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The effect of diet and polybrominated diphenyl ether (PBDE) exposure on adipocyte and whole body metabolism in male Wistar rats

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THE EFFECT OF DIET AND POLYBROMINATED DIPHENYL ETHER (PBDE) EXPOSURE ON ADIPOCYTE AND WHOLE BODY METABOLISM IN MALE WISTAR RATS

BY

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ABSTRACT

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PBDEs, lipophilic flame-retardant chemicals, are considered to be endocrine-disrupting compounds and potential obesogens. This study investigated PBDE exposure plus a high-fat/high-sugar diet in rats. Twenty-eight male Wistar rats were fed either a control or high-fat/high-sugar diet and gavaged with either 18 mg/kg PBDEs or corn oil daily for 4 weeks. Body weight and food intake were measured 3x/week. At 3 weeks, 24-hr whole-body metabolism was measured. At 4 weeks, blood was sampled for T_4 and insulin, epididymal adipose tissue was removed and adipocyte lipolytic response to varying concentrations of isoproterenol was measured. PBDE administration significantly increased weight gain, decreased T_4 levels and tended to increase glucose disappearance, increase energy production and decrease insulin levels. A dietxPBDE treatment interaction was noted for metabolic efficiency, protein disappearance, epididymal adipose weight, and insulin level. PBDEs disrupt macronutrient metabolism and energy balance in rats and the obesogenicity of PBDEs can be modulated by diet.

CHAPTER I

LITERATURE REVIEW

Preface

This literature review is written in three parts: a brief overview of characteristics of polybrominated diphenyl ethers (PBDEs) and their prevalence throughout the world, an in-depth investigation of the biochemical consequences associated with exposure to PBDEs and the potential connection between PBDE exposure and obesity.

PBDEs: Characteristics and Prevalence

Structure and Characteristics

PBDEs are a class of flame retardant chemicals commonly found in products such as carpeting, furniture and electronics (Danerud et al., 2001). PBDEs were introduced into manufacturing in the 1970's. North America is the largest consumer of PBDEs and in 2001, North American companies used 149 million pounds of PBDEs in manufacturing, accounting for approximately half of the total global consumption of PBDEs (Sharp and Lunder, 2007).

All PBDEs are composed of two phenyl rings connected by an ether bridge; however the number of bromine atoms present on the rings can vary (Figure 1), leading

Figure 1—General Structure of PBDEs and Thyroxine

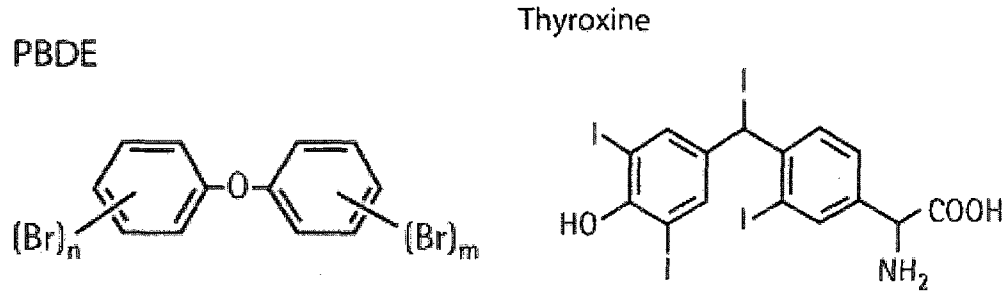

Figure 1: General structure of PBDEs and thyroxine (T_4). PBDEs are characterized by varying numbers of bromine atoms on the phenyl rings (Sightline, 2004). The structure of T_4 has two phenyl rings with iodine atoms attached. Iodine atoms and bromine atoms are both halogens with similar chemical properties. Hydroxylated PBDEs are even more similar in structure to T_4.

to 209 possible congeners of PBDEs (denoted BDE-1 through BDE-209) (Branchi et al., 2003). There are three major formulations of PBDEs: penta-, octa- and deca- (Hale et al., 2003). Penta- formulations are found in automobile upholstery and furniture, while deca- and octa- formulations are mainly found in electronics (Birnbaum and Staskal, 2004). The major congeners present in penta formulations are BDE-47, -99, -100, -153, -154, -85, while the major congeners in the octa formulations are BDE-183 and BDE-153. The major deca congener is BDE-209 (Hale et al., 2003).

PBDEs are incorporated into consumer products either by addition (in which PBDEs are not chemically bound to the products) or by chemical reaction. PBDEs incorporated via addition are more likely to leach into the environment than PBDEs incorporated via a chemical reaction (Kim et al., 2006). Once in the environment, PBDEs are resistant to physical or chemical degradation due to their chemical structure and high lipophilicity (Branchi et al., 2003).

Global Prevalence

Consumer products containing PBDEs are often disposed of by incineration (such as in electronic waste recycling sites) or by being deposited in a landfill (Danerud et al., 2001), allowing the PBDEs to leach into the soil or run off into the watershed.

Because PBDEs are a major component in electronics, high levels have been found in electronic waste (e-waste) recycling sites. In one study conducted by Liu et al, surface soil concentrations of PBDEs near an e-waste recycling site in China were found to be as high as 789.49 ng/g dry weight, whereas towns farther away from the e-waste site had levels of PBDEs as low as 0.40 ng/g dry weight (Liu et al., 2008). Another study conducted in China found concentrations of PBDEs to be 1.4 μg/g lipid weight in tree bark near an e-waste recycling site, which is indicative of high levels of PBDE contamination in that area (Wen et al., 2008). The authors did not compare tree bark from sites far removed from the e-waste recycling facility.

PBDE contamination is not limited to e-waste recycling sites. High levels of PBDEs have been documented in aquatic environments. Oros et al found PBDE levels as high as 513 pg/L in the San Francisco Bay estuary (Oros et al., 2005). Oysters, mussels and clams contained levels of PBDEs as high as 64 ng/g, 47 ng/g and 106 ng/g dry weight, respectively. Congeners found in the bivalves were BDE-47, BDE-99 and BDE-100.

There is also documentation of PBDEs in sewage sludge. Varying levels of PBDEs in sewage sludge have been documented in Sweden (Oberg et al., 2002),

Germany (Knoth et al., 2007) and China (Wang et al., 2007). Detectable levels in sewage sludge are indicative of contamination of the environment and of humans.

Routes of Exposure

Humans can come in contact with PBDEs once they are released into the environment. There are two major routes of human exposure to PBDEs: through dust and food. Whether it is in the home or in a landfill, PBDEs are released into the environment as consumer products start to wear down.

Indoor dust is a major contributor of PBDE contamination. In 2005, Schecter et al found that house dust from a sample of homes in the U.S. primarily contains deca- and penta PBDE formulations from computers and carpeting, respectively (Schecter et al., 2005). The predominant congener found in these dust samples was BDE-209.

Wilford et al also reported finding BDE-209 as the predominant congener in dust samples (Wilford et al., 2005). The average amount of PBDEs in dust samples was found to be 2000 ng/g dust. From this information, Wilford calculated that an average adult would be ingesting approximately 14% of his/her daily body burden of PBDEs from dust ingestion, 2% of his/her daily body burden of PBDEs from dust inhalation, while 84% of PBDE exposure would come from the diet. However, dust ingestion constitutes 80% of the body burden for toddlers, who generally spend more time close to dusty floors and carpeting.

Another important PBDE exposure pathway is through the diet. Ohta et al investigated the levels of PBDEs in fish, vegetables and meats from markets in Japan (Ohta et al., 2002). Mean PBDE concentrations were found to be as high as 1650 pg/g

fresh weight of edible tissue from fish, with the highest concentrations being found in yellowfin tuna, short-necked clam, salmon, yellowtail, mackerel and young yellowtail. Concentrations found in beef, pork and chicken were as high as 63.3 pg/g of fresh weight. Vegetables were found to have concentrations as high as 134 pg/g of fresh weight.

Researchers in Spain conducted a similar study to Ohta by obtaining various foodstuffs from markets in Catalonia (Bocio et al., 2003). Concentrations of PBDEs were found to be highest in oils and fats (587.7 ng/kg), with lower concentrations in fish and shellfish (333.9 ng/kg), meats (109.2 ng/kg) and eggs (64.5 ng/kg). Fruits, cereals and tubers did not have detectable levels of PBDEs. From this information, the researchers estimated the total dietary intake of PBDEs from foods for a 70 kg adult male to be 1.2-1.4 ng/kg body weight/day.

A study from the U.S. investigated PBDE concentrations in fish, meat and dairy foods (Schecter et al., 2004). Fish was found to have the highest concentration of PBDEs (median of 1725 pg/g wet weight) with the predominant congener being BDE-47. Meat had the second highest PBDE concentration (median of 283 pg/g wet weight) with BDE-99 being the predominant congener followed by BDE-47. Dairy products had the lowest levels of PBDEs (31.5 pg/g wet weight) with the predominant congener being BDE-47 followed by BDE-99.

Another group assessed the bioavailabiltity of ingested dust as compared to PBDEs dissolved in corn oil (Huwe et al, 2008). Rats were administered 1 or 6 μg/kg BW/ day of PBDEs for 21 days. PBDEs from the dust and oil mixture accumulated in

adipose tissue and the liver at similar rates, suggesting that both dust and diet contribute equally to PBDE body burden.

Humans are exposed to PBDEs through dust and diet, suggesting that virtually every animal and human in the world most likely has detectable levels of PBDEs in circulation, adipose tissue, in hair samples and in breastmilk.

Body Burden

Studies on land-based animals have uncovered high levels of PBDEs in animal tissues. A study from China illustrated the levels of PBDEs found in birds of prey (Chen et al., 2007). Multiple specimens were taken from each bird and subsequently analyzed for levels of various PBDE congeners. Common kestrels had the highest amount of PBDEs (12,300 ng/g lipid weight in liver and 12,200 ng/g lipid weight in muscle) when compared to the other species analyzed. The higher brominated congeners of PBDEs were abundant: BDE-209, BDE-153, BDE-207 and BDE-183. BDE-209 was found to be the predominant congener in buzzards and owls.

Many studies have focused on the amounts of PBDEs in marine organisms. Researchers in Italy investigated the amounts of PBDEs in Mediterranean swordfish caught from the South Tyrrhenian Sea (Corsolini et al., 2008). Liver and muscle tissue were analyzed by high resolution gas-chromatography and tandem mass spectrometry and PBDE congeners were detected. In liver, total PBDE concentrations were 2218 pg/g wet weight, while PBDE concentrations in the muscle tissue were 612 pg/g wet weight.

In another fish study, researchers examined two species of Antarctic fish (*Chionodraco hamatus* and *Trematomus bernachii*) for PBDE levels (Borghesi et al.,

2008). Congeners BDE-153 and BDE-154 were prevalent in both liver and muscle tissue. Hepatic PBDE levels in *C. hamatus* were on average higher than in *T. bernachii*. *C. hamatus* muscle contained 160.5 pg/g wet weight of PBDEs while *T. bernachii* muscle had 789.9 pg/g wet weight of PBDEs.

There are documented cases of PBDEs present in the tissues of higher organisms as well. A variety of PBDE congeners were found in the tissues of giant and red pandas in China (Hu et al., 2008). Total PBDE concentrations were found to be as high as 2158 ng/g lipid weight. The highest concentration of PBDEs was found in the gonadal tissue of male pandas. The predominant congener was BDE-209 with lower concentrations of BDE-206, BDE-207, BDE-208, BDE-203, BDE-47 and BDE-153.

PBDEs have been found in the tissue of killer whales in Puget Sound and Georgia Basin (Krahn et al., 2007). Blubber and epidermis biopsies were analyzed for PBDE content. Blubber PBDE concentrations ranged between 2500 and 15,000 ng/g lipid. The presence of PBDEs in higher animals suggests that PBDEs are biomagnifying through the food chain, most likely due to their lipophilic nature.

PBDEs are far-reaching environmental pollutants. Bentzen et al reported finding observable levels of PBDEs in the blood and adipose tissue of polar bears in Antarctica (2008). Since there are no humans living in Antarctica, and therefore no human products containing PBDEs, it is assumed that PBDEs are traveling through waterways and the air to settle in such remote places.

While wildlife studies illustrate the prevalence of PBDEs in the environment, human samples show that PBDEs are localizing within our own tissues as well. Because

there are detectable levels of PBDEs in wildlife, and PBDEs are in higher concentrations in animals higher up on the food chain, it is safe to assume that PBDEs are in high concentrations in humans and this is true, judging from the literature.

Choi et al reported on the increasing concentration of PBDEs in the adipose tissue of residents of Tokyo, Japan from the year 1970 compared to the year 2000, possibly a reflection of the increasing use of PBDEs in manufacturing over that time span (2003). The median concentration from 1970 was 29.2 pg/g lipid weight and the median concentration in 2000 was found to be 1288 pg/g lipid weight. There was also a shift in the types of PBDEs found from 1970 to 2000: BDE-47 comprised 56.2% of the total in 1970 and was 35.6% of the total in 2000, indicating a shift towards higher brominated PBDEs in commercial products.

Another study performed in Japan in 2007 showed high concentrations of PBDEs in the adipose tissue of human subjects and, when compared to a 2000 study on a similar cohort, showed that average PBDE levels had increased over the seven year span from 1.288 ng/g lipid weight in 2000 (Choi et al., 2003) to as high as 46 ng/g lipid weight (Kunisue et al., 2007). BDE-153 was the predominant congener found in adipose tissue.

Human hair samples collected from residents living near an e-waste recycling site in China were found to be significantly higher than a control group that lived far from the e-waste recycling site (Zhao et al., 2008). The highest levels found in the group living near the site were 29.64 ng/g dry weight, which was two times the amount of PBDEs found in the hair of the control group.

There is also documentation of PBDEs in the tissues of Europeans and Americans. Total PBDE concentrations in the adipose tissue of Belgians were found to be as high as 57.2 ng/g lipid weight (Naert et al., 2006). Americans tend to have very high tissue levels of PBDEs. A study done in California in 2002 showed that women, whose ages ranged from 28 to 62, in the San Francisco Bay area had high total PBDE levels, with BDE-47 being the most predominant congener (She et al., 2002). The average total PBDE levels were found to be 86 ng/g lipid weight in adipose tissue taken from the breast area. In another study, adipose tissue samples from residents of New York City had a mean PBDE concentration of 399 ng/g lipid weight (Johnson-Restrepo et al., 2005). A variety of congeners were found and BDE-47 was the major PBDE present in most subjects. The U.S. values reported are 10-100 times greater than those reported in many European countries, suggesting that Americans are more heavily exposed to PBDEs.

It has been shown that PBDEs are in detectable concentrations in animals and humans, but the biochemical consequences of PBDEs in the body are still being investigated.

PBDEs: Biochemical Consequences

The full extent to which PBDEs affect body processes is unknown, but studies have shown that PBDEs are metabolized by the body, can disrupt thyroid hormone, insulin and steroid hormone function.

PBDE Metabolism

Hydroxylated and methoxylated PBDEs have been found in human tissue, suggesting that PBDEs are being metabolized within the human body. Hamers et al

showed that PBDEs could be transformed into hydroxylated PBDEs when incubated with rat liver microsomes *in vitro* (Hamers et al., 2008). Six hydroxylated metabolites were produced and found to have higher transthyretin (thyroid hormone transport protein) binding-potential and estradiol-sulfotransferase inhibition than the parent compound, BDE-47 when used in an *in vitro* binding assay.

Lacorte and Ikonomou found detectable levels of hydroxylated and methoxylated PBDEs in human breast milk in women from Catalonia, Spain (Lacorte and Ikonomou, 2009). Hydroxylated and methoxylated PBDEs comprised 2.9% and 1.6% of the total PBDE concentration, respectively. Detectable levels of hydroxylated and methoxylated PBDEs in breast milk could constitute an important route of exposure for neonates.

Qiu et al also detected hydroxylated PBDEs in human blood samples (Qiu et al., 2009). BDE-47 was found in the form of 5-OH-BDE-47 and 6-OH-BDE-47 and BDE-99 was found in the form of 5-OH-BDE-99 and 6-OH-BDE-99. The ratio of total hydroxylated metabolites to their PBDE precursors ranged from 0.10 and 2.8 in blood samples. The researchers postulated that the high ratio of OH-PBDEs to their precursors meant that these metabolites were accumulating in the blood and believe that hydroxylation of the PBDEs is not a significant mechanism for excretion, as previously thought.

The presence of PBDEs and their metabolites in the body raises the question of how PBDEs may be interacting with enzymes, receptors, transcription factors or other biochemicals within the body, which could be causing a whole range of physiological effects.

Thyroid Hormone Disruption

PBDEs have been shown to decrease thyroid hormone levels in animals. In a study by Zhou et al, the researchers administered either DE-71 or DE-79 (commercial mixtures of PBDEs) at various doses (ranging from 0.30 to 300 mg/kg body weight/day) to Long Evans rats for four days (2001). There was a dose-dependent decrease in T_4, with an 80% decrease in T_4 levels seen with the highest dose of DE-71 and a 70% decrease in T_4 levels seen with the highest dose of DE-79. There was a modest reduction in T_3 levels (25-30%) seen with the highest doses of either mixture. Danerud et al saw a similar decrease in T_4 levels in rats treated with BDE-47 at a dose of 18 mg/kg body weight/day for four weeks. T_4 levels were 34% lower in PBDE-treated animals compared to control animals (2007).

Hydroxylated PBDEs have a very similar chemical structure to that of thyroid hormone, T_3 and T_4, and as such, have been implicated in thyroid hormone disruption in humans (Figure 1). Because of this similarity in structure, hydroxylated PBDEs have the ability to bind to transthyretin. Hamers et al incubated PBDEs with rat liver microsomes and subsequently collected hydroxylated PBDEs (2008). These hydroxylated PBDEs were used in bioassays to determine the ability of the OH-PBDEs to bind to transthyretin. The transformed OH-PBDEs bound 160-1600 times better to transthyretin than the non-hydroxylated counterpart.

Hydroxylated PBDEs have also been found to bind to thyroid hormone receptors *in vitro*. Researchers assessed the ability of PBDEs and hydroxylated PBDEs to bind to thyroid hormone alpha or thyroid hormone beta receptors *in vitro* (Marsh et al., 1998).

The PBDEs that exhibited the best binding to the thyroid hormone receptors were those that had bromines in the same positions as the iodines on the T_4 molecule.

Researchers in Japan investigated the thyroid-modulating potency of PBDEs found in dust samples taken from homes, offices and laboratories (Suzuki et al., 2007). All dust extracts produced significant competition curves for transthyretin binding. Because of the potency of PBDEs in dust to bind to transthyretin, there is concern that PBDEs will adversely affect thyroid hormone homeostasis.

Despite the clear effects of PBDEs on thyroid hormone function in animals and *in vitro*, human studies conflict with animal studies. Adult, male, sport fishermen showed a positive association between PBDE concentration and T_4 and reverse T_3 levels, but a negative association with T_3 and thyroid-stimulating hormone (TSH) (Turyk et al., 2008). The researchers also found an inverse relation between PBDE concentration and T_4 bound to T_4-binding globulin (TBG), but a positive correlation between T_4 bound to albumin and PBDE levels. This study illustrates a drastic difference between animal and human studies.

In another human study, Julander et al repeatedly sampled for 1.5 years the blood of workers in an electronic recycling facility for PBDE, total T_3, free T_4 and thyroid-stimulating hormone (TSH) levels (2005). The researchers found that there were fluctuations in the PBDE levels in the workers and there were small increases and decreases in T_3, T_4 and TSH levels, but these fluctuations were not significant. For three workers, there was a significant positive correlation between PBDE concentration and either T_3, T_4 or TSH concentrations, but the hormones were all found to be within normal

physiological range. It is possible that chronic low-level exposure to PBDEs would show decreased T_3, T_4 and TSH levels over a longer period of time, but the short period of time over which researchers sampled blood was not long enough to produce altered thyroid hormone status.

Researchers also investigated the correlation of PBDE concentrations to thyroid hormone levels in maternal and fetal blood samples from American women and fetuses (Madzai et al., 2003). The researchers found that maternal levels of PBDEs were reflected in fetal blood and that these levels of PBDEs were 20- 106 times higher than those found in pregnant women in Sweden; however, there was no correlation between PBDE levels in the pregnant mothers and thyroid hormone levels.

Animal studies show that PBDEs cause a decrease in T_3 and T_4 levels; however, studies show that human thyroid hormones may not be as sensitive to PBDE exposure as murine thyroid hormones. The differing results in these studies indicate that the relationship between PBDEs and thyroid hormone in humans is more complicated than what animal studies have lead scientists to believe. Animal studies generally reflect high doses of PBDEs given to animals over a relatively short period of time, whereas humans generally have chronic, low level exposure to PBDEs. More human studies are needed to characterize the effects of PBDEs *in vivo* over long periods of time. There may be a mechanism in place that prevents against substantially decreased thyroid hormone levels in humans.

Insulin Disruption

A cross-sectional study examining the prevalence of diabetes as associated with polybrominated biphenyls (PBBs) and PBDEs showed interesting results (Lim et al.,

2008). Researchers obtained information from NHANES data and looked for a correlation between diabetic condition (defined as fasting glucose levels of greater than 126 mg/dL, non-fasting glucose levels of 200 mg/dL or if the subjects were taking medications for previously diagnosed diabetes) and plasma concentrations of PBB-153 or PBDE-153. Covariates that were considered were age, sex, race/ethnicity, poverty income ratio and body mass index. A significant positive correlation was seen between PBB-153 and diabetes and a nonlinear association was seen with PBDE-153 and diabetes. While correlation does not constitute causation, it is provocative to think that PBDE body burden could be implicated in the diabetes epidemic in the United States.

In an *in vitro* study, PBDEs were shown to decrease insulin-stimulated glucose oxidation in adipocytes isolated from PBDE-treated rats (Hoppe and Carey, 2007). It is possible that PBDEs are causing disruption of insulin or glucose metabolism, which could lead to the development of diabetes. Despite this interesting result, this is an *in vitro* study that needs to be repeated while looking at whole body glucose disappearance and insulin levels to ascertain a more clear understanding of the potential connection between PBDEs and the diabetic condition.

Estrogen Disruption

PBDEs have been shown to affect estrogen signaling *in vitro* and *in vivo*. BDE-209 was found to significantly increase the 4-hydroxylation and 2-hydroxylation of estradiol in male rat liver microsomes *in vitro*, but caused no change in 4-hydroxylation and a decrease in 2-hydroxylation of estradiol in female rat liver microsomes (Segura-Aguilar et al., 1997). This research provides evidence that PBDEs are having an effect on

enzymes of the cytochrome P450 system that are involved in hydroxylation of estradiol. Increased hydroxylation of estradiol is linked with carcinogenicity, and could possibly be a mechanism for male estradiol-dependent cancers.

Researchers tested the ability of BDE-71 and its hydroxylated metabolites to displace estradiol from the estrogen receptor alpha *in vitro* in a recombinant cell line (Mercado-Feliciano and Bigsby, 2008). BDE-71 failed to displace estradiol from the estrogen receptor alpha; however, the six hydroxylated metabolites that were tested were all able to displace estradiol from its receptor. Meerts et al (2001) and Hamers et al (2006) also showed similar binding affinities for hydroxylated PBDEs *in vitro* using an estrogen-receptor element luciferase assay. If PBDEs are able to displace estradiol from its receptor, it is possible that PBDEs are acting as estrogen agonists or antagonists.

In another study, BDE-99 decreased the expression of the progesterone receptor, but slightly increased the expression of estrogen receptor alpha, and greatly increased the expression of estrogen receptor beta in developmentally exposed rats at a dose of 1 mg/kg body weight/day from gestational day 10-18 (Ceccatelli et al., 2006). A dose of 10 mg/kg body weight/day of BDE-99 decreased progesterone levels, but both estrogen receptor levels were the same as baseline at the higher dose. The upregulation of the estrogen receptors at the low dose is indicative that PBDEs are acting as estradiol agonists. Because PBDEs act as estradiol agonists, a high dose of PBDEs could cause the receptors to downregulate.

Pregnant Wistar rats were treated with 140 μg/ kg body weight or 700 μg/ kg body weight PBDEs from gestational day 7 through postnatal day 21. PBDEs or a control

vehicle and various estradiol factors were measured in the offspring (Talsness et al., 2008). PBDE treatment resulted in decreased ovarian weight and decreased serum estradiol concentrations in offspring. If PBDEs are acting as estradiol agonists, it is possible that PBDEs are mimicking estradiol and feeding back to cause less estradiol to be secreted.

PBDEs: Connection to Obesity

It is unknown the extent to which PBDEs affect bodily processes. However, studies have shown that PBDEs are metabolized by the body and can affect thyroid hormone, insulin hormone and steroid function. Because of the ways in which PBDEs affect biochemical processes in the body, it is possible that PBDEs could be implicated in the etiology of obesity.

Numerous scientists have contemplated the idea that endocrine-disrupting compounds are responsible in part for the obesity epidemic (Grun and Blumberg, 2009) (Heindel et al., 2003), but primary research studies investigating PBDEs for obesogenic properties are non-existent. It has been long recognized that xenobiotic chemicals do interfere with endocrine signaling within the human body, with a great deal of emphasis on steroid hormones such as estradiol and testosterone. However, research is revealing that these endocrine-disrupting compounds (EDCs) may be affecting other hormones, such as thyroid hormone in the case of PBDEs, which have distinct roles in controlling basal metabolic rate and adiposity (Silva, 1995).

In a seminal paper, Baillie-Hamilton outlined how environmental chemicals, now ubiquitous in the environment, could be partly responsible for the obesity epidemic

(Baillie-Hamilton, 2002). Her argument states that over the last few decades there has been a dramatic increase in the amount of chemicals that humans come in contact with on a daily basis and that these chemicals could be interfering with natural weight-regulatory mechanisms. She maintains that the current obesity epidemic cannot solely be explained by overeating, inactivity and genetic predisposition.

Two epidemiological studies involving environmental chemicals have shown correlations with body mass index and decreased basal metabolic rate. Pelletier et al found that organochlorines, another class of halogenated compounds, were positively associated with fat mass in endurance athletes and obese people (Pelletier et al., 2002). Endurance athletes had lower levels of fat mass and organochlorine concentrations, while the opposite was true for obese people. In a similar study from the same lab, researchers investigated the effect of organochlorine release into the blood after weight loss in obese men on T_3 and basal metabolic rate (Pelletier et al., 2002). Increased plasma organochlorine concentrations were negatively associated with T_3 levels and basal metabolic rate. Because PBDEs are halogenated compounds, similar to organochlorines, it is possible that PBDEs could also be correlated with increased body mass index and decreased basal metabolic rate, but this possibility has not yet been investigated.

There are four mechanisms by which PBDEs could be related to body weight gain and adiposity: by disrupting whole body metabolism, elevating circulating fatty acids levels, acting as an estrogen agonist, and by exacerbating the effects of diet-induced obesity.

Mechanism 1: Disruptors of Whole Body Metabolism

Thyroid hormones play an important role in mediating whole body basal metabolic rate (Silva, 1995) by increasing the expression of proteins involved in the electron transport chain, which ultimately causes an increase in oxygen consumption (Freake and Oppenheimer 1995). Basal metabolic rates tend to be 30% lower in hypothyroid individuals and 50% higher in hyperthyroid individuals (Freake and Oppenheimer 1995). It has been shown that circulating levels of thyroid hormone are severely reduced with PBDE exposure in animals (Zhou et al., 2001) (Danerud et al., 2007); therefore, it is possible that PBDEs are affecting the basal metabolic rate of an individual by inducing a hypothyroid state.

PBDEs have been shown to decrease T_3 and T_4 in animal studies (whether by mimicking or antagonizing T_3 and T_4 function is not yet established) and it is known that T_3 and T_4 are intricately involved in lipid metabolism (Pucci et al., 2000). T_3 and T_4 increase lipolysis while also increasing lipid oxidation. The precise mechanism by which this happens is still unknown, but may be related to increasing sensitivity of adipocytes to circulating catecholamines.

Contrary to what would be expected, hepatic lipogenesis is also stimulated by T_3 and T_4 (Pucci et al., 2000). The concurrent increases in lipolysis, lipid oxidation and lipogenesis represent an energy-consuming futile cycle and an increased basal metabolic rate may in part be a result of this futile cycle. By disrupting T_3 and T_4 levels, PBDEs could uncouple the futile cycle and thereby decrease basal metabolic rate.

Mechanism 2: Stimulators of Lipolytic Action

Evidence that PBDEs can increase lipolytic response comes from the Carey lab (Hoppe and Carey, 2007). In this study, rats were exposed to a penta-BDE mixture for four weeks. At the end of the four weeks, the epididymal adipose tissue was removed, adipocytes were isolated and exposed to isoproterenol, a lipolytic agent. After four weeks of treatment, adipocytes elicited an increased lipolytic response to isoproterenol. There was no evidence of increased body weight in the PBDE-treated rats. Increased lipolysis is associated with obesity and insulin resistance since fatty acids (FAs) will be mobilized and then deposited elsewhere in the muscle and the liver (Jensen et al., 1989). Increased FA release is also correlated with decreased insulin clearance by the liver, hypertriglyceridemia and impaired insulin secretion from the pancreas.

Mechanism 3: Estrogen Agonists

Estrogen receptors are important in mediating energy balance within the body. Silencing of the estrogen receptor alpha in the hypothalamus causes mice to develop an obese, hyperphagic, diabetic phenotype and results in decreased energy expenditure (Musatov et al., 2007). Heine et al has shown similar results in an estrogen receptor alpha knock out mouse; male mice showed increased epididymal, inguinal and perirenal adipose tissue, while female mice showed increased inguinal and perirenal adipose tissue and fat cell size in the absence of increased energy intake (Heine et al., 2000). Male mice also showed decreased energy expenditure. PBDEs could be mimicking estradiol and feeding back to the brain to signal that there are ample levels of estradiol circulating, which could lead to a silencing of the estrogen receptors alpha and beta in various tissues

over time. This would also result in decreased estradiol levels, which has been seen *in vivo* with PBDE exposure (Talsness et al., 2008). This mechanism therefore could implicate PBDEs as disruptors of estrogen function, leading to weight gain. The long term effects of PBDE administration on estrogen function have not yet been investigated. Future studies should focus on the role of PBDEs in estrogen disruption and should examine endpoints such as weight and increased adiposity over long periods of time.

Mechanism 4: Diet-Induced Obesity Instigators

It is well known and documented that overconsumption of calories can lead to increased weight gain. In general, foods that are highly palatable, (containing high amounts of fat and/or sugar) stimulate the mesolimbic dopamine pathway which functions as the reward center in the brain, causing increased energy intake (Lutter and Nestler, 2009).

A high fat diet can cause hyperphagia, weight gain and increased adiposity (Ghibaudi et al, 2002). Ghibaudi et al fed either 10, 32 or 45% fat by kcal diets to rats and assessed energy intake and adiposity. There was a dose-dependent increase in energy intake, weight gain, fat mass and free fatty acid levels with increased dietary fat. Feeding of a high-fat, refined-sugar diet has also been found to cause an increase in lipolytic action (Berger, 1999), which can predispose an individual to obesity as well.

Increased intake of simple carbohydrates can increase weight gain. Increased sweetened beverage consumption was associated with weight gain in a cohort of women from the Nurse's Health Study (Schulze et al., 2004). In a study by Stanhope and Havel (2008), it was shown that consumption of high-fructose drinks caused an increase in

blood triacylglycerol concentrations compared to individuals who consumed glucose-sweetened beverages. Increased serum triglycerides is a symptom of obesity.

PBDEs can cause increased sweetness preference (Hennigar et al., 2008). Male rats treated daily with 18 mg/kg BW PBDEs for four weeks showed increased consumption of sucrose water compared to controls, while there was no difference in distilled water intake. There were no differences seen in weight gain; however, sucrose intake correlated with adiposity, independent of food intake. This study suggests that administration of PBDEs not only alters taste preference, but increases calorie intake leading to increased adiposity.

The physiological response to environmental chemicals has been shown to vary with diet. Parathion is an organophosphate pesticide that is currently under investigation for endocrine-disrupting effects. Lassiter et al (2008) showed a parathion dose x diet interaction in neonatal rats. Rats were treated with parathion on postnatal days 1-4 at a dose of either 0.1 or 0.2 mg/kg body weight/day and then provided either a control or high-fat diet in adulthood. Weight gain and disordered lipid and glucose metabolism were seen in female rats on a high fat diet combined with parathion; however females on the control diet with parathion showed weight loss and greater disordered lipid and glucose metabolism.

In another study, Jin et al (2007) fed rats diets of soy oil, seal oil, docosahexaenoic acid (DHA), fish oil or lard for 28 days, then rats were administered differing doses of methylmercury (MeHg) for 14 days. Rats on the lard diet and high dose of MeHg showed increased liver and spleen weight compared to control, whereas rats fed

the oils displayed only slight increases or no change in liver and spleen weight. Rats on the lard diet also showed increased bilirubin levels when exposed to MeHg, while the oil treatments had no effect on bilirubin levels. The dietary composition (relative amount of saturated fat to mono- and polyunsaturated fat) appears to affect the outcomes of MeHg exposure in rats.

Summary

PBDE treatment has been shown to decrease thyroid hormone levels in animals, which could be related to decreased basal metabolic rate. Increased lipolytic action in the adipoctyes of PBDE-treated rats could lead to increased circulating fatty acid levels, which is correlated with obesity. Disruption of estrogen signaling has been shown to cause weight gain in animals and PBDEs have been shown to bind to estrogen receptors. Rats treated with PBDEs were shown to have an increased sweetness preference, which caused an increased consumption of calories. Taken together, these effects of PBDE exposure could potentially give rise to an obese phenotype and a diet high in fat and sugar could exacerbate this condition, given the effects of PBDEs on sweetness preference in rats.

Hypothesis

This study sought to explore the intersection between potentially obesogenic PBDEs and a high-fat, high-sugar diet. My primary hypothesis was that PBDE treatment could disrupt whole body metabolism and promote adiposity in growing rats. My secondary hypothesis was that PBDE treatment, along with a high fat/ high sugar diet, would further exacerbate the obese condition.

CHAPTER II

MATERIALS AND METHODS

Animals

Twenty-eight male weanling Wistar rats were used for this experiment. The rats weighed between approximately 75 to 100 grams and were purchased from Charles River Laboratory in Wilmington, MA. All rats were housed in the Animal Resource Facility in Rudman Hall, UNH under controlled pressure and timed 12 hour light/dark cycles. Rats received purified diets (Table 1) formulated by Research Diets, Inc in New Brunswick, NJ and water *ad libitum*. All procedures were approved by the University of New Hampshire's Animal Care and Use Committee, #080304.

Treatments

After arrival, rats adjusted to their new habitat for one week before the start of treatment. Four different treatments were administered to the rats for a four-week period: a high fat, high sugar (HF) diet plus corn oil gavage (HF-) (n=7), control (C) diet plus corn oil gavage (C-) (n=7), HF diet plus PBDE gavage (HF+) (n=7), and control diet plus PBDE gavage (C+) (n=7).

The C diet consisted of 20% protein, 70% carbohydrate and 10% fat of kilocalories. The HF diet consisted of 20% protein, 50% carbohydrate (with 40% of the

Table 1-- Diet Formulations

Macronutrients	Control (C) Diet		High-Fat/High Sugar (HF) Diet	
%	Gram	Kcal	Gram	Kcal
Protein	19.2	20	21.5	20
Carbohydrate	67.3	70	53.8	50
Fat	4.3	10	14.3	30
Total	90.8	100		100
kcal/gm	3.85		4.3	
Ingredient				
Casein, 80 mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	575	2300	0	0
Maltodextrin 10	125	500	9.25	370
Sucrose	0	0	405	1620
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	110	990
Mineral Mix S10026	10	0	10	0
Dicalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, H_2O	16.5	0	16.5	0
Vitamin Mix 10001	10	40	10	40
Choline Bitartrate	0	0	2	0

Table 1: Diet Formulations from Research Diets, Inc (New Brunswick, NJ). The C diet consisted of 20% of kcals from protein, 10% of kcals from fat and 70% of kcals from carbohydrates—a typical diet formulation for rats. The HF diet consisted of 20% of kcals from protein, 30% of kcals from fat and 50% of kcals from carbohydrates (40% of kcals from sucrose).

kcal coming from sucrose) and 30% fat from kilocalories. Further detail on the ingredients in the diet can be found in Table 1. The gavage solution was administered using a 22G-1" straight gavage tube with a 1 ml syringe. Corn oil and 18 mg/kg BW penta-PBDEs in corn oil solutions were prepared as described in "Solutions". The concentration of 18 mg/ml is the lowest dose of PBDEs that has been shown to cause thyroid hormone disruption in laboratory animals.

Three sets of parameters were measured throughout and at the end of the treatment period: (1) whole animal measurements, (2) blood measurements, and (3) fat tissue measurements.

Whole Animal Measurements

Initial body weights were obtained at the start of the treatment period. After that, body weight and energy intake were measured three times weekly. Body weights were measured by placing rats into a mass balance with a deep well and a top. Energy intake was measured in the same mass balance by placing food into a bag and then placing into the mass balance. The food was stored in a refrigerator to prevent the HF diet from going rancid. Food was thawed up to a week prior to being provided to the rats.

Indirect calorimetry was used to measure respiratory quotient, energy produced and macronutrient disappearance. Prior to the indirect calorimetry testing, rats were acclimated to the indirect calorimetry chambers for approximately 10-15 minutes on two separate occasions. At three weeks of treatment, four rats were placed into indirect calorimetry chambers for a 24 hour period on a reverse light/dark cycle, while one chamber served as a reference chamber. While in the chambers, rats were fed the diet and

water *ad libitum*. Ambient air was passed through each chamber continuously. O_2 and CO_2 production from the chambers were measured by gas analyzers (CO_2 Analyzer, Applied Electrochemistry Inc, Sunnyvale CA and S-3AF O_2 Analyzer, Applied Electrochemistry Inc, Ametek, Sunnyvale, CA) approximately every 13.5 minutes for 1 minute per chamber and recorded by a computer (Tagliaferro et al., 1996). Total gas volume over 24 hours was measured using a mass flow controller. Urine was collected over the 24 hour period and then sent for nitrogen analysis (Analab, Fulton IL). From O_2, CO_2 and urinary nitrogen information, the respiratory quotient, energy production and macronutrient disappearance were calculated using the following formulas (VO_2 = volume of O_2 produced in L/min; VCO_2= volume of CO_2 produced in L/min; N= g of nitrogen in urine in 24 hour period) (Simonson and DeFronzo, 1990):

Glucose disappearance (g/min): $4.57VCO_2 - 3.23VO_2 - 2.60N$

Lipid disappearance (g/min): $1.69\ VO_2 - 1.69\ VCO_2 - 2.03N$

Protein disappearance (g/24 hrs): $6.25N$

Energy production (kcal/min): $3.91VO_2 + 1.1VCO_2 - 1.93N$

Non-protein respiratory quotient: $(VCO_2 - 4.84N)/(VO_2 - 6.04N)$

Blood Measurements

After four weeks of treatment, rats were euthanized using carbon dioxide gas. Approximately 5 ml of blood was removed via cardiac puncture using an 18G-1.5" needle with a 1 ml syringe. The blood was then transferred to EDTA-treated tubes, kept on ice and transported back to the lab where it was centrifuged at 4°C for 15 minutes at 3000 rpm. Approximately 1 ml of plasma was removed, transferred to a cryovial and frozen until thyroid hormone and insulin analysis. Two hundred µl of plasma were sent to

AniLytics in Gaithersburg, MD for thyroid hormone analysis and insulin was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica MA).

Epididymal Adipose Tissue Measurements

The epididymal adipose tissue from the left and right sides were removed, combined and weighed. The adipose tissue was transported to the Carey lab in a warm saline solution (0.9% NaCl). Once in the lab, the epididymal adipose tissue was digested, adipocytes were isolated and fixed with osmium and incubated. Excess adipose tissue from select animals was frozen and analyzed for 11 PBDE congeners by using gas-chromatography-mass spectrometry (GC-MS). These analyses were performed by Dr. Janice Huwe at the USDA Biosciences Research Lab in Fargo, ND.

Epididymal Adipose Tissue Digestion

Adipose tissue was minced to 1x1 mm^2 pieces using sharp scissors and rinsed with warm saline (0.9% NaCl) over 1000 μm mesh. The mesh was blotted on a 90 mm diameter circular Whatman qualitative filter paper to remove excess moisture. The adipose tissue was then weighed into 1 g portions and rinsed into flasks with 3 ml of warm 2% BSA Krebs Ringer Buffer (KRB', see Solutions). The flasks were placed in a 37°C shaking water bath. Four mg of collagenase (Worthington, Lakewood NJ, Lot # X6C8693) were dissolved into 1 ml of warm 2% BSA KRB' buffer and added to each flask containing the fat. Each flask was gassed with 95% O_2:5% CO_2, corked and incubated on a shaker at 80 oscillations/min for 45 minutes.

Adipocyte Isolation

After 45 minutes, two flasks were removed from the water bath, their contents were rinsed through a 1000 μm mesh into a beaker with warm KRB, totaling to approximately 40 ml. The beaker contents containing cells were then transferred to a 50 ml polypropylene centrifuge tube and centrifuged at 300 rpm for one second. The infranate was removed via an 18 gauge needle attached to a 60 ml syringe pierced into the bottom side of the polypropylene centrifuge tube. The infranate was discarded and the cells leftover were resuspended in approximately 45 ml of warm 2% BSA KRB'. The centrifugation was repeated twice more. After the third centrifugation, the infranate was removed, the cells were decanted into a 15 ml polypropylene centrifuge tube, centrifuged, infranate was removed and the volume of the adipocytes was recorded. Warm 4% BSA KRB' (Solutions) was then added to yield a 10% cell suspension.

Osmium Fixation

Six hundred μl of the 10% cell suspension was aliquoted into each of two 20 ml plastic vials containing 2400 μl of osmium collidine (Solutions). These vials incubated at 37°C for approximately 1 hour, filled to the rim with osmium collidine and incubated for another 47-48 hours. At this time, the contents of each vial were rinsed with saline triton (Solutions) onto a 500 μm nylon screen and trapped on a 20 μm screen. Once the cells were sufficiently rinsed, the captured cells were transferred to glass vial with 4 ml of resuspension solution (Solutions). To count and size cells, 6 μl of the resuspensed osmium-fixed cells were pipetted using a cut pipette tip and placed onto a hemacytometer. The number of cells were manually counted in each of six fields and

averaged. Three hundred cells were sized using the CellSizer program to yield an average cell diameter (Sidmore and Carey, 1994).

Adipocyte Incubations

After aliquoting the adipocyte suspension for osmium fixation, 600 µl of the adipocyte solution were added to each incubation vial using a cut pipette tip. KRB'4 was added to each vial of adipocytes to bring the total volume of the vial up to 750 µl. The shaker was slowed to approximately 40 oscillations per minute while reagents and solutions were added at 30 second intervals. Each vial received either a) nothing, b) 1 unit of adenosine deaminase (an enzyme usually added to remove endogenous adenosine which has been shown to promote lipolysis in the absence of a lipolytic agent), or c) 25 µl of varying concentrations of isoproterenol (an epinephrine analog used to induce lipolysis) in a total volume of 750 µl (Solutions). The final concentrations of isoproterenol were $1x10^{-6}$, $1x10^{-7}$, $5x10^{-8}$, $3x10^{-8}$, $1x10^{-8}$ and $5x10^{-9}$M (Solutions). After reagents were added, each vial was gassed with 95% O_2:5% CO_2 for 3 seconds and the vial was capped and incubated at 80 oscillations per minute at 37°C for 90 minutes.

After the 90 minute period, each vial was decanted into a 1.5 ml microcentrifuge tube containing 50 µl of 60% perchloric acid at 30 second intervals. The tubes were vortexed and placed on ice for at least 15 minutes. The tubes were then centrifuged in a 4°C microcentrifuge for 30 minutes. The tubes were transported back to the lab on ice and 500 µl of the infranate was removed and transferred to a second microcentrifuge tube containing 50 µl of 10N KOH. These tubes were kept on ice for 1 hour or refrigerated at 4°C overnight. The tubes were centrifuged for 15 minutes at 4°C. The infranate was

transferred to a new, clean 1.5 ml microcentrifuge tube and either used the following day for a glycerol assay or frozen until assayed at a later date.

Glycerol Assay

Glycerol assay samples and standards were thawed (if necessary) and vortexed. Three ml disposable plastic cuvettes (Sarstedt, Newton NC) were used for the glycerol assay (Weiland, 1985). There was a total volume of 2040 µl in each cuvette containing 1416 µl of glycine buffer, 50 µl of 50 mM ATP, 50 µl of 20 mM NAD (Solutions), 400 µl of double distilled water for vials containing samples, 100 µl of sample or standard and 20 µl of glycerol-3-phosphate dehydrogenase (GPDH) (from rabbit muscle, Roche Applied Science, Indianapolis IN). All cuvettes were thoroughly mixed and sat on the lab bench for at least 10 minutes. After at least 10 minutes had elapsed, the cuvettes were mixed again and then measured in a spectrophotometer (Beckman Instruments, Inc., Irvine, CA or Spectronic Genesys 5) at a wavelength of 340 nm. Four µl of glycerokinase (from bacillus *stearothermophilus*, Sigma Chemical Co., St. Louis, MO) was added to each cuvette. Each cuvette was mixed thoroughly and left to sit on the lab bench for at least 15 minutes. The cuvettes were mixed again and read at 340 nm in the spectrophotometer.

In order to determine the amount of glycerol that was released by the adipocytes, Beer's Law was used: A= Ebc, where A is the change in OD, E is the extinction coefficient (6200 L/ (mol x cm), b is the path length for the cuvette (1 cm) and c is the concentration (mol/ L). Using Beer's Law and correcting for dilutions, the concentration

of glycerol present after the 90 minute incubation, as expressed per 10^5 cells, was determined.

Statistics

Statistical analysis was performed on Systat 10 software. A two-way ANOVA—general linear model analysis was performed for these measurements using PBDE treatment and diet as main effects, diet x PBDE treatment interaction and treatment start date as a covariate. A Tukey's test was used for between group comparisons. For indirect calorimetry data, the covariate of light vs. dark period was added. Significance was set at $P < 0.05$; however, P values that were approaching significance were also reported.

Solutions and Reagents

Rat Treatment

- PBDE Gavaging Solution

  The PBDE gavaging solution was made by dissolving 2 g of penta-PBDEs (Cambridge Isotope Labs) into 1200 µl of hexane. The hexane was added directly into the PBDE container and stirred until the PBDEs were completely dissolved (approximately 5 minutes). After the PBDEs were totally dissolved, the solution was poured into a glass vial containing 60 ml of corn oil. An additional 51 ml of corn oil were then added. The vial was then vortexed for 30-60 seconds to thoroughly mix the contents. The solution was then placed under the hood and the hexane was evaporated using compressed nitrogen for 5 hours or longer. Previous GCMS analysis has shown that PBDE gavaging solution prepared in this manner has a concentration of 14 mg/ml (Hoppe and Carey, 2007), even though it is

prepared to yield a concentration of 18 mg/ml. This solution was stored at room temperature in an amber bottle.

- Corn Oil Gavaging Solution

  The corn oil gavaging solution was made by adding 1200 µl of hexane to 111 ml of corn oil in an amber bottle. The vial was then vortexed for 30-60 seconds to thoroughly mix the contents. The solution was then placed under the hood and the hexane was evaporated using compressed nitrogen for 5 hours or longer. This solution was stored at room temperature in an amber bottle.

Adipocyte Isolation and Adipocyte Tissue Digestion Solutions and Reagents

- Stock Solutions for Krebs-Ringer-Bicarbonate Buffer

  The stock solutions for Kreb's Ringer Buffer (KBR) were made ahead of time and were stable for 3 months at 4°C. The 2 M NaCl was prepared by dissolving 116.88 g of NaCl in double distilled water and was brought up to 1L. The 1 M KCl was prepared by dissolving 7.46 g of KCl into double distilled water and the volume was brought up to 100 ml. The 1 M $CaCl_2$-$6H_2O$ was made by dissolving 14.702 g of $CaCl_2$-2H2O in double distilled water and was brought up to 100 ml. The 1 M $MgCl_2$-$6H_2O$ was made by dissolving 20.33 g into double distilled water and was brought up to 100 ml. The 1 M KH_2PO_4 was made by dissolving 2.7218 g into double distilled water and was brought up to 20 ml. The 1 M HEPES was prepared by dissolving 119.15 g of HEPES into double distilled water and the volume was brought up to 500 ml, pH of 7.76. The 1 M $NaHCO_3$ was prepared by dissolving 42.005 g of $NaHCO_3$ into double distilled water and the volume was brought up to 500 ml.

- Stock Krebs-Ringer-Bicarbonate Buffer (KRB)

  Stock KRB is stable for up to one week at 4°C. For two experiments performed in one week, two liters of stock KRB buffer were made. To make the stock KRB buffer, approximately one liter of double distilled water was added to a beaker and the following stock solutions were added while stirring, in this order: 500 ml of 1 M HEPES pH of 7.76, 125 ml of 2 M NaCl, 10 ml of 1 M KCl, 2 ml of 1 M KH_2PO_4, 2.5 ml of 1 M $MgCl_2$-$6H_2O$, 2.5 ml of 1 M $CaCl_2$-$2H_2O$, 50 ml of $NaHCO_3$. The solution was brought up to 2 L with double distilled water in a graduated cylinder and the pH was adjusted to 7.4 with 1 N NaOH.

- Working Krebs-Ringer-Bicarbonate with 2% BSA (KRB2')

  KRB2' was prepared the morning of the experiment. KRB2' was made by mixing: to 400 ml of KRB, 1 ml of 0.5 M glucose and 44 mg of ascorbic acid. The solution was then gassed with 95% O_2:5% CO_2 for at least ten minutes and then 10 g of bovine serum albumin (BSA) (Sigma, St. Louis MO, Lot # 057K0737) was added, mixed until dissolved and then warmed to 37°C. The pH was adjusted to 7.4 and the volume was brought up to 500 ml with KRB.

- Working Krebs-Ringer-Bicarbonate with 4% BSA (KRB4')

  KRB4' was prepared the morning of the experiment. KRB4' was made by mixing: to 80 ml of KRB, 0.2 ml of 0.5 M glucose and 8.8 mg of ascorbic acid was added. The solution was then gassed with 95% O_2:5% CO_2 for at least ten minutes and then 4 g of BSA (Sigma, St. Louis MO, Lot # 057K0737) was added, mixed until dissolved and then warmed to 37°C. The pH was adjusted to 7.4 and the volume was brought up to 100 ml with KRB.

- 0.5 M Glucose

  The 0.5 M glucose was prepared by dissolving 18.02 g of glucose in double distilled water and by bringing up the volume to 200 ml. The solution was then separated into 10 ml aliquots and frozen until needed. This solution is stable for 3 months.

Adipocyte Incubation Solutions

- Isoproterenol Solutions

  A parent isoproterenol solution was prepared by dissolving 2.16 mg of isoproterenol (Sigma) into 20 ml of double distilled water to result in a concentration of $3x10^{-4}$M.

  Isoproterenol solutions were prepared according to the following table:

Table 2—Preparation of Isoproterenol Solutions

Starting soln (M)	REMOVE from starting soln (µl)	ADD double distilled water (µl)	TO YIELD working soln (M)	FINAL CONC in vial (M)
$3x10^{-4}$ M	100	900	$3x10^{-5}$	$1x10^{-6}$
$3x10^{-5}$ M	100	900	$3x10^{-6}$	$1x10^{-7}$
$3x10^{-6}$ M	500	500	$1.5x10^{-6}$	$5x10^{-8}$
$1.5x10^{-6}$ M	500	335	$9x10^{-7}$	$3x10^{-8}$
$3x10^{-6}$ M	100	900	$3x10^{-7}$	$1x10^{-8}$
$3x10^{-7}$ M	500	500	$1.5x10^{-7}$	$5x10^{-9}$

Table 2: Preparation of isoproterenol solutions for adipocyte incubations. Concentrations of starting solutions are provided, along with volumes required for the starting solution and distilled water are provided to yield a working solution. The final concentration in the vial is also provided.

- Adenosine Deaminase (ADA)

  In order to assess the lipolytic potential of adenosine deaminase on the adipocytes in the absence of isoproterenol, adenosine deaminase (from calf intestine, Roche

Applied Science, Indianapolis IN) was added in duplicate to vials during incubation. Adenosine deaminase was prepared by adding 5 µl of adenosine deaminase to 295 µl of saline which yields 1 unit per 30 µl. This solution was prepared the morning of the experiment and kept on ice throughout the procedure.

Osmium Fixation Solutions and Reagents

- 2 M Stock Collidine Buffer

  To make the stock collidine buffer, 26.4 ml of 2,4,6 trimethylpyridine was dissolved in double distilled water under the hood. The volume was brought to 1 L. Because the 2,4,6 trimethylpyridine takes 24 hours to dissolve, the solution was left stirring overnight under the hood and then stored in the refrigerator.

- 0.05 M Working Collidine Buffer

  To make the working collidine buffer, 225 ml of 0.01 N HCl was added to 250 ml of 2 M stock collidine buffer. The volume was brought to 1 L with saline and stored at 4°C for no more than three months.

- 1% Osmium Collidine Solution

  The 1% (w/v) osmium tetroxide solution was prepared no more than one week before the experiment. The solution was made by dissolving 1 g of osmium tetroxide in 100 ml of 0.05 M working collidine buffer while stirring and then stored in a dark bottle away from direct sunlight. The osmium tetroxide was allowed 2 hours to dissolve completely and then stored at room temperature for no more than a week.

- 0.01% Saline-Triton Solution

  The saline-Triton solution was prepared by adding 10 ml of 1% (v/v) of Triton X-100 to 990 ml in saline and stored at 4°C.

- Resuspension Solution

  The resuspension solution was prepared by dissolving 19.25 g of NaCl in approximately 100 ml of double distilled water. To this solution, 5 ml of 1% Triton X-100 and 277.5 ml of glycerol were added. The solution was stirred until the glycerol and water were mixed thoroughly. The volume was brought up to 500 ml with double distilled water. The solution was stored at 4°C and is stable at this temperature indefinitely.

Glycerol Assay Solutions and Reagents

- Stock Glycine/ $MgCl_2$ Solution (0.4M glycine/4mM $MgCl_2$-6(H_2O))

  The stock glycine/ $MgCl_2$ solution was prepared by adding 30 g of glycine and 4 ml of 1 M $MgCl_2$-6(H_2O) to double distilled water and the volume was brought up to 1 L. The solution was stored at 4°C.

- 0.2 M Glycine/ 1 M Hydrazine/ 2 mM $MgCl_2$ Buffer

  The 0.2 M Glycine/ 1 M Hydrazine/ 2 mM $MgCl_2$ buffer was prepared by combining 50 mls of the stock glycine/ $MgCl_2$ solution, 30ml ddH_2O and 5 ml hydrazine (added under the hood). The pH was adjusted to 9.8 with 10 N KOH and the volume was brought up to 100 ml.

- 20 mM NAD

The 20 mM NAD was prepared by dissolving 74.283 mg of NAD in 5 ml of double distilled water. This solution was stored in a 20 ml scintillation vial in the freezer.

- 50 mM ATP

The 50 mM ATP was prepared by dissolving 142.99 mg of ATP in 5 ml of double distilled water. This solution was stored in a 20 ml scintillation vial in the freezer.

- Glycerol Standards

Three glycerol standards were used in the glycerol assay: 10 nmol/ 0.1 ml, 20 nmol/ 0.1 ml and 40 nmol/ 0.1 ml. A parent solution of 10mM was prepared first by adding 92.1 mg of glycerol to 100 ml of double distilled water. Dilutions were then performed to make the standards. Forty µl of the parent solution was added to 3960 µl of distilled water to make the 1:100 dilution (10 nmol/ 0.1 ml). Eighty µl of the parent solution was added to 3920 µl of double distilled water to make the 1:50 dilution (20 nmol/ 0.1 ml). One hundred and sixty µl of the parent solution was added to 3840 µl of double distilled water to make the 1:25 dilution (40 nmol/ 0.1 ml). These standards were frozen in 250 µl aliquots until the assay was performed. One aliquot of each standard was used per assay.

CHAPTER III

THE EFFECT OF DIET AND POLYBROMINATED DIPHENYL ETHER (PBDE) EXPOSURE ON ADIPOCYTE AND WHOLE BODY METABOLISM IN MALE WISTAR RATS

INTRODUCTION

The traditional etiology of obesity has focused on increased calorie consumption, with the majority of excess calories coming from fat and sugar. While it is known that a high fat diet can cause hyperphagia, weight gain and increased adiposity (Ghibaudi et al., 2002) and excess intake of carbohydrates can cause partitioning of the macronutrients that is conducive to body weight gain (Brand-Miller et al., 2002), increased caloric consumption cannot solely explain the exponential increase in obesity (Baille-Hamilton, 2002). To explain the recent rise in obesity, a new hypothesis has emerged within the scientific community that implicates endocrine disrupting compounds (EDCs) as obesogens— compounds that can promote obesity in laboratory animals and possibly humans.

A number of EDCs have recently been designated as obesogens such as organotins (Grun and Blumberg, 2006), polychlorinated biphenyls (Arsenescu) and bisphenol A (Hugo). One class of EDCs that previously unexamined for obesogenic properties until now are polybrominated diphenyl ethers (PBDEs).

PBDEs are flame-retardant chemicals used widely in the manufacturing of commercial products such as carpeting, furniture and electronics (Danerud et al., 2001). Since they were introduced into manufacturing in the 1970's, the use of PBDEs has increased. Exposure of animals to PBDEs has been found to cause four adverse physiological effects including disrupted isoproterenol and insulin effects *in vitro*, decreased thyroid hormone levels *in vivo*, binding to estrogen receptors *in vitro* and increased sweetness preference. Interestingly, all of these effects are implicated in the etiology of obesity. Thus, it is possible that PBDEs are potential obesogens.

First, Hoppe and Carey reported that PBDEs caused an increase in isoproterenol-stimulated lipolysis and a decrease in insulin-stimulated glucose oxidation in adipocytes isolated from rats treated with PBDEs (Hoppe and Carey, 2007). Increased lipolysis is associated with obesity and insulin resistance because fatty acids (FAs) will be mobilized and then deposited elsewhere in the body such as in the muscle and the liver (Jensen et al., 1989). Increased FA release is also correlated with decreased insulin clearance by the liver, hypertriglyceridemia and impaired insulin secretion from the pancreas.

With these perturbations in lipolytic action and insulin response, it is possible that PBDEs could be affecting whole body macronutrient utilization. Increased carbohydrate utilization results in a higher respiratory quotient, which is correlated with obesity (Zurlo et al., 1990), most likely due to preferential oxidation of carbohydrates at the expense of lipid oxidation. The macronutrient composition of the diet will also play a role in macronutrient utilization.

Second, PBDEs are also notorious for disrupting thyroid hormone function and metabolism. PBDEs are similar in structure to thyroxine (T_4) and triiodothyronine (T_3) and hydroxylated metabolites of PBDEs bind to transthyretin (Hamers et al., 2006) and to thyroid hormone nuclear receptors (Marsh et al., 1998) *in vitro*.

Thyroid hormones are essential in controlling the basal metabolic rate in the body possibly by promoting the gene expression of uncoupling proteins (Lanni, et al., 2003), resulting in futile cycles, or by increasing ion flux into the mitochondria, resulting in increased thermogenesis (Kim, 2008). T_3 and T_4 are known to increase lipid utilization in target tissues by mobilizing stored triglycerides. Hepatic lipogenesis is also stimulated by T_3 and T_4 and the concurrent increase in lipid oxidation and synthesis may cause futile cycles that lead to increased basal metabolic rate (Pucci et al., 2000). If PBDEs decrease levels of T_3 and T_4 in experimental animals, it is possible that PBDEs could be responsible for decreased metabolic rate and disordered lipid metabolism in humans.

Third, PBDEs can disrupt estrogen metabolism by binding to estrogen receptors (Mercado-Feliciano and Bigsby, 2008). In a recombinant cell line, hydroxylated PBDEs were able to bind to the estrogen receptor alpha. Meerts et al (2001) and Hamers et al (2006) also showed similar binding affinities for hydroxylated PBDEs *in vitro* using an estrogen-receptor element luciferase assay. PBDE exposure will increase estrogen receptor expression at low doses, but PBDEs have no effect on receptor expression at a higher dose (Ceccatelli et al., 2006). If PBDEs are acting as agonists, the PBDEs could acutely increase estrogen receptor expression, but high levels over time would lead to the downregulation of receptors and decreased circulating estradiol levels, as has been seen previously (Talsness et al., 2008), which would downregulate receptor expression over

time. It is known that estrogen receptors are important in mediating energy balance within the body and silencing the estrogen receptor in the hypothalamus or knocking out the estrogen receptor globally promotes an obese phenotype and causes decreased energy expenditure (Musatov et al., 2007) (Heine et al., 2000).

Lastly, PBDEs can increase sweetness preference in animals. Hennigar et al (2008) showed that male rats treated daily with 18 mg/kg BW PBDEs for four weeks showed increased consumption of sucrose water compared to controls, while there was no difference in distilled water intake between the treatments. No difference in body weight gain was observed, but a significant correlation was found between sucrose and adiposity, independent of food intake. This study suggests that administration of PBDEs not only alters taste preference, but increases calorie intake and adiposity. In general, foods that are highly palatable (containing high amounts of fat and/or sugar) stimulate the mesolimbic dopamine pathway which functions as the reward center in the brain, causing increased energy intake (Lutter and Nestler, 2009). Feeding of a high-fat, refined-sugar diet has also been found to cause an increase in lipolytic action (Berger and Barnard, 1999), which can predispose an individual to obesity.

Because of the effects of PBDEs *in vivo* and *in vitro* and because a high-fat/high-sugar diet can predispose an individual to obesity, it is possible that the obesogenic effects of PBDE exposure could be magnified by a high-fat/high-sugar diet. Our primary hypothesis was that PBDE treatment could disrupt whole body metabolism and promote adiposity in growing rats. A secondary hypothesis was that PBDE treatment, along with a high fat/ high sugar diet, would further exacerbate the obese condition.

MATERIALS AND METHODS

Animals

Twenty-eight male weanling (approximately 3 weeks of age) Wistar rats were used for this experiment. The rats weighed between approximately 75 to 100 grams (Charles River Laboratory, Wilmington, MA). Rats were housed under controlled pressure and timed 12 hour light/dark cycles. Rats received purified diets (Table 1) formulated by Research Diets, Inc (New Brunswick, NJ) and water *ad libitum*. All procedures were approved by the University of New Hampshire's Animal Care and Use Committee, #080304.

Treatments

Rats adjusted to their new habitat for one week before the start of treatment. Four different treatments were administered to the rats for a four-week period: a high fat, high sugar (HF) diet plus corn oil gavage (HF-) (n=7), control (C) diet plus corn oil gavage (C-) (n=7), HF diet plus PBDE gavage (HF+) (n=7), and control diet plus PBDE gavage (C+) (n=7).

The C diet consisted of 20% protein, 70% carbohydrate and 10% fat of kilocalories. The HF diet consisted of 20% protein, 50% carbohydrate (with 40% of the kcal coming from sucrose) and 30% fat from kilocalories. Further detail on the ingredients in the diet can be found in Table 1. Corn oil and 18 mg/kg BW penta-PBDEs

in corn oil solutions were prepared as described previously (Hoppe and Carey, 2007) and administered daily using a 22G-1" straight gavage tube with a 1 ml syringe. The 18 mg/ml concentration of PBDEs was chosen because it is the lowest dose of PBDEs that have been shown to cause thyroid hormone disruption.

Three sets of parameters were measured throughout and at the end of the treatment period: (1) whole animal measurements, (2) blood measurements, and (3) adipose tissue measurements.

Whole Animal Measurements

Initial body weights were obtained at the start of the treatment period. Body weight and energy intake were measured three times weekly. Indirect calorimetry was used to measure respiratory quotient, energy produced and macronutrient disappearance. Prior to the indirect calorimetry testing, rats were acclimated to the indirect calorimetry chambers for approximately 10-15 minutes on two separate occasions. After three weeks of treatment, four rats were placed into individual indirect calorimetry chambers for a 24 hour period on a timed 12 hour light/dark cycle, while one chamber served as a reference chamber. While in the chambers, rats were fed the diet and water *ad libitum*. Ambient air was passed through each chamber continuously. O_2 and CO_2 production from the chambers were measured by gas analyzers (CO_2 Analyzer, Applied Electrochemistry Inc, Sunnyvale CA and S-3AF O_2 Analyzer, Applied Electrochemistry Inc, Ametek, Sunnyvale, CA) approximately every 13.5 minutes for 1 minute per chamber and recorded by a computer (Tagliaferro et al., 1996). Total gas volume over 24 hours was measured using a mass flow controller. Urine was collected over the 24 hour period and

then sent for nitrogen analysis (Analab, Fulton IL). From O_2, CO_2 and urinary nitrogen information, the respiratory quotient, energy production and macronutrient disappearance were calculated using the following formulas (VO_2 = volume of O_2 produced in L/min; VCO_2= volume of CO_2 produced in L/min; N= g of nitrogen in urine in 24 hour period) (Simonson and DeFronzo, 1990):

Glucose disappearance (g/min): $4.57VCO_2 - 3.23VO_2 - 2.60N$

Lipid disappearance (g/min): $1.69\ VO_2 - 1.69\ VCO_2 - 2.03N$

Protein disappearance (g/24hr): $6.25N$

Energy production (kcal/min): $3.91VO_2 + 1.1VCO_2 - 1.93N$

Non-protein respiratory quotient: $(VCO_2 - 4.84N)/(VO_2 - 6.04N)$

Blood Measurements

After four weeks of treatment, rats were euthanized using carbon dioxide gas. Approximately 5 ml of blood was removed via cardiac puncture using an 18G-1.5" needle with a 1 ml syringe and transferred to an EDTA-treated tube. Plasma was isolated and frozen for thyroid hormone and insulin analysis. Plasma was sent to AniLytics in Gaithersburg, MD for thyroid hormone analysis. Insulin was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica MA).

Epididymal Adipose Tissue Measurements

The epididymal adipose tissue from the left and right sides were removed, combined, weighed and placed in a warm saline solution (0.9% NaCl) for transport back to the laboratory. The epididymal adipose tissue was subjected to digestion and adipocyte isolation, and adipocytes were osmium fixed and incubated, as described previously (Meservey and Carey, 1994). Excess adipose tissue from select animals was

frozen and analyzed for 11 PBDE congeners by using gas-chromatography-mass spectrometry (GC-MS) (Huwe et al., 2005).

Epididymal Adipose Tissue Digestion and Adipocyte Isolation

Adipose tissue was minced, weighed into 1 g portions and rinsed into flasks with 3 ml of warm Krebs Ringer Buffer containing 2% BSA (KRB'2) (Sigma, St. Louis MO, Lot # 057K0737) and four mg of collagenase (Worthington, Lakewood NJ, Lot # X6C8693). Each flask was gassed with 95% O_2:5% CO_2, corked and incubated at 80 oscillations/min for 35 minutes at 37°C. Adipocytes were isolated as previously described (Sidmore and Carey, 1994) and resuspended to 10% (v/v) in KRB buffer containing 4% BSA (KRB4').

Osmium Fixation

For each experiment, duplicate samples of the 10% adipocyte resuspension was incubated at 37°C for 47 to 48 hours with 1% osmium tetroxide in collidine to fix the adipocytes for sizing and counting, as described previously (Sidmore and Carey, 1994).

Adipocyte Incubations

Six hundred µl of the adipocyte solution were incubated with KRB'4 and either a) nothing, b) 1 unit adenosine deaminase (an enzyme usually added to remove endogenous adenosine which has been shown to promote lipolysis in the absence of a lipolytic agent), or c) varying concentrations of isoproterenol (an epinephrine analog used to induce lipolysis) in a total volume of 750 µl. The final concentrations of isoproterenol were $1x10^{-6}$, $1x10^{-7}$, $5x10^{-8}$, $3x10^{-8}$, $1x10^{-8}$ and $5x10^{-9}$M. After reagents were added,

each vial was gassed with 95% O_2:5% CO_2 for 3 seconds and the vial was capped and incubated in a shaker at 80 oscillations per minute at 37°C for 90 minutes.

Glycerol Assay

After the 90 minute period, each vial was decanted into a 1.5 ml microcentrifuge tube containing 50 µl of 60% perchloric acid. The tubes were vortexed and placed on ice for at least 15 minutes, then centrifuged at 4°C for 30 minutes. The infranate was neutralized with 50 µl of 10N potassium hydroxide, kept on ice for 1 hour or refrigerated at 4°C overnight, then centrifuged for 15 minutes at 4°C. The supernatant was frozen until assayed for glycerol by an enzyme-coupled spectrophotometric technique, as previously described (Weiland, 1985). Results are expressed in µmol of glycerol released per 10^5 adipocytes/ 90 minutes.

Statistics

Statistical analysis was performed using Systat 10 software. A two-way ANOVA—general linear model analysis was performed for these measurements using PBDE treatment and diet as main effects, diet x PBDE treatment interaction and treatment start date as a covariate. A Tukey's test was used for between group comparisons. For indirect calorimetry data, the covariate of light vs. dark period was added. Insulin concentration data was log transformed and analyzed statistically as stated above. Significance was set at $P < 0.05$; however, P values that were approaching significance are also reported.

RESULTS

Whole Animal Measurements

The average weight gain over the treatment period was higher for PBDE-treated animals than non-PBDE treated animals (231.9 vs 212.5 g, respectively, $P<0.05$) (Figure 2). Energy intake over the treatment period tended to be higher for animals on the HF diet (2424.4 kcal) than for rats on the control diet (2237.0 kcal) ($P=0.053$) (Figure 3). Metabolic efficiency (defined as the 4 week body weight gain over 4 week food intake) was lower in animals on the HF diet compared to animals on the C diet ($P<0.05$); however, a diet x PBDE treatment interaction effect that approached significance ($P=0.08$) revealed that PBDE treatment caused an increase in metabolic efficiency in the C-fed rats, but decreased metabolic efficiency in HF-fed rats (Figure 4).

Glucose disappearance was significantly higher for rats on the C diet than the HF diet ($P<0.05$) (Figure 5). Also, PBDE treatment tended to increase glucose disappearance compared to non-PBDE treated rats ($P=0.062$). Lipid disappearance was significantly higher for HF-fed rats than C-fed rats ($P<0.05$) (Figure 6). A significant diet x PBDE treatment interaction effect was seen in protein disappearance. PBDE treatment in the C-fed rats decreased protein disappearance, but increased protein disappearance in HF-fed rats ($P<0.05$) (Figure 7). Animals on the C diets tended to have higher protein disappearance than animals on the HF diet ($P=0.058$).

Animals on the C diet tended to have higher energy production than animals on the HF diet (P=0.066) (Figure 8). PBDE-treated rats tended to have increased energy production compared to non-PBDE treated rats (P=0.081). When energy production was normalized for body weight at 3 weeks, there was no effect of PBDE treatment, however, there was a diet effect that was approaching significance (P=0.077). Non-protein respiratory quotient was higher in the C-fed animals than in the HF-fed animals (P<0.05) (Figure 9); C-fed animals were utilizing more carbohydrates for fuel, while HF-fed rats were utilizing less carbohydrates and more lipid for fuel.

Blood Measurements

There was a significant decrease in plasma thyroid hormone (T_4) levels in PBDE-treated animals when compared to non-PBDE treated animals (P<0.05). T_4 levels were decreased by 87% with PBDE treatment of C-fed rats (from 6.75 in C- animals to 0.90 μg/dL in C+ animals), while T_4 levels were decreased by 78% by PBDE treatment in HF-fed rats (from 4.33 in HF- animals to 0.97 μg/dL in HF+ animals) (Figure 10). Regression analysis showed that T4 levels were weakly correlated with the four week average weight gain (P=0.084).

Plasma insulin levels tended to be lower in PBDE-treated animals compared to non-PBDE treated animals (P=0.075) (Figure 11); animals treated with PBDEs had an average of 30% lower insulin levels when compared to non-PBDE treated counterparts. Also, there was a diet x PBDE treatment interaction effect that approached significance (P=0.081), where PBDE treatment caused a decrease in insulin levels in C-fed animals, but caused an increase in insulin levels in HF-fed animals.

Epididymal Adipose Tissue Measurements

Eleven PBDE congeners were measured in epididymal adipose tissue taken from 3 PBDE-treated rats and 3 non-PBDE treated rats. The predominant congener in both PBDE-treated and non-PBDE treated rats was BDE-47, followed by BDE-99 (Table 3). These 2 congeners comprised an average of 79.5% of the total PBDEs found in epididymal adipose tissue. The next most abundant congeners were BDE-100 & 153, comprising 16.0% of total PBDEs found in epididymal adipose tissue. PBDE concentrations were 1107 times higher in PBDE-treated animals as compared to non PBDE-treated animals (626,327 ng/g lipid in PBDE-treated rats vs. 566 ng/g lipid in non-PBDE-treated rats). There were traces of PBDEs in non-PBDE treated animals, which displays the ubiquitous nature of PBDEs within even a controlled environment and/or the corn oil gavage solution.

Average epididymal adipose tissue weight of C+ animals was 4.4 g while average epididymal adipose tissue weight of C- animals was 5.0 g (Figure 12). HF+ animals had an average epididymal adipose tissue weight of 5.1 g and HF- animals had an average epididymal adipose tissue weight of 4.1 g. There was no significant effect of diet or PBDE treatment on adipose tissue weight, but there was a significant diet x PBDE treatment interaction effect in epididymal adipose tissue weight. In C-fed animals, PBDE treatment caused a 12% decrease in epididymal adipose tissue weight, while PBDE treatment in HF-fed animals caused an 19% increase in epididymal adipose tissue weight ($P<0.05$).

Cell size, as measured by cell diameter and cell volume, showed no significant differences between treatments. However, a diet effect for cell diameter that is approaching significance was found (P=0.057) (Table 4); the average cell diameter for C-fed animals was 6% higher than HF-fed animals. The average cell volume for C-fed animals was 12% higher than HF-fed animals. Cell sizes for all treatment groups were normally distributed.

Contrary to previous research, there was no significant difference in the amount of glycerol released from incubated adipocytes across the treatments (Figure 13). When data were expressed as glycerol released as a percent of maximum lipolysis, the lack of a significant difference between treatments persisted (Figure 14). The amount of glycerol released when the adipocytes were incubated with adenosine deaminase (ADA) in the absence of isoproterenol also showed no significant difference between treatments (Figure 15) and the lack of significance persisted when the results were expressed as glycerol released as a percent of maximum lipolysis (Figure 16).

Figure 2—Average Four Week Weight Gain

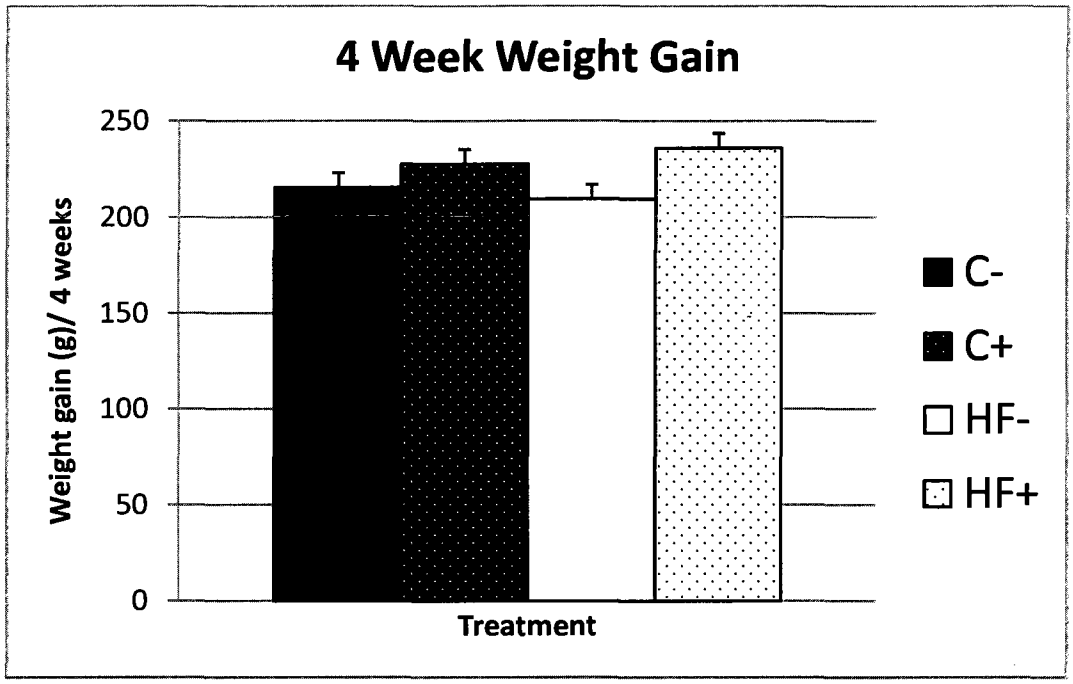

Figure 2: Average weight gain over the four week treatment period. PBDE-treated animals had significantly increased weight gain compared to non-PBDE treated animals (N=14 per group) ($P<0.05$). Results are expressed as means ± SE. N=7 per treatment.

Figure 3— Average Energy Intake Over Four Weeks

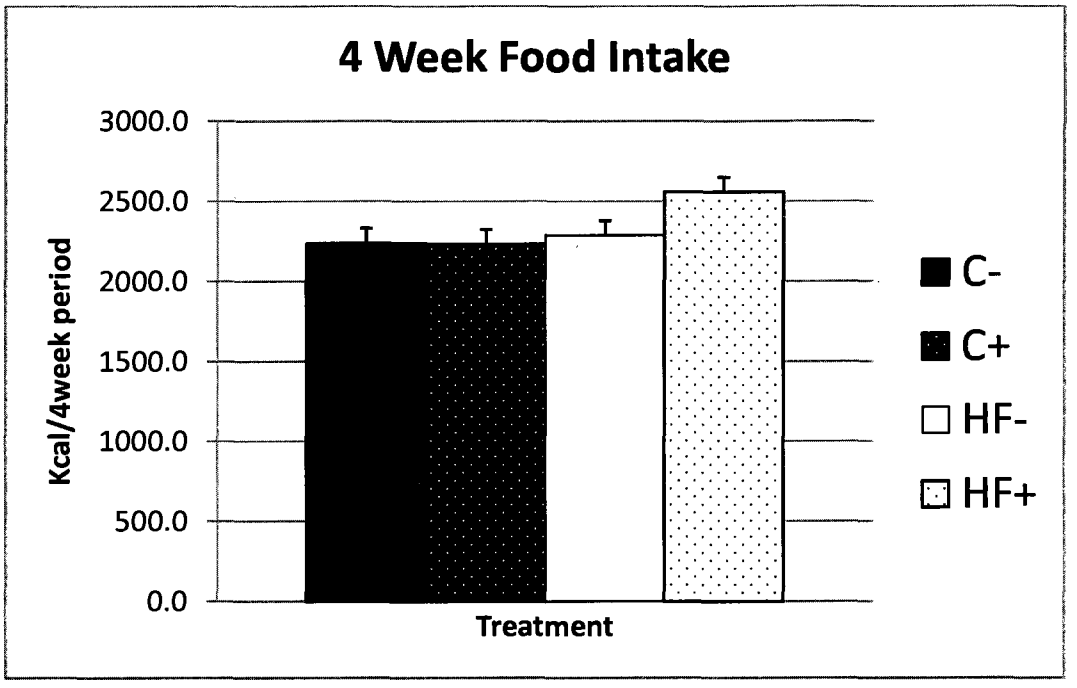

Figure 3: Average energy intake over the four week treatment period. HF-fed animals tended to have higher energy intake than C-fed animals (N=14 per group) (P=0.053). Results are expressed as means ± SE. N=7 rats per treatment.

Figure 4— Average Metabolic Efficiency Over Four Weeks

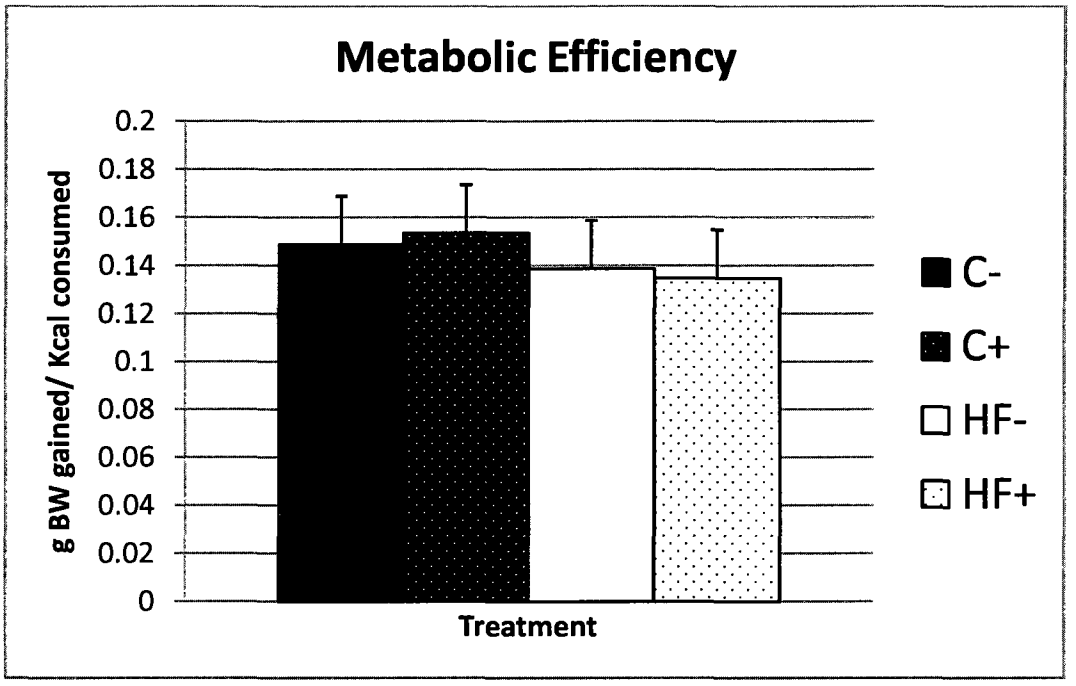

Figure 4: Average metabolic efficiency over the four week treatment period. Metabolic efficiency was significantly lower in HF-fed animals compared to C-fed animals ($P<0.05$) ($N=14$ per group). PBDE treatment tended to increase metabolic efficiency in C-fed rats, but tended to decrease metabolic efficiency in HF-fed rats ($P=0.08$) ($N=7$ per group). Results are expressed as means ± SE. $N=7$ rats per treatment.

Figure 5—*In Vivo*- 24 Hour Average Glucose Disappearance

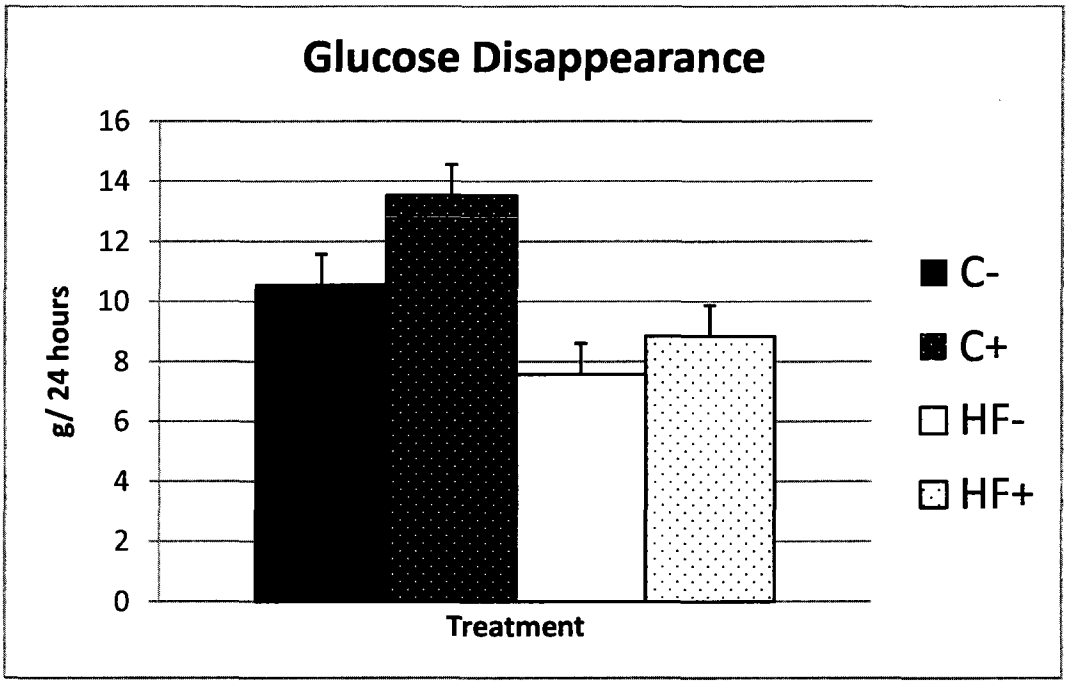

Figure 5: Average glucose disappearance over a 24 hour period. C-fed rats showed significantly higher glucose disappearance than HF-fed rats ($P<0.05$) (N=12 per group), while PBDE-treated rats tended to have increased glucose disappearance compared to non-PBDE treated rats ($P=0.062$) (N=12 per group). Results are expressed as means ± SE. N=6 rats per treatment.

Figure 6— *In Vivo*- 24 Hour Average Lipid Disappearance

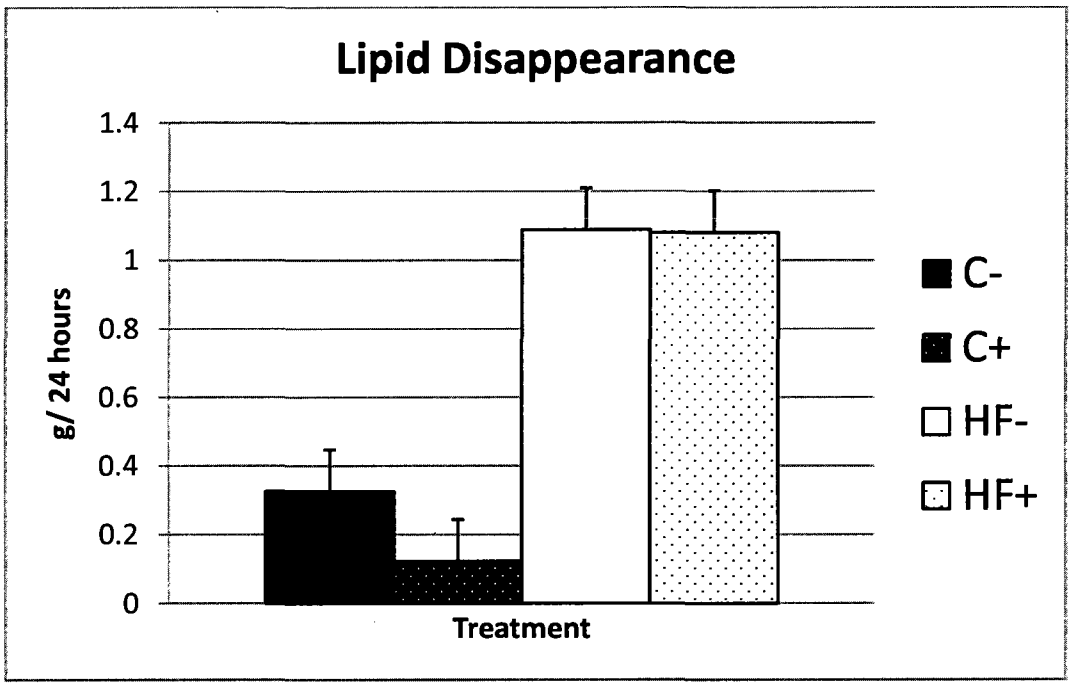

Figure 6: Average lipid disappearance over a 24 hour period. Lipid disappearance was significantly higher in HF-fed rats compared to C-fed rats (N=12 per group) ($P<0.05$). Results are expressed as means ± SE. N=6 rats per treatment.

Figure 7— *In Vivo*- 24 Hour Average Protein Disappearance

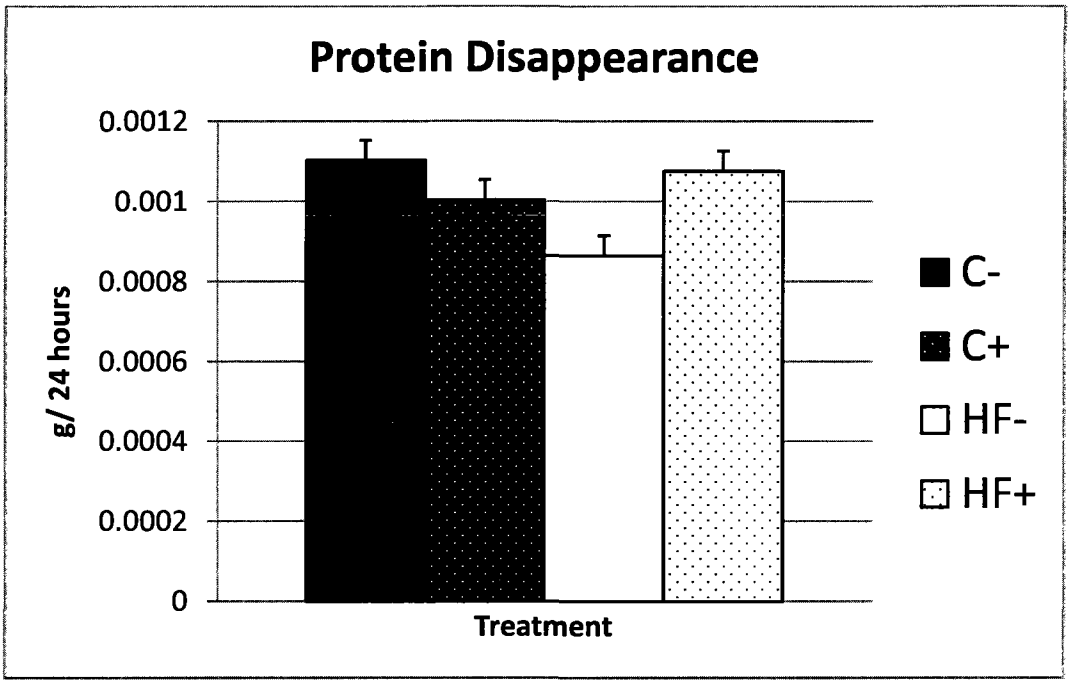

Figure 7: Average protein disappearance over a 24 hour period. PBDE treatment caused a decrease in protein disappearance in C-fed rats, but increased protein disappearance in HF-fed rats ($P<0.05$) (N=6 per group). C-fed rats tended to have higher protein disappearance than HF-fed rats ($P=0.058$) (N=12 per group). Results are expressed as means ± SE. N=6 per group

Figure 8— *In Vivo*- 24 Hour Average Energy Produced

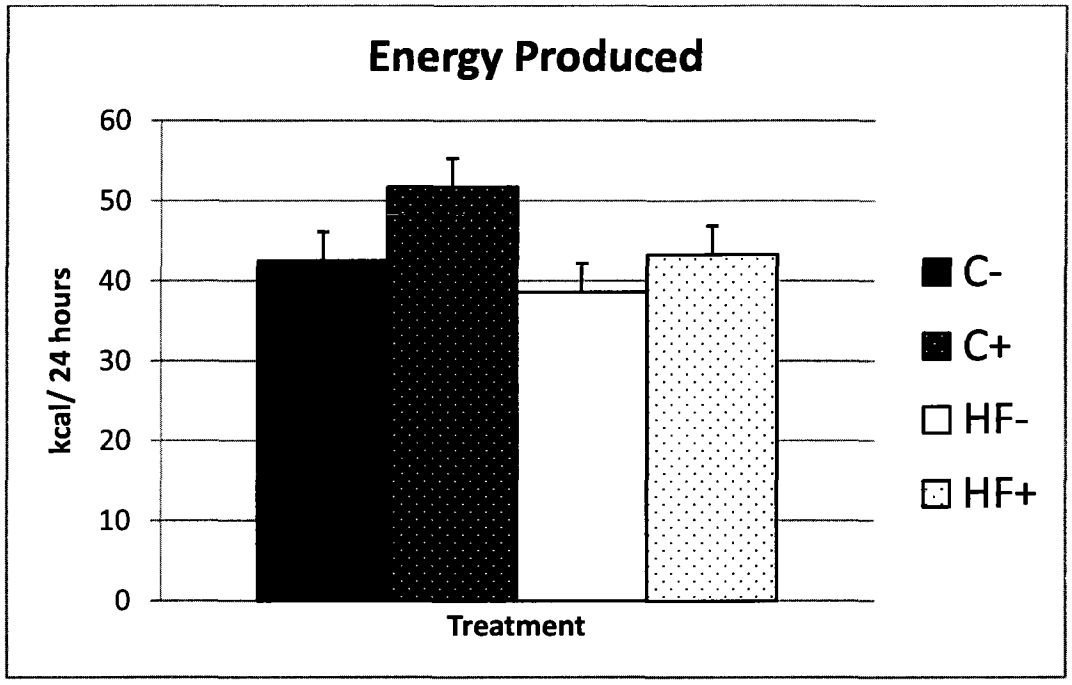

Figure 8: Average energy produced over a 24 hour period. PBDE-treated rats tended to have increased energy production compared to non-PBDE treated animals (P=0.081) (N=12 per group). C-fed rats tended to have higher energy production than HF-fed rats (P=0.066) (N=12 per group). Results are expressed as means ± SE. N=6 rats per treatment.

Figure 9— *In Vivo*- 24 Hour Non-Protein Respiratory Quotient

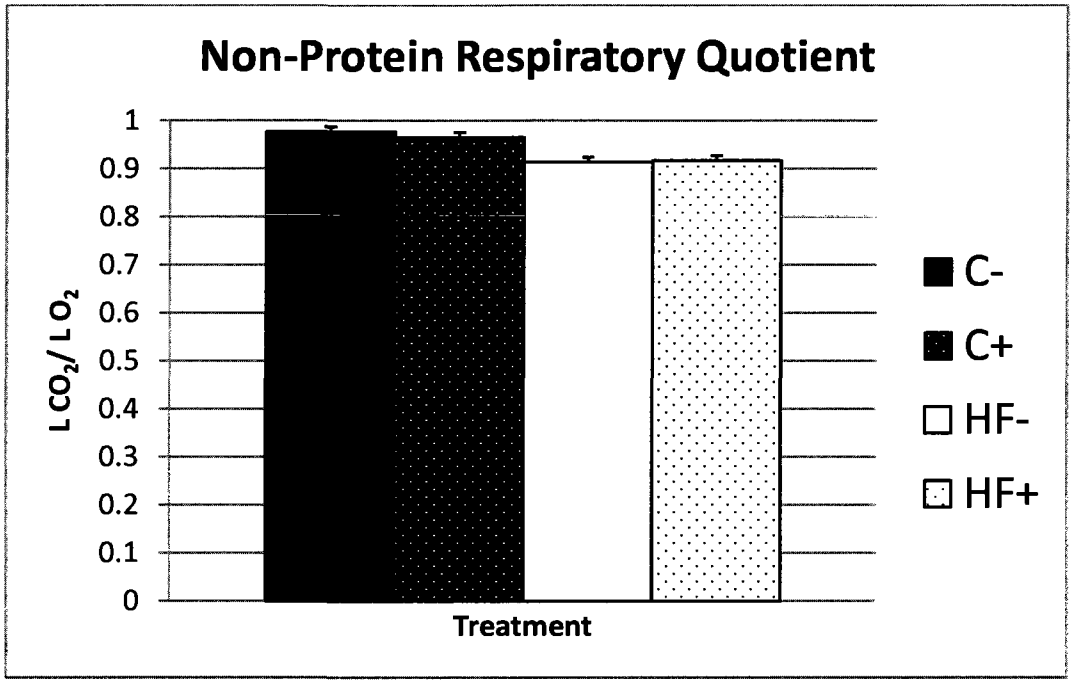

Figure 9: Average non-protein respiratory quotient over a 24 hour period. Non-protein respiratory quotient was significantly higher in C-fed animals than HF-fed animals ($P<0.05$) (N=12 per group). Results are expressed as means ± SE. N=6 rats per treatment.

Figure 10—Fasting Plasma T_4 Levels

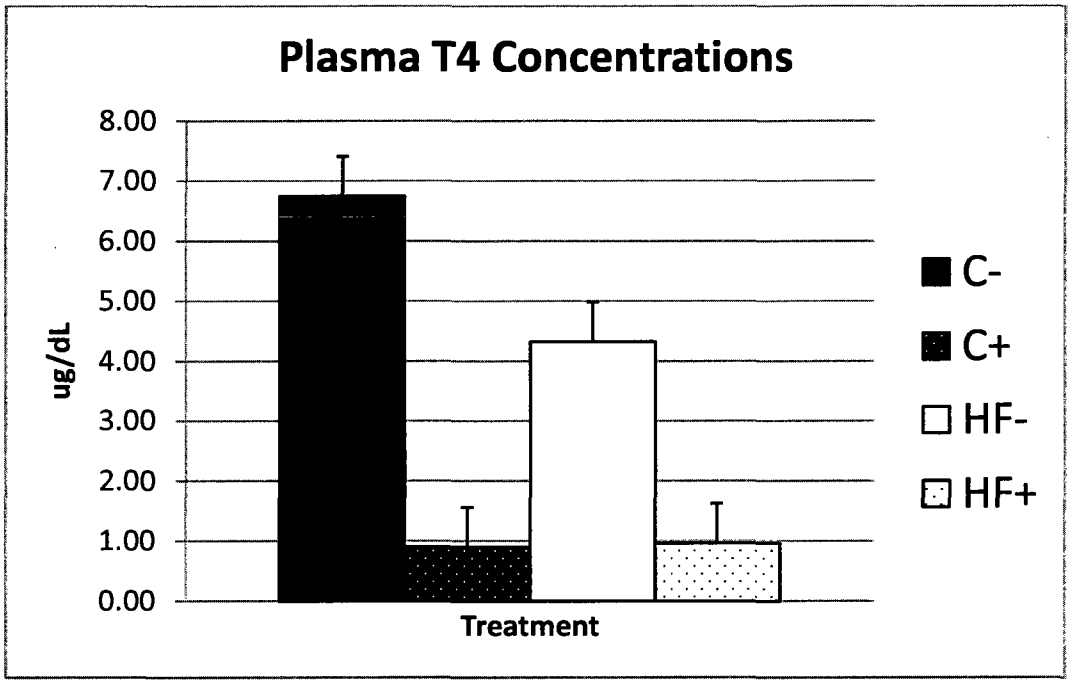

Figure 10: Plasma T_4 concentrations. There was a significant decrease in PBDE-treated rats compared to non-PBDE treated rats ($P<0.05$). C+ animals had 87% lower T_4 levels than C-. HF+ animals had 78% lower T_4 levels than HF- animals ($P<0.05$). Results are expressed as means ± SE. N=6 rats per treatment.

Figure 11—Fasting Plasma Insulin Levels

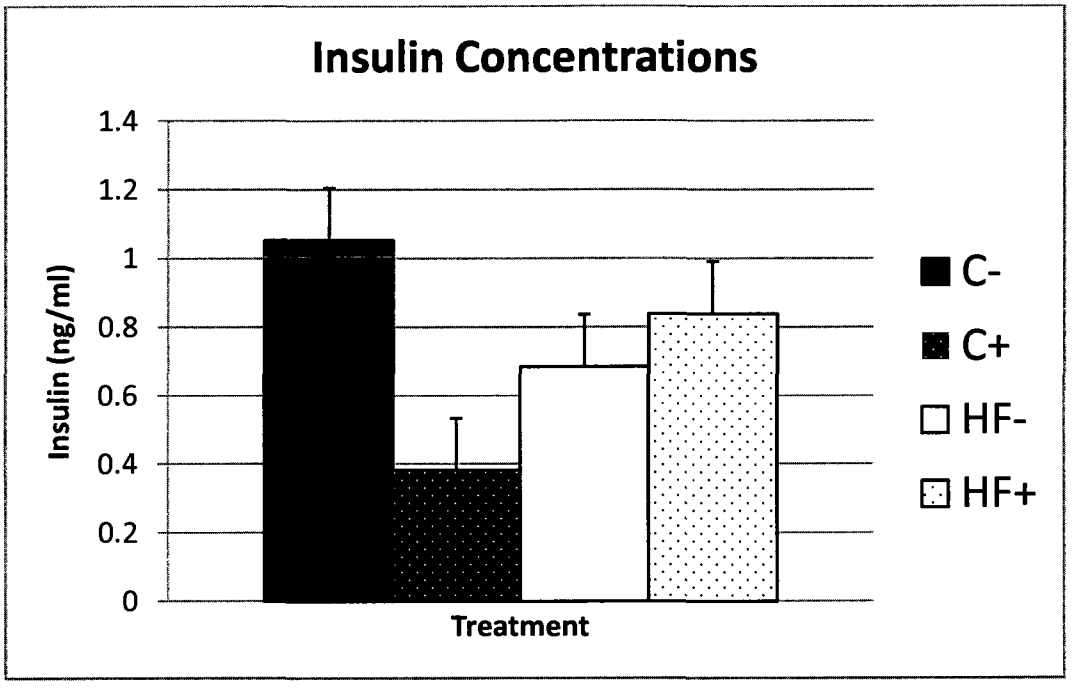

Figure 11: Plasma insulin concentrations. PBDE treatment tended to lower insulin levels (P=0.075) (N=14 per group). A diet x PBDE treatment interaction effect approaching significance (P=0.81) was observed, where PBDE treatment tended to lower insulin in C- fed rats, but tended to increase insulin levels in HF-fed rats (N=7 rats per treatment). Results are expressed as means ± SE. N=7 rats per treatment. Statistics were performed a logarithmic transformation of the data, but data is reported as total concentrations of insulin.

Table 3: PBDE Concentrations as Percent of Total PBDE Concentration

PBDE	C-	C+	HF-	HF+
28&33	0.52	0.09	0.94	0.09
47	41.80	45.99	45.33	45.24
66	0.39	0.06	0.39	0.06
100	11.37	11.01	10.20	11.28
99	36.69	34.07	34.93	34.06
154	2.09	1.02	1.52	1.25
85	1.96	1.67	1.85	1.77
153	4.57	5.45	4.48	5.70
138	0.00	0.59	0.00	0.53
183	0.61	0.04	0.36	0.04
TOTAL (ng/g lipid)	316	636,894	816	615,760

Table 3: PBDE concentrations in epididymal adipose tissue for select animals. PBDE concentrations for various congeners were measured as ng/g lipid and expressed as a percent of total PBDE concentration. N=1-2 rats for each treatment.

Figure 12—Epididymal Adipose Tissue Weight

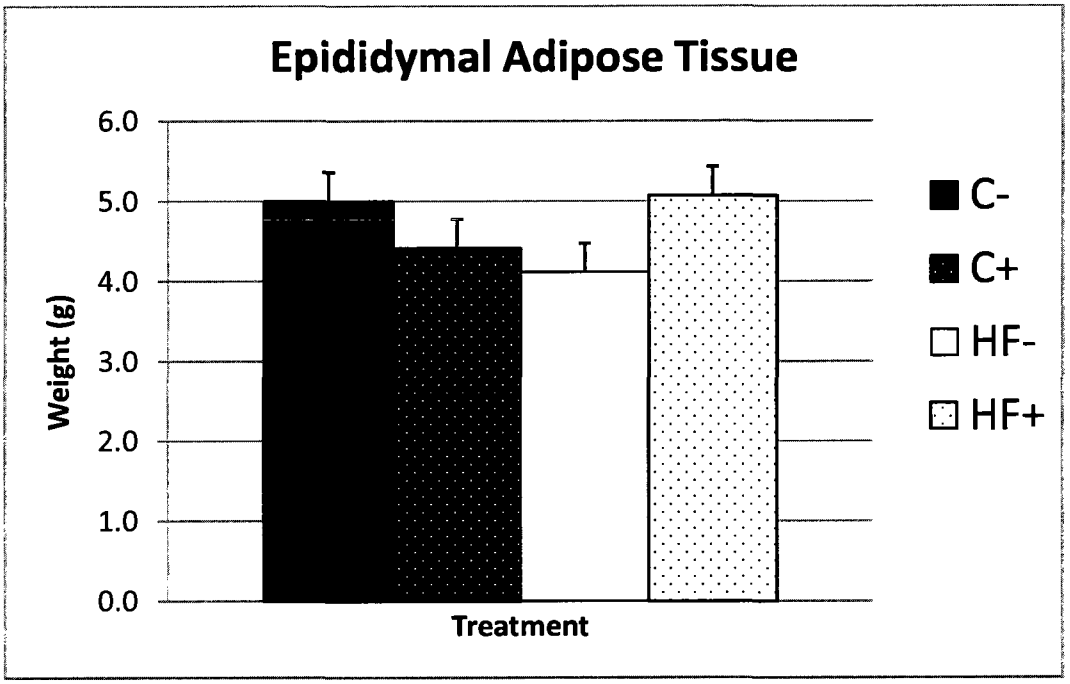

Figure 12: Epididymal adipose tissue weight as measured at euthanasia. There was a significant diet and PBDE treatment interaction effect on epididymal adipose tissue weight ($P<0.05$) (N=7 per group). PBDE treatment decreased epididymal adipose tissue weight in C-fed rats, but increased epididymal adipose tissue in HF-fed rats. Results are expressed as means ± SE. N=7 per group

Table 4: Average Cell Diameter and Volume

Treatment	Cell Diameter	Cell Volume
C-	95 ± 1.29	457441 ± 18700
C+	91 ± 1.29	395152 ± 15937
HF-	85 ± 2.91	343723 ± 28867
HF+	90 ± 2.84	402064 ± 31664

Table 4: Average cell diameter and volume for adipocytes. There were no significant differences in cell diameter or cell volume across the treatments. However, there was a p value that was approaching significance (P=0.057) for diet effect for cell diameter, with the C-fed rats having higher cell diameters than the HF-fed rats. Results are expressed as means ± SE. N=7 rats per treatment.

Figure 13: Glycerol Release

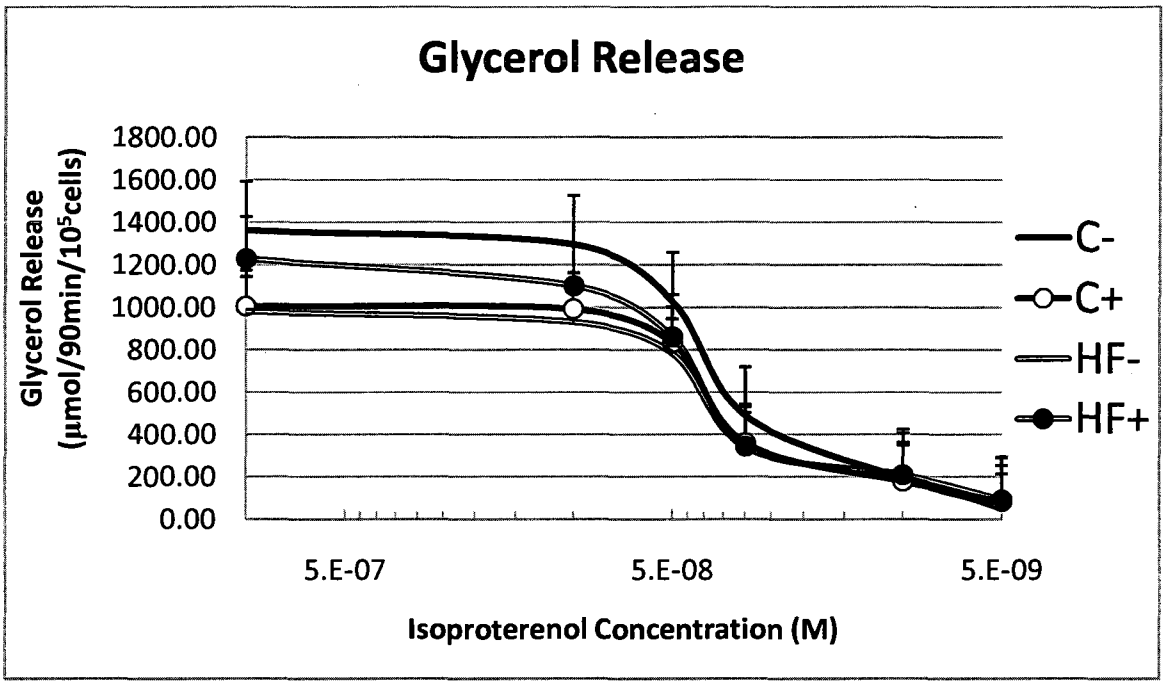

Figure 13: Absolute glycerol release from isoproterenol-treated adipocytes as expressed as µmol per 90 minute incubation per 10^5 cells. There were no significant differences between treatments in glycerol release ($P>0.05$). Results are expressed as means ± SE. N=7 rats per treatment.

Figure 14—Glycerol Release as Expressed as a Percent of Maximum Lipolysis

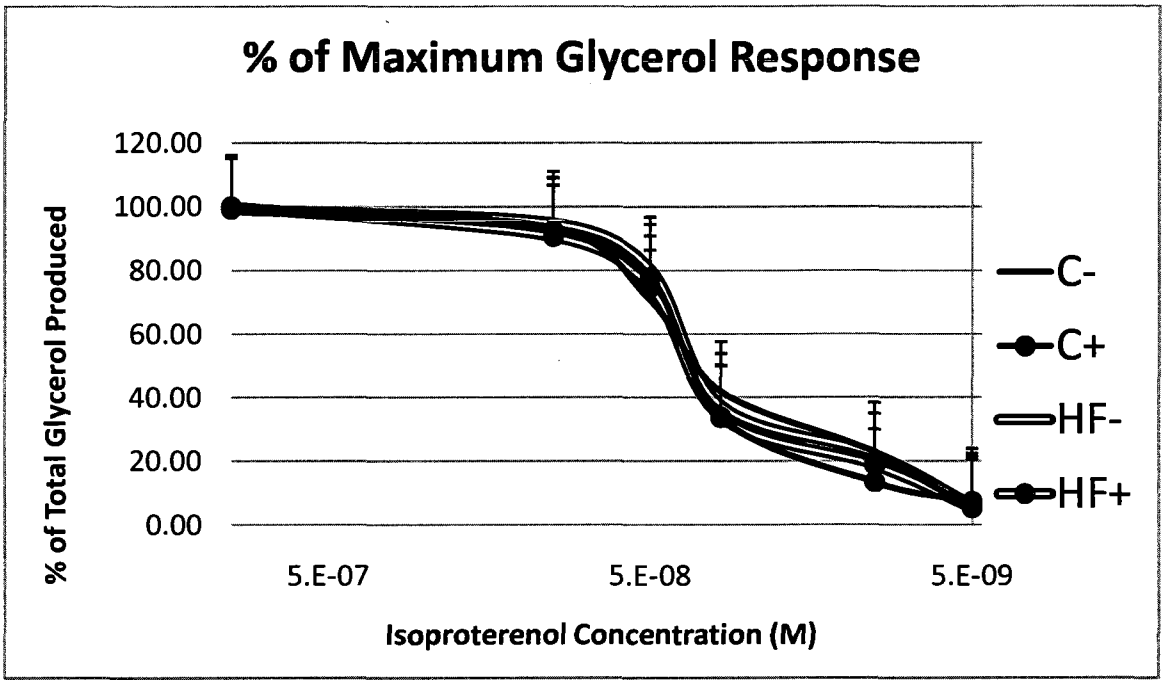

Figure 14: Glycerol produced as a percent of maximum glycerol release from isoproterenol-treated adipocytes. This transformation of the data was performed to standardize the amount of glycerol produced from experiment to experiment. When expressed as glycerol produced as a percent of max lipolysis, the glycerol data showed no significant difference between treatments ($P>0.05$). Results are expressed as means ± SE. N=7 rats per treatment.

Figure 15—Glycerol Release by ADA stimulation

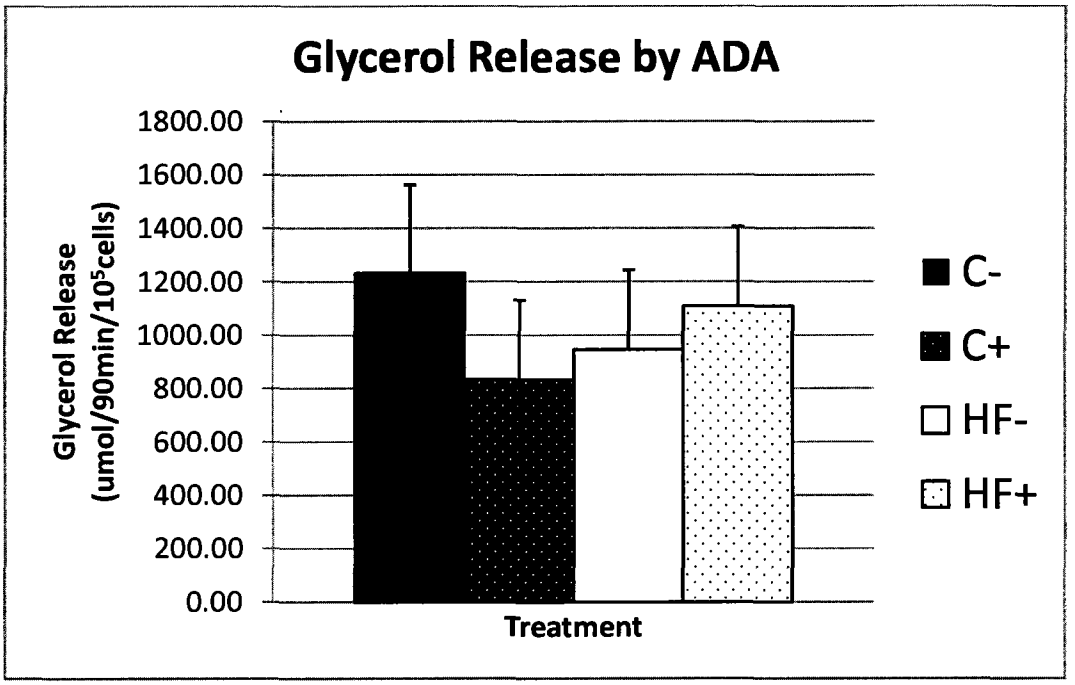

Figure 15: Glycerol release from ADA-stimulated adipocytes over a 90 minute incubation per 10^5 cells. There was no significant difference in the amount of glycerol produced by ADA stimulation across the treatments ($P>0.05$). Results are expressed as means ± SE. N=7 rats per treatment.

Figure 16—Glycerol Release by ADA as Expressed as Percent of Maximum Lipolysis

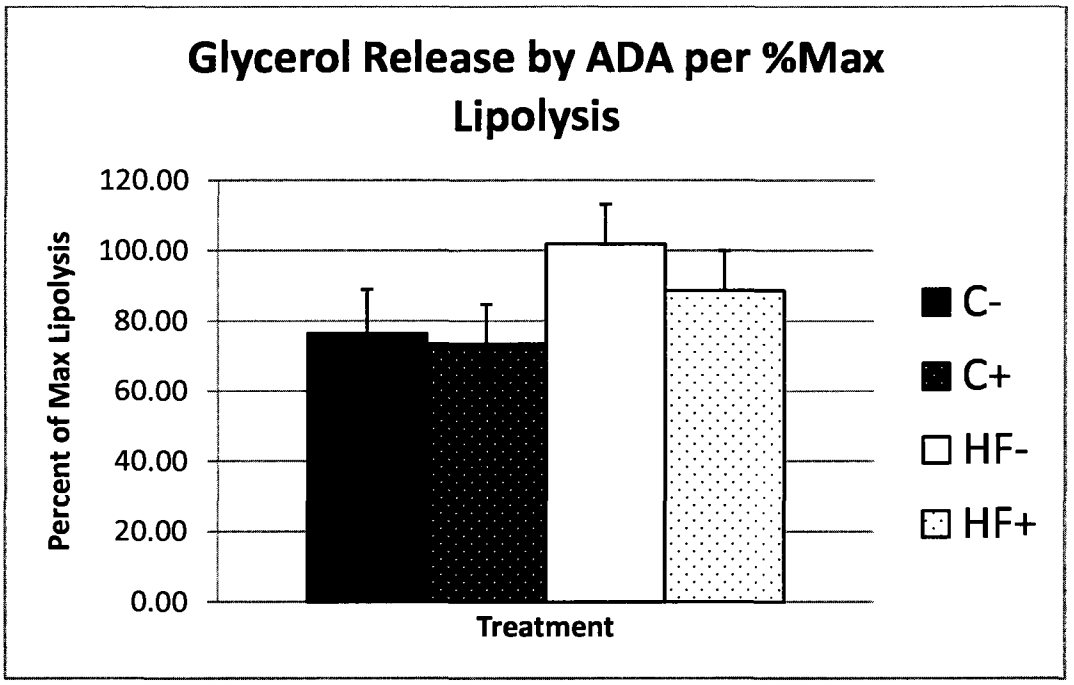

Figure 16: Glycerol released by ADA-stimulated adipocytes as a percent of max lipolysis expressed per 90 minute incubation per 10^5 cells. There was no significant difference in the amount of glycerol produced by ADA stimulation across the treatments. Results are expressed as means ± SE. N=7 rats per treatment.

Table 5: Statistical Summary

Measurement	P values for diet effect	P values for PBDE treatment effect	P values for Diet x PBDE Intx effect
Whole animal measurements			
Weight gain over 4 weeks		<0.05	
Energy intake over 4 weeks	0.053		
Metabolic efficiency	<0.05		0.080
Glucose disappearance	<0.05	0.062	
Lipid disappearance	<0.05		
Protein disappearance	0.058		<0.05
Energy Produced	0.066	0.081	
Non-protein Respiratory Quotient	<0.05		
Blood measurements			
Thyroid hormone		<0.05	
Insulin		0.075	0.081
Glucose			
C-peptide			
Epididymal adipose Tissue			
Weight			<0.05
Cell diameter	0.057		
Cell volume			
Lipolytic sensitivity to isoproterenol			
Lipolytic sensitivity to ADA			

Table 5: Summary of significant and approaching significant findings. P values are listed for each datum that is significant ($P<0.05$) or approaching significance.

DISCUSSION

This study is the first to investigate the effects of diet and PBDE exposure on whole body and adipocyte metabolism in rats. We hypothesized that PBDE exposure would cause male Wistar rats to gain weight and exhibit disordered metabolism and that the addition of a HF diet would exacerbate these physiological effects. Our findings implicate PBDEs, in conjunction with diet, as potential obesogens.

First, we provide evidence of PBDE-induced weight gain (Figure 2). PBDE-exposed animals also tended to have increased energy expenditure, perhaps due to their increased size compared to non-PBDE treated animals. A high fat diet (Schrauwen and Westerterp, 2000) or high sugar diet will provide excess calories and can cause increased adiposity humans (Nicklas et al., 2001). However, our results imply that PBDE exposure can also cause increased weight gain. To date and to our knowledge, this is the first study that documents an increase in weight gain with a four week exposure to PBDE treatment.

Other endocrine-disrupting compounds (EDCs) have been shown to cause weight gain *in vivo*. Arnesecu et al observed an increase in weight gain in C57BL6 mice when administered polychlorinated biphenyl-77 (PCB-77) daily for 5 weeks, similar to our study (Arnesecu et al., 2008). In another study, pregnant ICR mice dams were exposed to 1 μg/ml or 10 μg/ml bisphenol A in drinking water and then their pups were exposed postnatally to the same doses of bisphenol A in drinking water (Miyawaka et al., 2007).

A significant increase in body weight was observed in both the low and high dose groups. A cross-sectional study using NHANES data has found that higher levels of phthalate metabolites are associated with higher body mass index and waist circumference (Hatch et al., 2008). Men aged 20-59 years old showed a significant correlation between increased urinary mono-benzyl phthalate and body mass index and waist circumference, whereas adolescent females (aged 10-19 years old) showed a positive correlation with monoethyl phthalate. This correlation was not as strong of a correlation in females aged 20-59.

While not significant, it is interesting that animals fed the HF diet and given PBDE treatment had the highest body weight gain and the highest epididymal adipose tissue weight. This result was also seen in a study performed by Grun et al using organotins (Grun et al., 2006). Pups exposed to organotins *in utero* showed a 20% greater epididymal adipose tissue weight when compared to controls. There were no significant differences in body weight gain. In the bisphenol A study by Miyawaka et al., adipose tissue weight in animals on the 1 μg/ml dose of bisphenol A in drinking water increased 132% as compared to controls (2007).

Contrary to previous work done in our lab, no differences were observed between PBDE-treated animals and non-PBDE treated animals in *in vitro* isoproterenol-stimulated lipolysis, (Hoppe and Carey, 2007). There may be several reasons for this discrepancy. Wistar rats were used in the current study in place of Sprague-Dawley rats, as the latter gave highly variable responses. Next, in the current study, adenosine was not used in any of the buffers used for adipocyte isolation or incubation as was done in the previous study because ADA was found to induce lipolysis in the absence of isoproterenol in pilot

studies. Lastly, the diets in both studies were different. In the current study, rats were fed a purified diet with 20% protein, 10-30% fat and 0-40% sucrose by kcal. In contrast, rats in the previous study were fed a lab chow which is 26% protein, 14% fat and 60% carbohydrates by kcal (Pro-lab, Lab-Diet 5P00).

A second critical finding confirms not only that T_4 but also insulin levels are disrupted with *in vivo* PBDE treatment. Our PBDE-treated rats showed an 83% decrease in T_4 levels compared to controls and a 30% decrease in insulin levels. In a study by Danerud et al, there was a significant decrease in free T_4 levels in animals treated with a commercial PBDE mixture. Animals were treated for two weeks and exhibited a 70% decrease in T_4 levels compared to control animals (Danerud et al., 2007), which is a similar result to what we found in this study. We hypothesized that decreased T_4 levels could lead to a dampened basal metabolic rate (BMR), which would explain the increased weight gain. However, this was not borne out by the energy expenditure measurements; in fact, there is a trend towards increased energy expenditure in PBDE-treated animals. A study by Verreault et al examined the effects of circulating PBDE, hydroxylated PBDE and thyroid hormone levels and corresponding BMR in glaucous gulls (Verreault et al., 2007). This study revealed that circulating T4 levels were not predictors of overall BMR and that PBDE levels were not associated BMR of the glaucous gulls. Because there are many hormones that interact to maintain basal metabolic rate, it is possible that there is a hormonal compensation for the lower T_4 levels.

The lower levels of insulin and increased glucose disappearance seen in PBDE-treated animals are indicative of disordered glucose metabolism. A cross-sectional study examining prevalence of diabetes as associated with polybrominated biphenyls (PBBs)

and PBDEs showed interesting results (Lim et al., 2008). Researchers obtained information from NHANES data and looked for a correlation between diabetic condition (as defined as fasting glucose levels of greater than 126 mg/dL, non-fasting glucose levels of 200 mg/dL or if the subjects were taking medications for previously diagnosed diabetes) and plasma concentrations of PBB-153 or PBDE-153. A significant positive correlation was seen between PBB-153 and diabetes and a monotonic association was seen with PBDE-153 and diabetes. While correlation does not constitute causation, it is provocative to think that PBDE body burden could be implicated in the diabetes epidemic in the United States.

A third critical finding is that four diet x PBDE-treatment interaction effects were either significant or approaching significance: metabolic efficiency, protein disappearance, insulin concentrations and epididymal adipose tissue weight. These results suggest that response to PBDE treatment will vary depending on the diet that is provided. The data in this study support an altered global hormonal response within the body to PBDE exposure that is complicated by the diet. Because various hormones modulate basal metabolic rate, macronutrient uptake and metabolism, influence body fat stores and body weight gain, and that diet can also cause changes in these regulatory processes, it is possible that PBDEs and the diet are causing widespread hormonal disruption. At this time, it is premature to propose a mechanism for this diet x PBDE interaction; more studies are needed to elucidate precisely what is being disrupted in these signaling and metabolic pathways.

Recent literature supports the notion that diet modulates toxicological outcomes in lab animals. Parathion is an organophosphate pesticide that is currently under

investigation for endocrine-disrupting effects. Lassiter et al (2008) showed a parathion dose x diet interaction on neonatal rats treated with parathion on postnatal days 1-4 at a dose of either 0.1 or 0.2 mg/kg body weight/day and then provided either a control or high-fat diet in adulthood. Weight gain and disordered lipid and glucose metabolism were seen in female rats on a high fat diet combined with parathion; however females on the control diet with parathion showed weight loss and greater disordered lipid and glucose metabolism.

In another study, Jin (2007) et al fed rats diets of soy oil, seal oil, docosahexaenoic acid (DHA), fish oil or lard for 28 days, then rats were administered differing doses of methylmercury (MeHg) for 14 days. Rats on the lard diet and high dose of MeHg showed increased liver and spleen weight compared to control, whereas rats fed the oils displayed only slight increases or no change in liver and spleen weight. Rats on the lard diet also showed increased bilirubin levels when exposed to MeHg, while the oil treatments had no effect on bilirubin levels. The dietary composition (relative amount of saturated fat to mono- and polyunsaturated fat) appears to affect the outcomes of MeHg exposure in rats.

Taken together, this research confirms our hypotheses and provides evidence that PBDE exposure causes an increase in weight gain, affects glucose disappearance and energy expenditure *in vivo* and decreases T_4 and insulin levels. The diet x PBDE treatment interaction effects are indicative that the diet plays an important role in mediating the actions of PBDEs in Wistar rats. Therefore, careful consideration of the animal's diet is necessary in order to assess true toxicological effects when working with PBDEs and other EDCs. Future research should focus on testing various types of lipids

and carbohydrates in the diet to elucidate precisely the varying responses to the PBDE exposure as well as investigating further the hormonal mechanism behind PBDEs obesogenic effects.

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APPENDIX: IACUC APPROVAL

University *of* New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

02-Apr-2008

Carey, Gale B
Animal & Nutritional Sciences, Kendall Hall
Durham, NH 03824

IACUC #: 080304
Project: Flame Retardants as Obesogens
Category: B
Approval Date: 28-Mar-2008

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.* The IACUC made the following comment(s) on this protocol:

1. As part of the process of training process, the investigator and study personnel should review the gavage procedure/techniques with ARO staff.
2. Erin Allgood needs to complete the occupational program for animal handlers prior to handling any vertebrate animals.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,
Jessica Bolker

Jessica A. Bolker, Ph.D.
Chair

cc: File