribution criteria for parametric statistical analyses. However, for visual clarity, graphs generated in the results section display non-log transformed values unless otherwise indicated.

Chapter 3

Results

3.1. Mean Penh Calculations

To verify that methacholine exposure induced labored breathing, Penh values were plotted for each methacholine dose (Baseline, PBS, D1-D4) at each age of administration (T1-T6) for each experimental group (Control, Low, Med, High Meth). **Fig. 6** shows these data for T6 (PND 57), the last week of methacholine exposure. Penh values for the experimental treatment groups did not deviate from control mice until the second methacholine dose or higher (D2-D4; highlighted in green). (See **Appendix A** for T1-T5 data.)

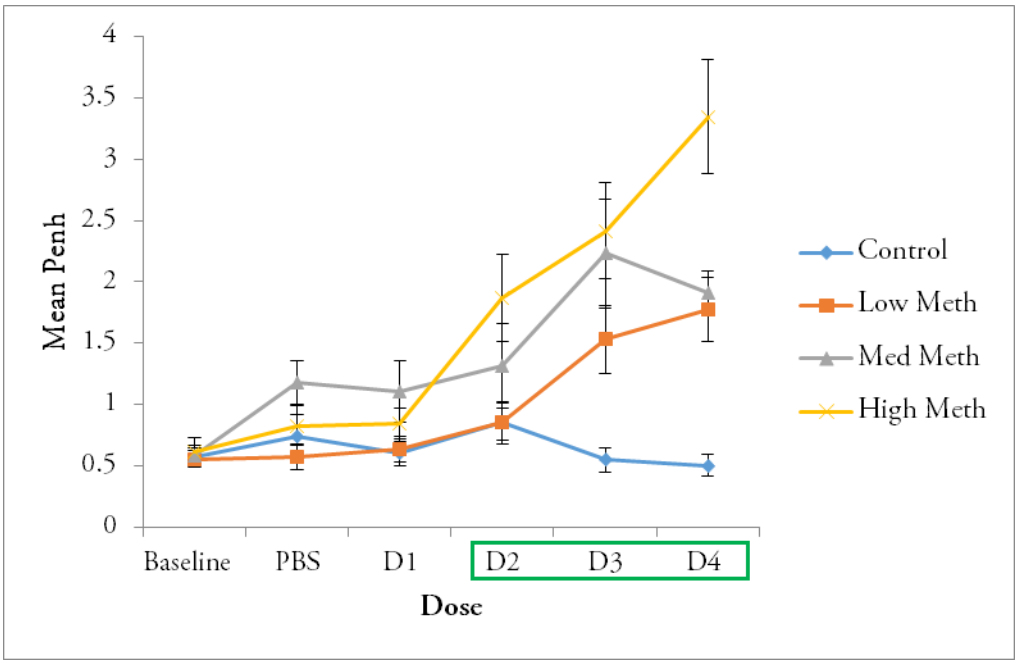


Figure 6: Mean Penh for Each Experimental Group at Each Dose at Week 6 (T6; PND 57). Average Penh values recorded for animals in the control (n=10), low- (n=12), medium- (n=12), and high- (n=13) methacholine groups are shown for baseline and during PBS and methacholine doses (D1-D4). A distinct

separation in mean Penh values between treatment and control groups does not occur until D2-D4. (Low Meth D2-D4 doses were **3.75**, **7.5**, and **15** mg/mL; Med Meth D2-D4 doses were **7.5**, **15**, and **30** mg/mL; High Meth D2-D4 doses were **7.5**, **15**, **30**, and **60** mg/mL).

To determine how methacholine induced labored breathing at each administration (T1-T6), mean Penh values for D2-D4 were examined for each week (**Fig. 7)**.

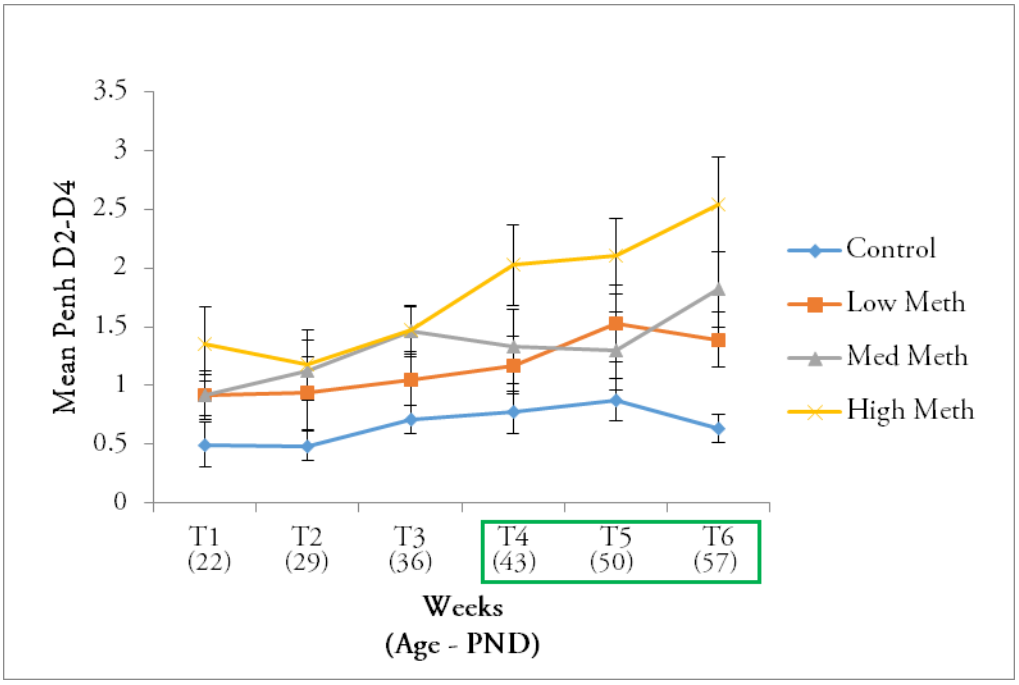


Figure 7: Mean Penh at D2-D4 for Each Experimental Group Across Weeks (T1-T6). The mean of the D2-D4 Penh averages recorded for animals in the control (n=10), low (n=12), medium (n=12), and high (n=13) experimental groups across administrative weeks (T1-T6).

The greatest differences in mean Penh among experimental treatment groups appeared after T4 (PND 43; highlighted in green on **Fig. 7**). Instrument malfunctions and missing data during week 5 may have made T5 data unreliable. Therefore, a single overall "mean Penh value" was calculated as the average of D2-D4 Penh values for weeks T4 and T6 only. This overall measure was used for all subsequent analyses.

3.2. Effect of Experimental Group on Mean Penh

No sex or cohort effects were present for Mean Penh (t_{45}=1.552, p=0.128; $F_{2,44}$=0.435, P=0.650) and thus, because of low sample size, these variables were not included as factors in the ANOVA. Experimental treatment group had a significant effect on Penh values ($F_{3,43}$=21.041, P=0.000). Mice receiving higher methacholine doses had increased Penh values compared to vehicle control animals (**Fig. 8**).

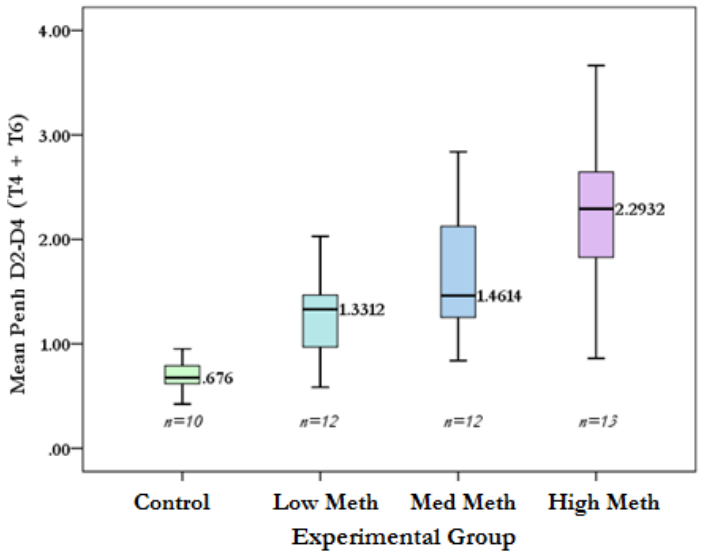


Figure 8: Mean Penh for Each Experimental Group. Mean Penh values are labeled with standard error bars for control (n=10), low (n=12), medium (n=12), and high animals (n=13). Upper and lower quartiles are defined by boxplot boundaries. (Note: non-handled control animals are not depicted because they were not placed in the plethysmograph for Penh measures.)

3.3. Effect of Body Weight on Penh

Since the plethysmography instrument appeared to have difficulty detecting Penh measures for juvenile mice before T4, we retrospectively analyzed whether body weight (as an estimate of lung size) had an effect on recorded Penh measurements as a potential confound of this study. As shown in **Fig. 9-11**, animals across all three cohorts appeared to rapidly increase in

weight before plateauing around PND 40, approximately the age at which Penh measurements noticeably increased.

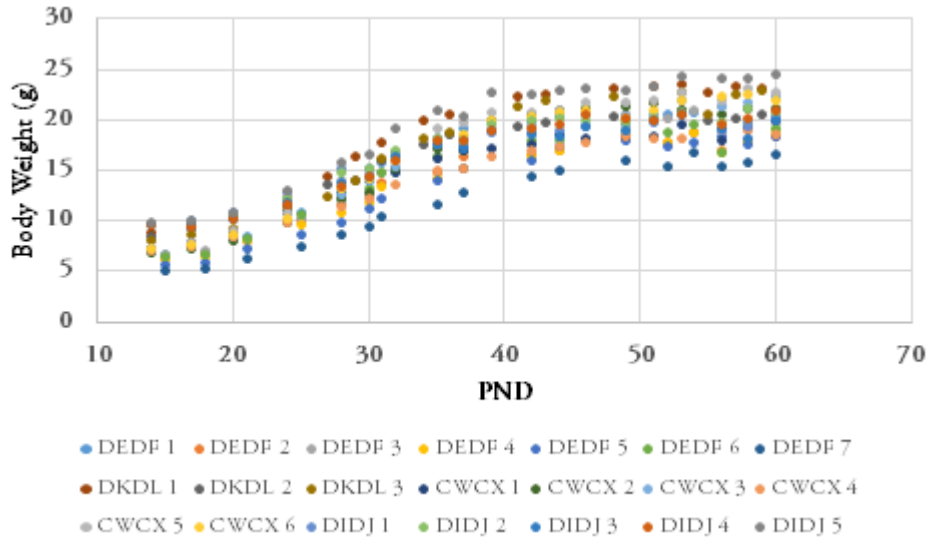


Figure 9: Cohort 1 Animal Weights. Estimated body weights were plotted for cohort 1 mice on PND 22-59.

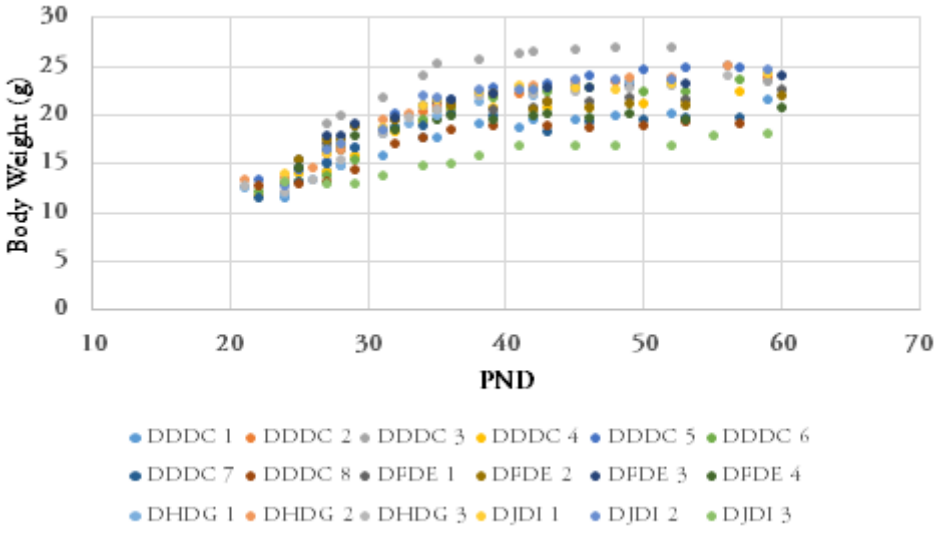


Figure 10: Cohort 2 Animal Weights. Estimated body weights were plotted for cohort 2 mice on PND 22-59.

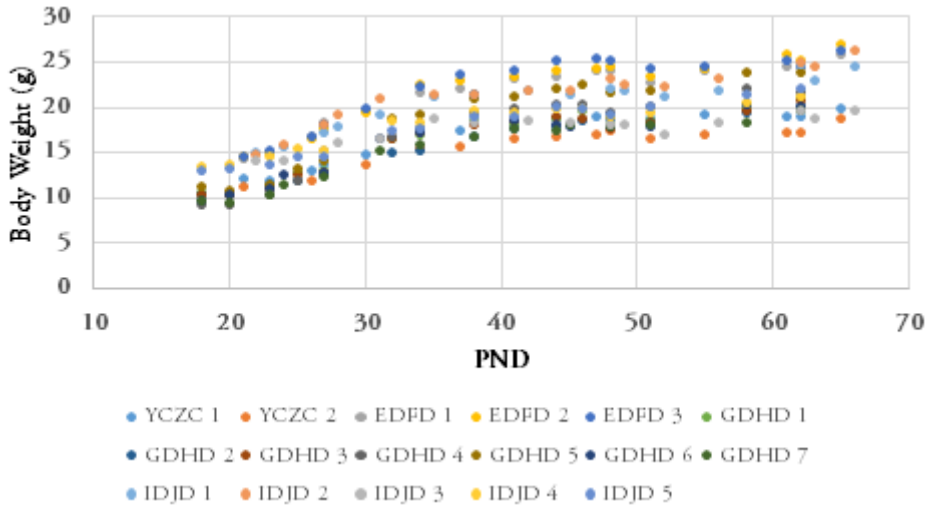


Figure 11: Cohort 3 Animal Weights. Estimated body weights were plotted for cohort 3 mice on PND 22-59.

Since not every animal was weighed on the exact dates of methacholine treatment, estimate weights for weeks T1-T6 were calculated based on available weight data through a best-fit line (see **Appendix B** for table of weight estimates). As shown in **Fig. 12**, an increase in body weight was correlated with an increase in Penh measurements for PND 22, 29, and 36 (corresponding to T1-T3). However, after PND 43, body weight was less strongly correlated with Penh.

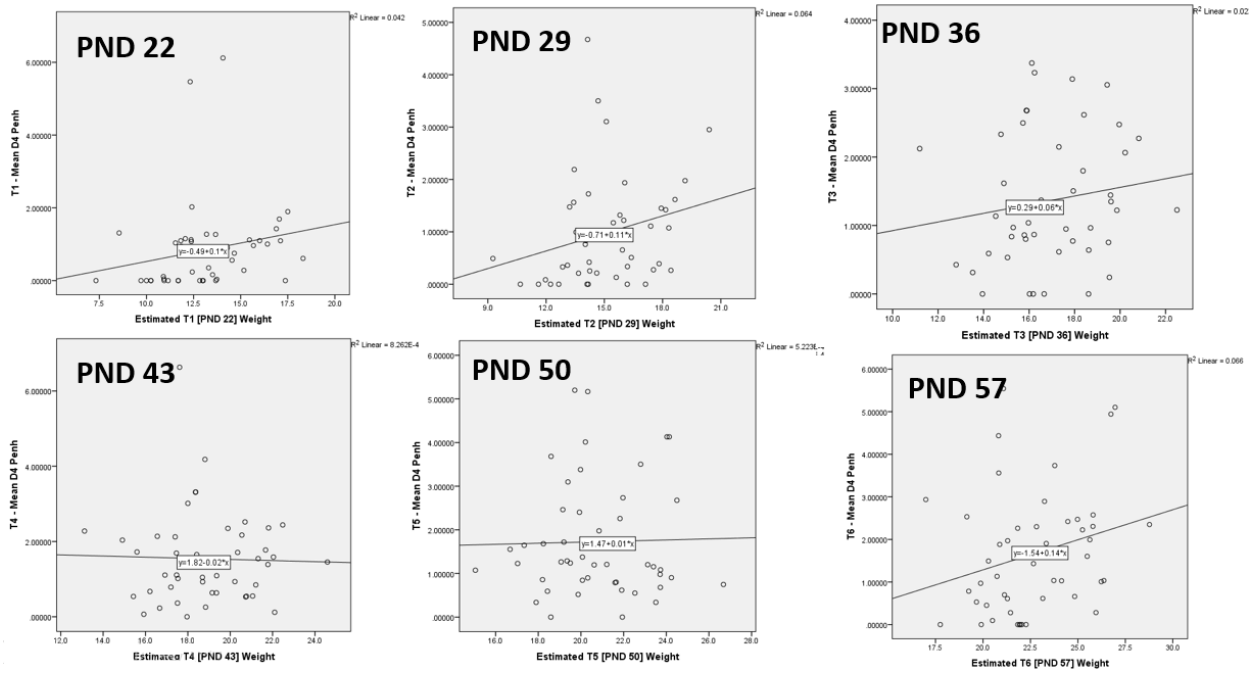


Figure 12: Estimated Weights relative to D4 Penh. The estimated weight of each animal on PND 22, 29, 36, 43,50, and 57 (T1-T6) was plotted relative to their recorded D4 Penh value. Penh graphs for D4 (the highest methacholine dose level) was included because we reasoned that if the mice were going to elicit a methacholine-induced Penh response, they would do so at the highest dose level.

3.4. USV Classification

High- vs. Low- USV mice did not have significantly different mean Penh values (t_{45}=0.519, p=0.607, **Fig. 13**) or adult EPM Open Arm Times (t_{54}=0.573, p=0.569, **Fig. 14**). Since no USV-dependent effect was found, USV classification was not included as a random factor in outcome variable analyses.

MEAN PENH

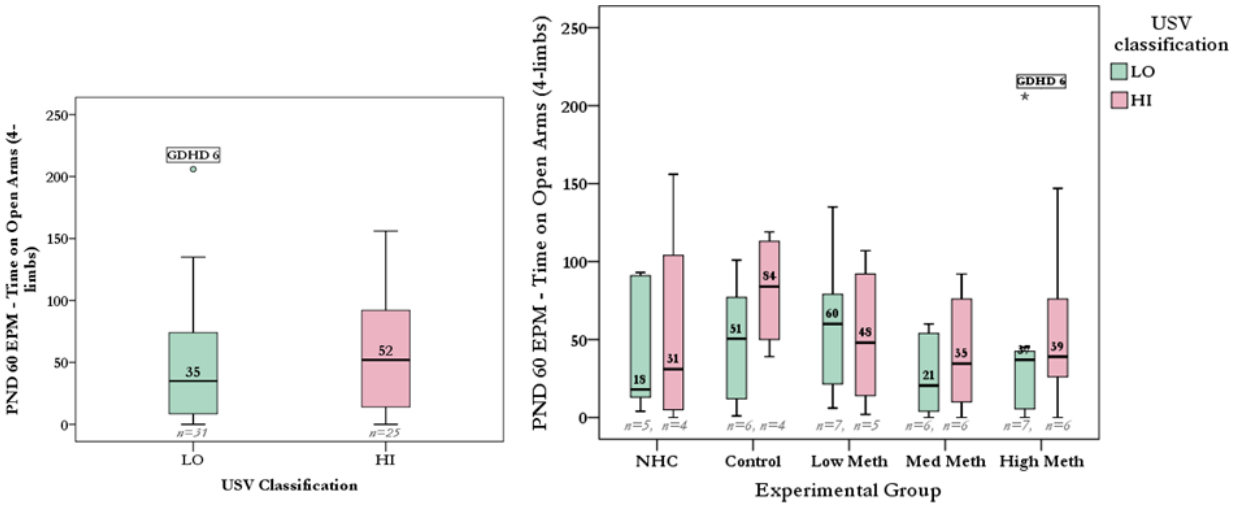


Figure 13: Mean Penh following methacholine administration for High- vs. Low- USV mice. Left: overall, Right: broken down by experimental group. Mean Penh values are labeled with standard error bars. Upper and lower quartiles are defined by boxplot boundaries. (**Note:** lower total *n* because Penh was not measured for NHC animals).

EPM OPEN ARM TIME

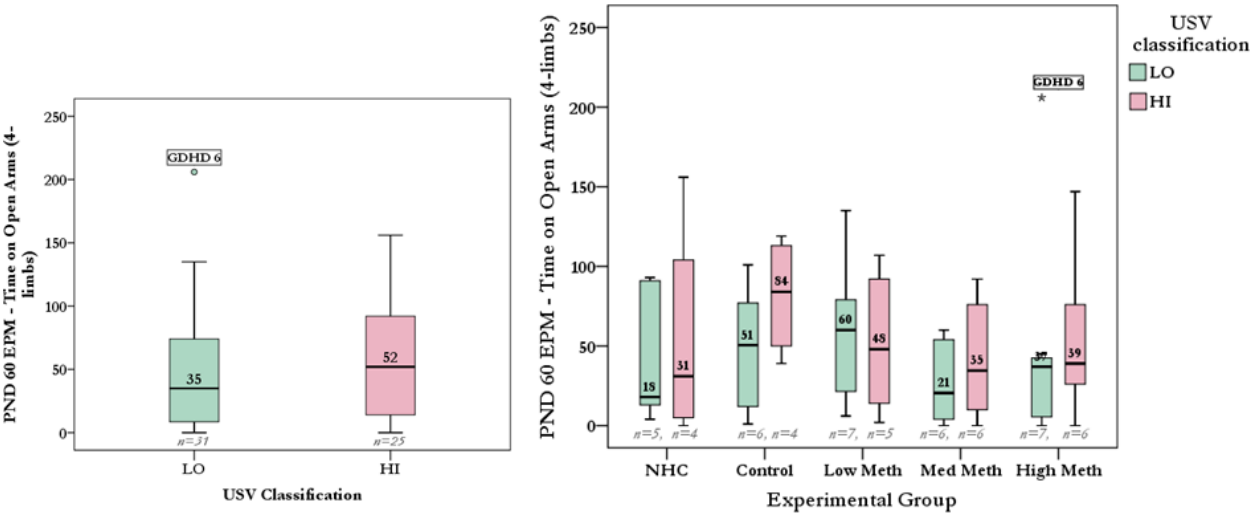


Figure 14: PND 60 EPM Open Arm Time for High and Low- USV mice. Left: overall, Right: broken down by experimental group. Average time spent on EPM Open Arms (4-limb) are labeled with standard error bars. Upper and lower quartiles are defined by boxplot boundaries.

3.5. Effect of Experimental Group on Adult EPM Open Arm Time (PND 60)

No sex effects were present for adult EPM Open Arm Time (t_{54}=0.541, p=0.591). However, significant differences in EPM Open Arm Time were seen across the three cohorts of mice ($F_{2,53}$=5.006, P=0.010). Therefore, cohort-specific mean EPM Open Arm Times were used as a covariate in the ANOVA analysis. Mice treated with methacholine spent less time on EPM Open Arms compared to controls, but this effect was not statistically significant ($F_{4,50}$=0.736, P=0.572) (**Fig. 15**).

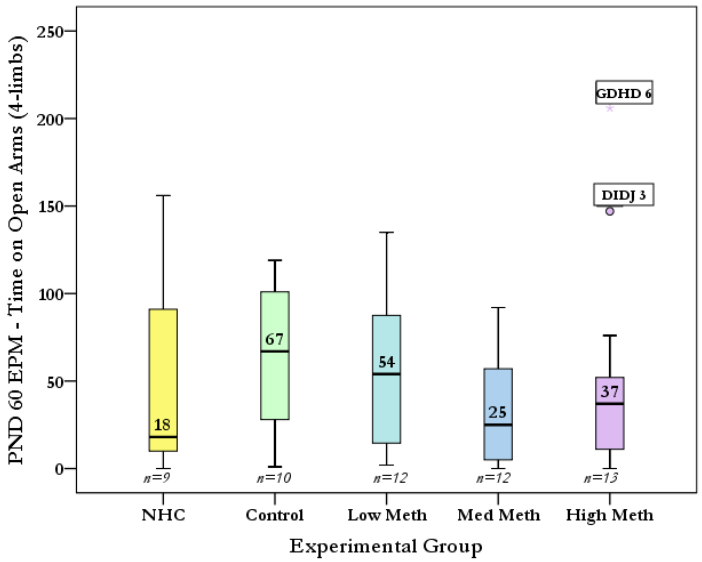


Figure 15: EPM Open Arm Time for Each Experimental Group. Average time spent on EPM Open Arms are labeled with standard error bars for the NHC (n=9), control (n=10), low- (n=12), medium- (n=12), and high-Meth animals (n=13). Upper and lower quartiles are defined by boxplot boundaries. Outlier animals: ○ = DIDJ 3 and * = GDHD 6 in the High-Meth group are identified.

When the two High Meth outlier animals were excluded, there was still no significant difference in EPM Open Arm Time among experimental groups ($F_{4,48}$=1.104, P=0.365).

3.6. Effect of Mean Penh on EPM Open Arm Time (PND 60)

Mean Penh had a significant linear relationship with EPM Open Arm Time when controlling for cohort mean EPM Open Arm Time (Adj.-R^2=0.190, β = -0.294, P=0.041). Mice that had greater Penh values at D2-D4 in T4 and T6 spent less time on the open arms of the EPM as adults (**Fig. 16**). Log-transformed Penh explained 19.0% of the variance in time spent on open arms.

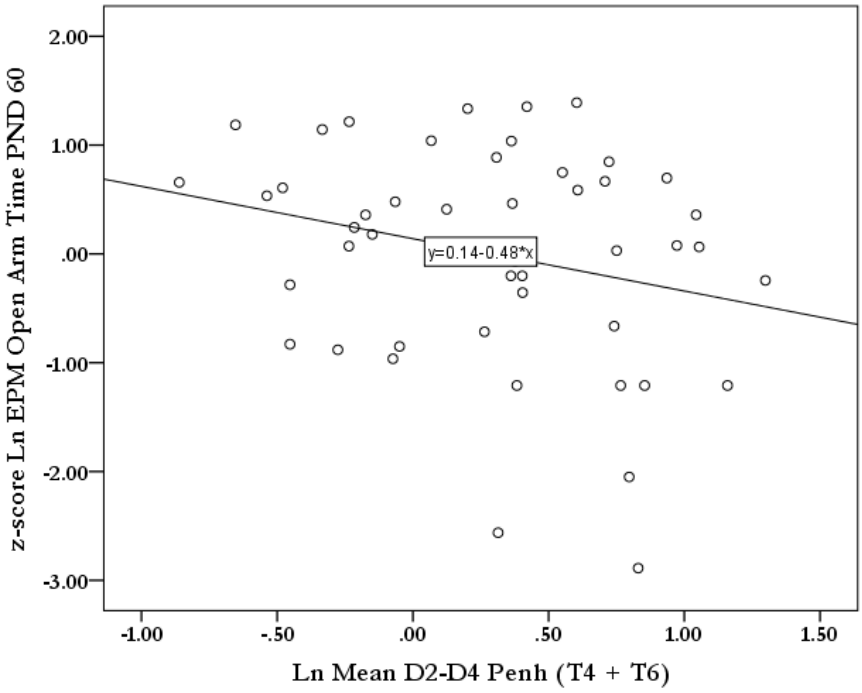


Figure 16: EPM Open Arm Time relative to Mean Penh. Adult mice that have experienced higher Penh values during adolescence spent less time on open arms of EPM. (Z-scores are shown to control for cohort effects on EPM Open Arm Time and adjusted values are presented because both variables were not normally distributed).

3.7. Effect of Experimental Group on Percent Sucrose Consumption (PND 66)

One outlier animal, GDHD 2 from the low methacholine condition, was excluded in the SPT analysis (n=55). There was no sex, cohort, or USV effect on percent sucrose consumption in

the SPT (t_{53}=0.854, P=0.397; $F_{2,52=}$0.898, P=0.414; t_{53}=0.794, P=0.431). There was no statistically significant difference in percent sucrose consumption among experimental treatment groups ($F_{4, 50}$=0.650, P=0.629). There was also no linear effect of mean Penh on percent sucrose consumption (Adj-R^2= -0.014, β = -0.090, P=0.552). To test the face validity of sucrose preference as a measure of anhedonia in our study, we conducted a one-sample t-test to evaluate the null hypothesis that mice show no preference for sucrose (with a 50% sucrose consumption rate indicating no preference). The mice in this study showed a slight (59%) but significant preference for the 1% sucrose solution ($|t|_{55}$=5.403, P=0.000). However, the amount of preference did not differ among experimental groups ($F_{4, 50}$=0.650, P=0.629).

3.8. Effect of Experimental Group on Fecal Corticosteroid Metabolites (PND 58-59)

There was a significant sex effect for all fCORT collections (PND 58-59): 20:00, 24:00, 8:00, and 12:00 (t_{53}=8.780, 0.000; t_{40}=6.219, 0.000; t_{54}=16.145, P=0.000; t_{54}=14.548, P=0.000); females had higher levels than males. There was no cohort effect for 24:00, 8:00, or 12:00 ($F_{2,42}$=1.516, P=0.232; $F_{2,53}$=1.649, P=0.202; $F_{2,53}$=2.619, P=0.082); however, there was a cohort effect at 20:00 ($F_{2, 52}$=3.395, 0.041). There was no USV effect at any time point (t_{53}=0.665, P=0.509; t_{40}=0.615, P=0.542; t_{54}=0.836, P=0.407; t_{54}=0.869, P=0.389). Therefore, sex was included as a random factor for all fCORT analyses, and cohort-specific mean 20:00 fCORT was added as a covariate for analysis of the 20:00 fCORT data.

One animal was missing data at 20:00 and 14 animals were missing data at 24:00. Two outlier animals, GDHD 1 (female) from the control group and DDDC 1 (male) from the NHC group, were excluded from the 8:00 fCORT analyses (n=54). DDDC 1 and IDJD 3 (female) from

the NHC condition were similarly excluded from the 12:00 fCORT analyses (n=54). DIDJ 2 (female) from the low methacholine condition was excluded as an outlier for the 20:00 fCORT analyses (n=54). DDDC 1 was excluded as an outlier for the 24:00 fCORT analyses (n=41).

Mean fCORT was higher during the *lights off* cycle (8:00 and 12:00) for both male (**Fig. 17)** and female (**Fig. 18**) mice in all treatment groups.

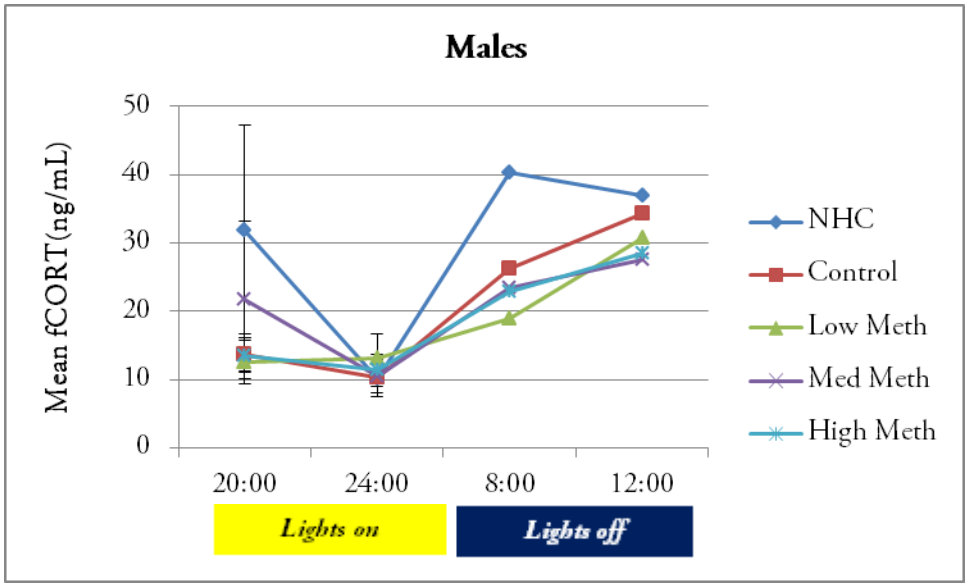


Figure 17: Mean fCORT for MALES in Each Experimental Group. The mean fCORT values (ng/mL) for male mice in the NHC (n=5), control (n=5), low (n=6), medium (n=6), and high (n=7) experimental groups are shown for the 20:00, 24:00, and 8:00, 12:00 time points (PND 58-59). As indicated by the yellow and dark blue blocks, lights on occurred at 20:00 on PND 58 and lights off occurred at 8:00 on PND 59.

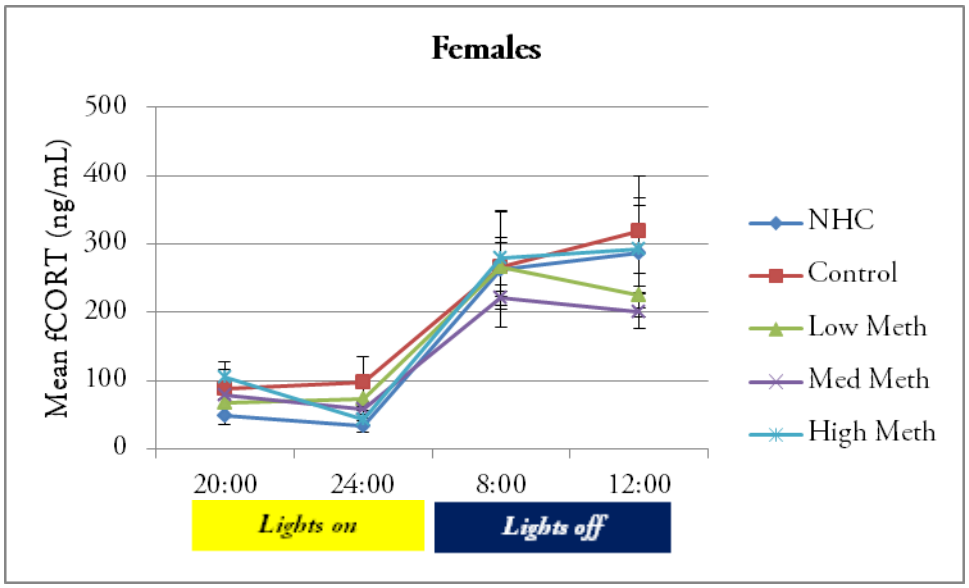


Figure 18: Mean fCORT for FEMALES in Each Experimental Group for All Time Points. The mean fCORT values (ng/mL) for female mice in the NHC (n=4), control (n=5), low (n=6), medium (n=6), and high (n=6) experimental groups are shown for the 20:00, 24:00, and 8:00, 12:00 time points (PND 58-59). As indicated by the yellow and dark blue blocks, lights on occurred at 20:00 on PND 58 and lights off occurred at 8:00 on PND 59.

There was no statistically significant sex-dependent experimental group effect on fCORT at 20:00 ($F_{4,43}$=0.240, P=0.902), 24:00 ($F_{4,31}$=0.711, P=0.625), 8:00 ($F_{4,46}$=2.070, P=0.100), or 12:00 ($F_{4,46}$=0.165, P=0.955). There was no linear effect of mean Penh on fCORT at 20:00, 24:00, 8:00, and 12:00 (Adj.-R^2= 0.703, sex: β = 0.847, P=0.000; mean Penh: β = -0.011, P=0.901; Adj.-R^2= 0.621, sex: β = 0.799, P=0.000; mean Penh: β = -0.090, P=0.458; Adj.-R^2= 0.902, sex: β = 0.953, P=0.000; mean Penh: β =0.001, P=0.977; Adj.-R^2= 0.850, sex: β = 0.914, P=0.000; mean Penh: β = -0.046, P=0.436). Boxplots in **Fig. 19** show mean fCORT values across all four time points by treatment group and sex.

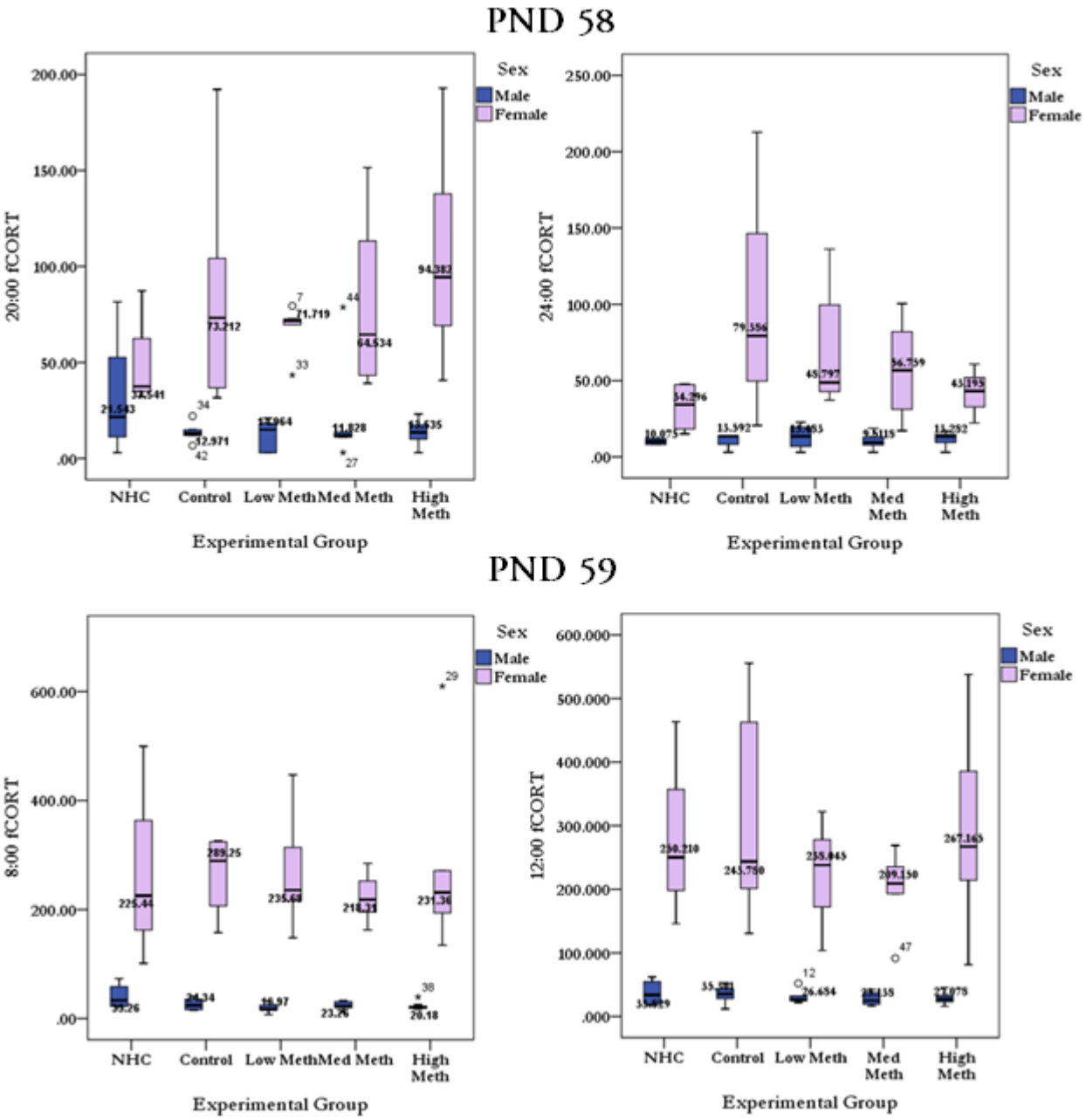


Figure 19: Mean fCORT for Each Experimental Group. The mean fCORT values (ng/mL) for males and females in each experimental group are shown for the 20:00, 24:00, and 8:00, 12:00 time points (PND 58-59).

3.8. Serum CORT (PND 80)

There was a significant sex effect on Serum CORT (t_{53}=3.534, P=0.001); females had higher levels than males. There was a significant cohort effect (χ^2=19.288, df=2, P=0.000). There was no USV effect (t_{53}=0.651, P=0.518). There was no statistically significant effect of experimental group on Serum CORT levels at necropsy when controlling for cohort mean Serum CORT values ($F_{4,49}$=1.322, P=0.275). There was also no linear effect of mean Penh on Serum CORT (Adj.-R^2= 0.300, cohort: β = 0.571, P=0.000; mean Penh: β = -0.061, P=0.630). The boxplot in **Fig. 20** shows mean Serum CORT by treatment group and sex.

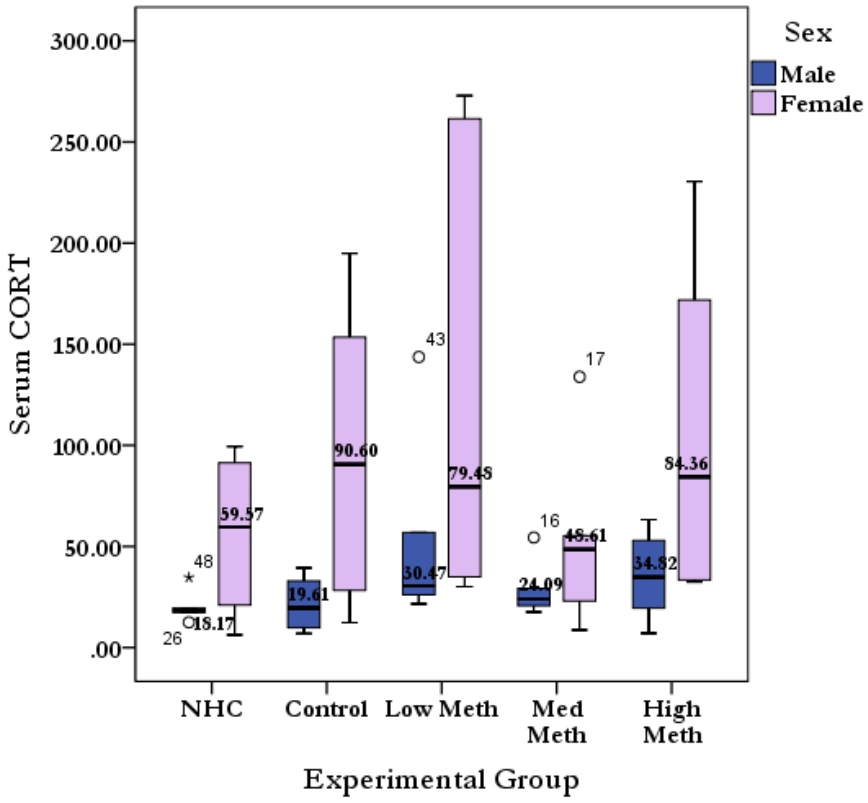


Figure 20: Mean Serum CORT for Each Experimental Group. Standard error bars for the NHC (n=9), control (n=10), low- (n=12), medium- (n=12), and high-Meth animals (n=13) are shown. Upper and lower quartiles are defined by boxplot boundaries.

3.9. *Sert* gene expression (PND 80)

There was no sex or USV effect on *Sert* gene expression (t_{53}=1.196, P=0.237; t_{53}=1.370, P=0.176). However, there was a significant cohort effect ($F_{2, 51}$=8.145, P=0.001). Therefore, cohort mean *Sert* was used as a covariate in ANOVA analyses. When controlling for cohort mean *Sert*, there was no statistically significant effect of experimental group on adult *Sert* gene expression ($F_{4,49}$=0.548, P=0.702) and there was no linear effect of mean Penh on *Sert* expression (Adj.-R^2= 0.358, cohort: β = -0.062, P=0.000; mean Penh: β = -0.081, P=0.504).

Chapter 4

Discussion

The goal of this study was to utilize a mouse model of asthma to experimentally determine the effect of recurring adolescent asthmatic labored breathing on neuroendocrine physiology and susceptibility to anxiety- and depressive-related behavior in adulthood. Specifically, we manipulated methacholine treatment doses to successfully induce asthma-like symptoms of severe, labored breathing in adolescent BALB/cJ mice.

In light of strong co-morbidity between adolescent asthma and adult internalizing disorders in humans, we tested the causal hypothesis that greater methacholine-induced labored breathing during adolescence leads to higher anxiety- and depressive-like symptoms in adulthood using our mouse model. We further hypothesized that individual differences in pre-asthmatic affective behavior (measured by neonatal USV, i.e. "basal affective behavior") would further affect susceptibility to labored breathing effects. Moreover, since stress-induced glucocortoid dysregulation and decreased serotonergic function have been previously associated with internalizing disorders (Ross, 2007; Zhang, 2012), we predicted that mice with greater labored breathing would demonstrate long-term flattening of glucocorticoid rhythm and reduced *Sert* gene expression as potential mediating factors for the co-morbidity between asthma and adult anxiety and depression.

Methacholine administration induced labored breathing in a dose-dependent fashion – mice that received higher methacholine doses had higher Penh measures, an index of labored

breathing. A key finding of this study was that mice with greater labored breathing had elevated adult anxiety-like behavior, demonstrated by the elevated plus maze test (EPM), compared to mice with less periadolescent labored breathing. This effect also followed a dose-dependent relationship as seen with Penh. However, basal affective behavior as measured by High- vs. Low- USV categorization did not predict significant differences in labored breathing or any behavioral or physiological outcome variables. Additionally, induced labored breathing was not associated with a categorical or linear effect on anhedonic behavior in the sucrose preference test (SPT), glucocorticoid dysregulation, or *Sert* gene expression as initially predicted.

Labored Breathing and Adult Anxiety-Like Behavior

The results of this study indicate that the adolescent asthma-like symptom of labored breathing had a significant linear influence on adult anxiety behavior. Mice with higher Penh values spent significantly less time on the open arms of the EPM, indicating increased anxiety-related behavior in a standard mouse behavioral test of anxiety. After controlling for cohort effects, labored breathing explained 19% of the variance in exploratory activity in the EPM. A similar study of 20 BALB/cJ mice pre-exposed to the allergen, ovalbumin, also reported an association between Penh and increased anxiety behavior in the EPM after aerosolized methacholine administration (Cheng, 2013). These findings provide support for our hypothesis that severe asthmatic labored breathing may be associated with increased anxiety-related behavior in adulthood.

Co-Morbidity of Asthma and Anxiety

Previous studies have established the importance of early life experiences in brain and behavioral development. In particular, significant psychological stressors during rapid developmental periods – including adolescence – can permanently affect glucocorticoid regulation and increase risk for internalizing disorders (Heim, 2001; Caspi, 2003; Feder, 2004; Sullivan, 2006; Taylor, 2009). With recurring episodes of severe, potentially life-threatening labored breathing associated with asthma, we reasoned that adolescent asthmatics may experience a significant chronic psychological stressor that could trigger glucocorticoid dysregulation and predispose individuals to develop internalizing disorders later in life. Studies of children with other recurring health problems also demonstrate increased likelihood for developing psychiatric conditions. For instance, individuals with childhood epilepsy, a condition characterized by unexpected periodic seizures, experience increased anxiety-related behavior (Williams, 2003; Vega, 2011). These findings provide support for the hypothesis that chronic health-related psychological stress during development may play an important role in the development of anxiety.

While our mouse model of labored breathing resulted in increased adult anxiety-related behavior as expected, there was no effect of labored breathing on sucrose preference in the sucrose preference test (SPT), a test used for studying depression-like symptoms of anhedonia in mice (Willner, 1987). Interestingly, sucrose preference in the BALB/cJ mice in this study was relatively low (59%). Upon further research, our findings are not inconsistent with other studies. In comparison to other strains such as the black C57BL6/J mice, BALB/cJ mice are a relatively low sweet preference strain. For example, in one study that directly compared the effects of social stress on sucrose preference (1% sucrose solution like our study) in BALB/cJ and

C57BL6/J mice, BALB/cJ mice demonstrated 49% sucrose preference in control vs. 44% for stressed mice, while C57BL6/J mice demonstrated 81% in control vs. 71% sucrose preference in stressed mice (Razzoli 2011). Given these findings, it is possible that the sucrose preference test may not be the most robust measure for anhedonia in BALB/cJ mice due to their comparatively low preference for sucrose.

Another possibility for the association between asthmatic symptoms and anxiety-related behavior but not depressive-like behavior may be that the underlying mechanism linking asthma with anxiety may be different than the mechanisms linking asthma and depression. Specifically, because depression is often co-morbid with anxiety (Noyes, 2001; Goodwin, 2008; Lamers, 2011; Goodwin, 2014), anxiety may be the significant driving factor linking the co-morbidity between asthma and depression. (In other words, instead of depression itself being directly linked to asthma, asthma may increase susceptibility to anxiety, which in turn may increase susceptibility to depression.) For instance, one longitudinal cohort study found that 67% of individuals currently diagnosed with depression also concurrently suffered from an anxiety disorder and that 75% of the depressive patients had an anxiety disorder in their lifetime (Lamers, 2011). Interestingly, the strongest correlations have been found between asthma and anxiety as opposed to internalizing disorders overall (Goodwin, 2008). For instance, children with respiratory disorders had a significantly elevated risk of receiving treatment for anxiety, but not depression alone (Goodwin, 2008).

No Effect of Labored Breathing on Glucocorticoid Regulation

Corticosterone (CORT) metabolite concentration in feces collected at the end of the adolescent period on PND 58-59 (one to two days after last methacholine administration) did not differ between methacholine treatment conditions and control mice. However, there was a circadian effect with high fCORT at 8:00 and 12:00 and low fCORT at 20:00 and 24:00 for both male and female mice. Serum CORT levels collected at necropsy (several weeks after the last methacholine administration) showed no significant difference between experimental groups and controls.

Our fecal CORT data replicate previous findings with higher CORT production for both males and females during the active dark phase and lower CORT production during the light phase (Jiang, 2006; Verma, 2010). In addition, in our study, female mice exhibited significantly higher fecal CORT and serum CORT levels than males, with a roughly 10-fold magnitude difference during peak fCORT production during the dark phase and roughly 3-fold magnitude difference in circulating basal CORT levels at necropsy. Our finding of significantly higher female serum CORT levels than males is not inconsistent with findings from previous literature (Handa, 1994), although the magnitude of fecal CORT difference between males and females is much higher in our study than reported elsewhere (10-fold difference vs. 2-3 fold difference) (Touma, 2003; Verma, 2010).

No Effect of Labored Breathing on *Sert* Gene Expression

Since previous studies have suggested that responses to chronic stressors may be regulated by serotonin, we originally hypothesized that asthmatic labored breathing stress may

alter glucocorticoid and serotonin regulation to increase risk for internalizing disorders. In our study, there was no significant difference in *Sert* mRNA gene expression in the dorsal raphe nuclei of the brainstem among experimental and control groups. However, this finding does not exclude the possibility that serotonin may influence the development of adult internalizing behaviors. Since there are multiple steps in the pathway between *Sert* mRNA gene expression and functionally expressed protein (i.e. protein translation, transport, activation, localization to the membrane, etc), it is possible that downstream factors later in the pathway may be critical in influencing serotonin and transporter levels. To confirm this possibility, future studies could directly measure levels of translated *Sert* protein or circulating serotonin levels at synapses in addition to mRNA gene expression.

Study Limitations

Limitations of this study should be noted, including a small sample size, imperfect instrument sensitivity, and technical difficulties. In total, the study included only 56 animals (n=29 male, n=27 female), which may have limited the statistical power of our findings. In addition, for certain physiological measures, such as fecal CORT during the inactive light cycle 24:00 hr time point, some animals did not defecate so no measure was obtained, which further reduced the sample size.

In this study, Penh was utilized as a noninvasive repeated measure of labored breathing. Whole-body barometric plethysmography has historically been used to estimate airway hyperresponsitivity in mice for longitudinal studies because of the benefit of obtaining data from conscious mice non-invasively and minimizing the potential confound of restraint stress (Dohi,

1999). However, the use of Penh is not without its criticisms. In our study, the plethysmograph instrument had difficulty picking up Penh readings for younger mice up to PND 35, despite visibly labored breathing. Since Penh recordings rely on changes in airflow and pressure as the mouse inhales and exhales, the instrument may not have been sensitive enough to detect small breathing changes due to small lung size in young mice. Previous studies have also reported difficulties in recording accurate Penh measurements for juvenile mice using barometric plethysmography (Hamada, 2000; Irvin, 2003). To evaluate this possibility, we retrospectively analyzed the effect of body weight (as an estimate of lung size) on Penh readings. An increase in body weight was correlated with an increase in Penh measurements during T1-T3. However, after PND 43 (T4), when recorded Penh measurements increased, body weight appeared to be less strongly correlated with Penh. It is notable that by PND 43, the majority of mice in all three cohorts appeared to be above a certain body weight threshold (approximately 17g). This finding suggests that mice need to reach a certain age and adequate lung size in order for a plethysmograph to be able to reliably detect recordings of Penh.

Additionally, instrument malfunctions during week 5 of the methacholine manipulation resulted in unreliable data for PND 49, so averages of week 4 and week 6 data were used to determine mean Penh values after methacholine administration.

Implications and Future Directions

In our study, initial plots of Penh by experimental condition indicated that mice that received the Low Methacholine Treatment condition (1.9, 3.75, 7.5, 15 ng/mL) were not significantly different from mice that received the Medium Methacholine condition (3.75, 7.5,

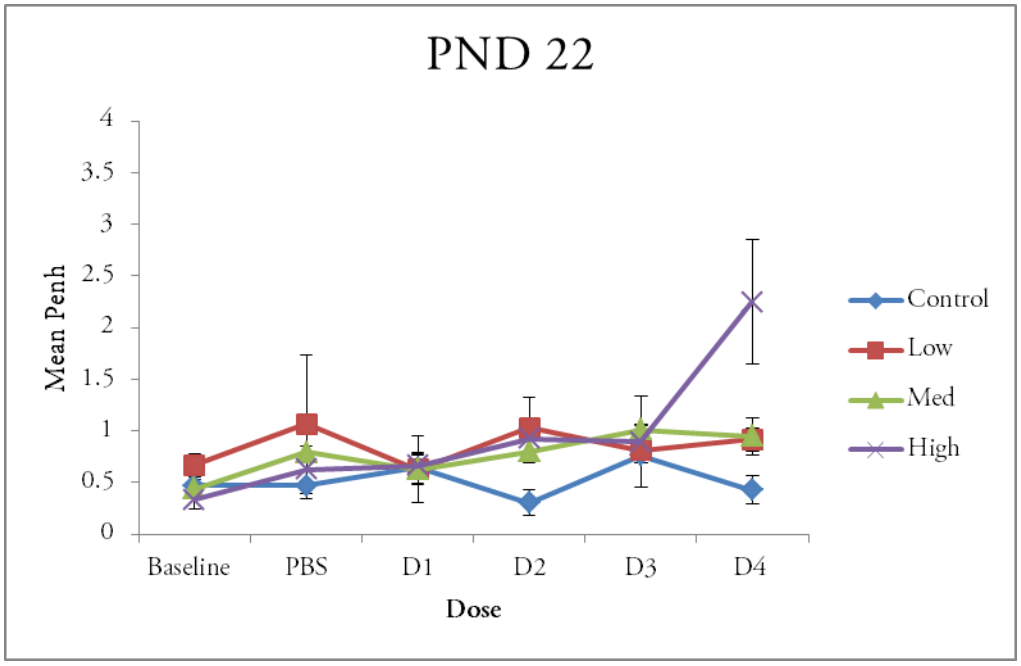
15, 30 ng/mL). In addition, body size may also play an important role in plethysmography detection of Penh. Therefore, for future studies, researchers may want to use higher incremental doses of methacholine (i.e. 7.5, 15, 30, 60 ng/mL) and take into account body size in order to significantly induce detectable labored breathing. Significant effects of our manipulation were not seen on CORT, SPT, or *Sert* data. It is possible that the strength of our manipulation was not high enough to mimic the asthma condition in humans.

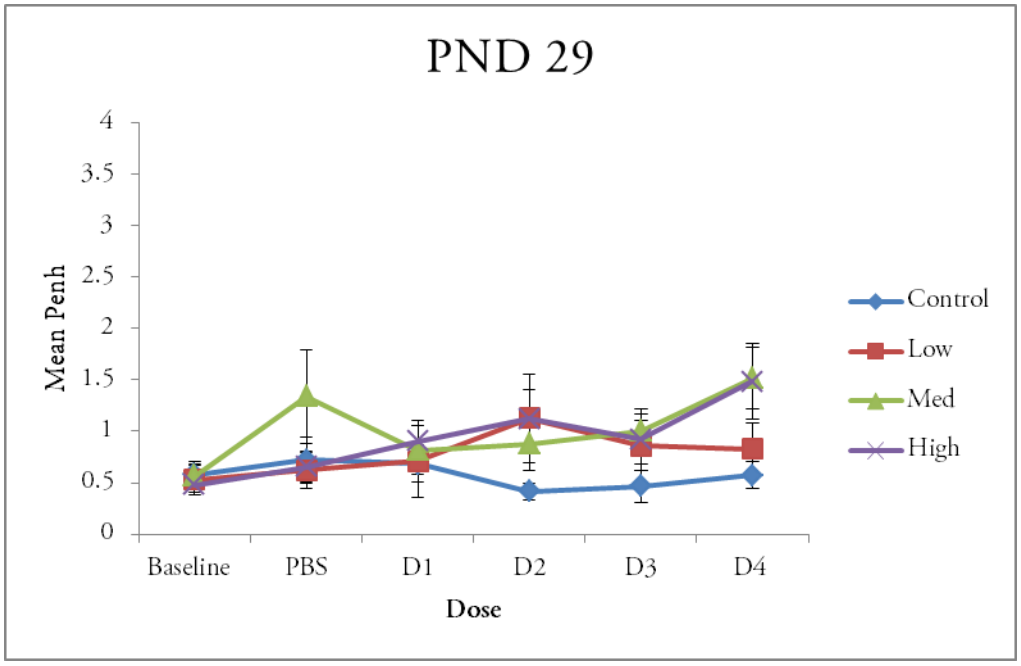
The major finding of this study is that increased intensity of adolescent labored breathing was associated with increased adult anxiety-like behavior. While asthmatic adolescents have a nearly doubled risk for developing internalizing disorders in adulthood, studies show that children with well-controlled asthma do not have an increased anxiety risk compared to non-asthmatic children (Letitre, 2014). In addition, poorer asthma control is correlated with increased anxiety-related symptoms (Letitre, 2014). This suggests that the chronic psychological stressor of severe, asthmatic labored breathing may play an important manageable – and potentially preventable – clinical role in the manifestation of anxiety risk. Going forward, the findings from this study may also help researchers develop more accurate mouse models for studying the relationship between adolescent asthma and adult anxiety.

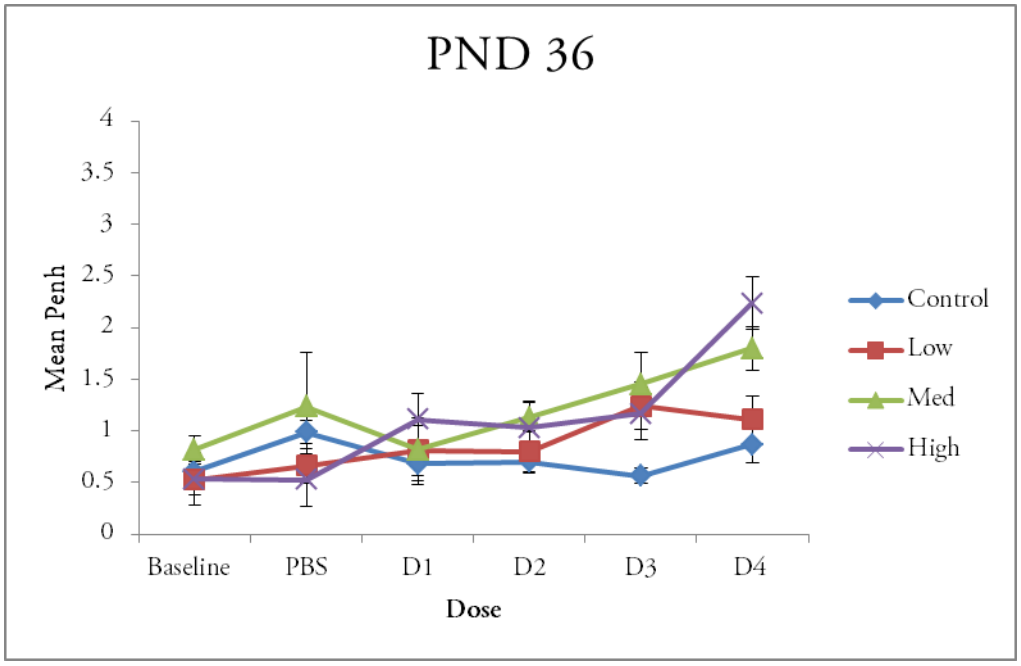
Appendix A

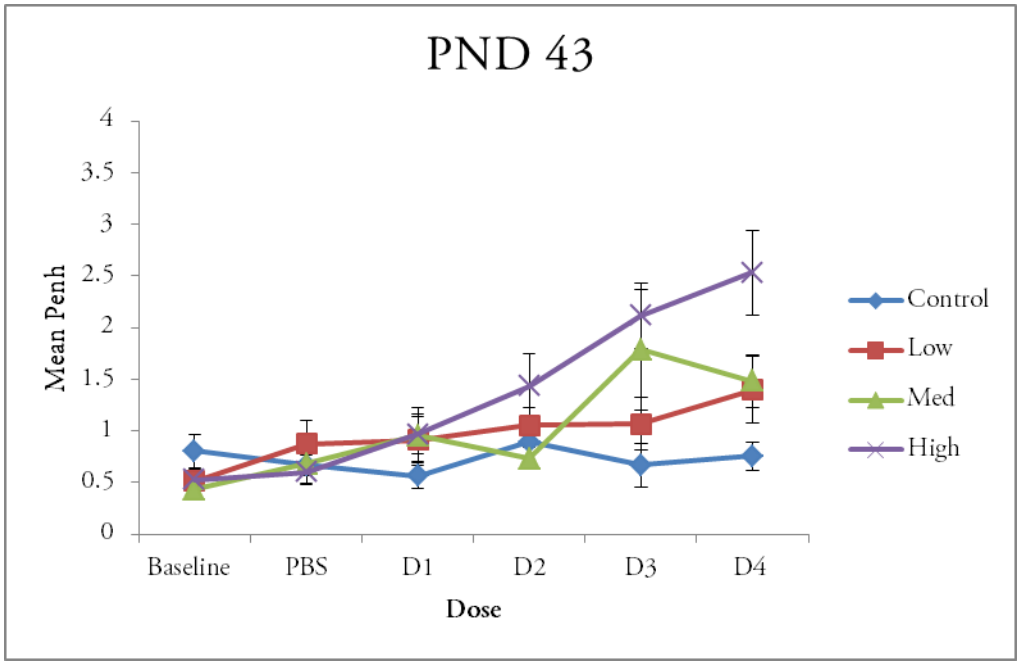
T1-T5 Penh

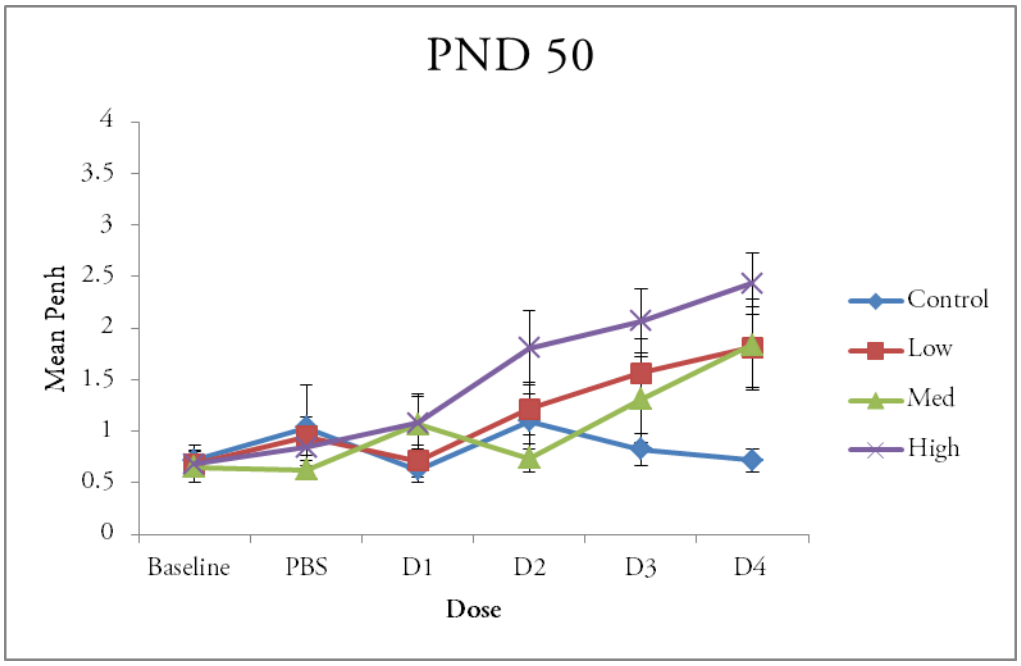
Graphs of Penh values for PND 22-59 (T1-T5).




PND 29
Mean Penh
4
3.5
3
2.5
2
1.5
1
0.5
0
Baseline
PBS
D1
D2
D3
D4
Dose
Control
Low
Med
High


PND 36
Mean Penh
4
3.5
3
2.5
2
1.5
1
0.5
0
Baseline
PBS
D1
D2
D3
D4
Dose
Control
Low
Med
High


PND 43
Mean Penh
4
3.5
3
2.5
2
1.5
1
0.5
0
Baseline
PBS
D1
D2
D3
D4
Dose
Control
Low
Med
High


PND 50
Mean Penh
4
3.5
3
2.5
2
1.5
1
0.5
0
Baseline
PBS
D1
D2
D3
D4
Dose
Control
Low
Med
High

Appendix B

Estimated Weight Data

Estimated PND 22, 29, 36, 43, 50, and 57 body weights were calculated. Experimental Group 1=NHC, 2=Control, 3=Low Meth, 4=Med Meth, 5=High Meth.

Mouse ID	*Cohort*	*Sex*	*Exp Group*	*T1 (PND 22)*	*T2 (PND 29)*	*T3 (PND 36)*	*T4 (PND 43)*	*T5 (PND 50)*	*T6 (PND 57)*
DEDF 1	1	M	4	10.9	13.1	15.3	17.5	19.7	21.9
DEDF 2	1	F	4	9.7	11.6	13.5	15.4	17.3	19.2
DEDF 3	1	M	3	10.2	12.6	15.1	17.5	19.9	22.3
DEDF 4	1	F	2	9.3	11.3	13.3	15.3	17.2	19.2
DEDF 5	1	F	1	8.6	10.7	12.8	14.9	17.0	19.2
DEDF 6	1	F	5	10.2	12.2	14.2	16.2	18.2	20.2
DEDF 7	1	F	3	7.3	9.3	11.2	13.1	15.1	17.0
DKDL 1	1	M	2	13.0	15.5	17.9	20.4	22.8	25.2
DKDL 2	1	F	4	12.1	14.0	16.0	18.0	20.0	22.0
DKDL 3	1	M	1	11.7	14.2	16.7	19.2	21.6	24.1
CWCX 1	1	F	3	10.9	12.8	14.8	16.7	18.6	20.5
CWCX 2	1	M	3	10.7	13.2	15.7	18.2	20.7	23.2
CWCX 3	1	M	2	10.9	13.4	15.9	18.4	20.9	23.3
CWCX 4	1	F	5	10.0	12.0	13.9	15.9	17.9	19.9
CWCX 5	1	M	5	11.7	14.2	16.8	19.4	21.9	24.5
CWCX 6	1	M	4	11.2	13.7	16.2	18.7	21.2	23.7

Mouse ID	*Cohort*	*Sex*	*Exp Group*	*T1 (PND 22)*	*T2 (PND 29)*	*T3 (PND 36)*	*T4 (PND 43)*	*T5 (PND 50)*	*T6 (PND 57)*
DIDJ 1	1	F	4	13.0	14.6	16.2	17.9	19.5	21.1
DIDJ 2	1	F	3	12.8	14.7	16.5	18.4	20.2	22.0
DIDJ 3	1	F	5	12.4	14.1	15.8	17.5	19.1	20.8
DIDJ 4	1	F	2	12.3	14.2	16.1	18.0	19.9	21.8
DIDJ 5	1	M	5	13.7	16.2	18.6	21.1	23.5	26.0
DDDC 1	2	M	1	13.4	15.1	16.9	18.7	20.5	22.3
DDDC 2	2	M	3	15.5	17.5	19.6	21.7	23.7	25.8
DDDC 3	2	M	4	18.3	20.4	22.5	24.6	26.7	28.8
DDDC 4	2	M	5	14.1	16.0	17.9	19.9	21.8	23.8
DDDC 5	2	M	1	14.8	17.3	19.8	22.3	24.7	27.2
DDDC 6	2	M	4	13.7	16.0	18.4	20.8	23.1	25.5
DDDC 7	2	M	2	14.5	15.9	17.3	18.7	20.1	21.4
DDDC 8	2	F	5	13.7	15.1	16.5	18.0	19.4	20.8
DFDE 1	2	F	2	17.1	18.3	19.5	20.7	21.9	23.1
DFDE 2	2	F	4	17.4	18.4	19.5	20.5	21.6	22.7
DFDE 3	2	M	5	17.0	18.6	20.2	21.8	23.4	25.0
DFDE 4	2	F	3	16.4	17.4	18.4	19.3	20.3	21.3
DHDG 1	2	M	2	13.2	15.9	18.6	21.3	24.0	26.7
DHDG 2	2	M	3	14.7	17.1	19.6	22.0	24.5	27.0
DHDG 3	2	M	1	13.7	16.2	18.7	21.2	23.7	26.2
DJDI 1	2	M	4	16.0	17.9	19.9	21.8	23.7	25.6
DJDI 2	2	M	5	15.7	17.8	20.0	22.1	24.2	26.4

Mouse ID	*Cohort*	*Sex*	*Exp Group*	*T1 (PND 22)*	*T2 (PND 29)*	*T3 (PND 36)*	*T4 (PND 43)*	*T5 (PND 50)*	*T6 (PND 57)*
DJDI 3	2	F	1	12.5	13.7	14.8	16.0	17.1	18.3
YCZC 1	3	F	4	13.5	14.7	16.0	17.2	18.4	19.7
YCZC 2	3	F	5	12.4	13.5	14.5	15.6	16.7	17.8
EDFD 1	3	M	2	17.1	18.6	20.2	21.8	23.4	25.0
EDFD 2	3	M	3	17.0	18.7	20.5	22.2	24.0	25.7
EDFD 3	3	M	4	17.5	19.2	20.8	22.5	24.1	25.8
GDHD 1	3	F	2	12.4	14.1	15.9	17.6	19.3	21.1
GDHD 2	3	F	3	11.9	13.5	15.2	16.9	18.6	20.3
GDHD 3	3	F	4	12.4	14.1	15.7	17.4	19.1	20.7
GDHD 4	3	M	1	11.8	14.0	16.2	18.4	20.6	22.8
GDHD 5	3	M	5	13.3	15.6	17.9	20.2	22.5	24.8
GDHD 6	3	F	5	12.5	14.2	15.9	17.5	19.2	20.9
GDHD 7	3	F	1	11.5	13.2	14.9	16.6	18.2	19.9
IDJD 1	3	M	5	16.9	18.2	19.4	20.7	22.0	23.2
IDJD 2	3	M	3	17.3	18.7	20.1	21.5	22.9	24.2
IDJD 3	3	F	1	15.4	16.1	16.8	17.5	18.2	18.9
IDJD 4	3	F	2	15.2	16.4	17.6	18.8	20.1	21.3
IDJD 5	3	F	3	14.3	15.8	17.3	18.8	20.3	21.8

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ACADEMIC VITA

MARY CHEN

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EDUCATION

B.S. in Biology – Vertebrate Physiology, with Honors in Biobehavioral Health | May 2016
The Pennsylvania State University, University Park, PA

Summa Cum Laude
Wissahickon Senior High School, Ambler, PA | June 2012

RESEARCH EXPERIENCE

Penn State Behavioral Neuroendocrinology Laboratory
Undergraduate Research Assistant | Spring 2014 - Spring 2016

- Completed honors thesis research under the supervision of Dr. Cavigelli
- Involved in animal handling, specimen collection, and data analysis

Fox Chase Cancer Center, Philadelphia, PA
Summer Research Intern | Summer 2014

- Studied racial disparities in prostate cancer among underrepresented minority populations in the US SEER database
- Involved in community recruitment of Haitian-American study participants
- Assisted with organizing meeting logistics and abstract submissions for the African-Caribbean Cancer Consortium conference held in October 2014

COMMUNITY INVOLVEMENT

Mount Nittany Medical Center, State College, PA
Emergency Department Volunteer | Spring 2014 - Spring 2016

- Transported patients to testing locations
- Assisted hospital staff with room preparation and supply stocking

Hershey Regional Campus Career Observation Program, State College, PA
Student Observer, Spring 2016-Present

- Shadowed physician during evening clinic hours

Penn State Eberly College of Science
BIOL473 Mammalian Physiology Lab Co-Teaching Assistant | Spring 2016

- Demonstrated lab dissections, held weekly office hours, and graded assignments
- Assisted students with survival rodent surgery, suturing, and maintaining sterile surgical fields

Science U Curriculum Mentor | Summer 2015

- Led small groups of students in grades 2-8 during summer science camp sessions

Chemistry Department Tutor | Spring 2015 - Spring 2016

- Tutored inorganic and organic chemistry

BIOL110 Learning Assistant | Fall 2014

- Assisted students during lecture and maintained office hours twice a week

Penn State Schreyer Honors College
Career Development Mentor | Fall 2015 - Spring 2016
- Mentored underclassmen in pre-health academic and career programs

Faculty Liaison Gateway Orientation Leader | Fall 2014
- Coordinated faculty dinner discussions for the Gateway Orientation program for new students entering the Schreyer Honors College

Penn State African Library Project
Secretary & Web Coordinator | Fall 2012 - Fall 2015
- Organized book drive logistics and fundraising to collect and ship 5,000+ books to start libraries in Sierra Leone and Malawi
- Created and managed club website and mailing list

St. Mary Manor Catholic Health Care Services Center, Lansdale, PA
Volunteer | Summer 2013
- Escorted and performed at musical recitals for residents

Circle K International
Scrapbook Chair | Fall 2012 – Spring 2013
- Volunteered in local State College area at *The Village* retirement community and *Shaver's Creek Environmental Center*
- Photographed service and club events for end-of-year scrapbook

HONORS AND AWARDS

- Evan Pugh Senior Award (awarded to students in the top 0.5% of the senior class)
  The Pennsylvania State University | Spring 2016
- Edward C. Hammond Memorial Scholarship for Science
  The Pennsylvania State University | Fall 2015
- College of Health and Human Development Undergraduate Research Award
  The Pennsylvania State University | Summer 2015
- Schreyer Honors College Summer Research Grant
  The Pennsylvania State University | Summer 2015
- Evan Pugh Junior Award for Academic Excellence
  The Pennsylvania State University | Spring 2015
- FCCC Undergraduate Summer Research Fellowship
  Fox Chase Cancer Center, Philadelphia, PA | Summer 2014
- Penn State Montgomery County Alumni Association Scholarship (for academic achievement, community service, and leadership)
  The Pennsylvania State University | Summer 2014
- President Sparks Sophomore Award for Academic Excellence
  The Pennsylvania State University | Spring 2014
- President's Freshman Award for Academic Excellence
  The Pennsylvania State University | Spring 2013
- Thomas J. Cassidy Memorial Award (for community service and leadership)
  Blue Bell Rotary International Club, Blue Bell, PA | 2012
- Dean's List
  The Pennsylvania State University | Fall 2012 - Spring 2016