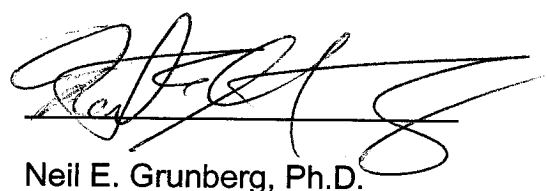


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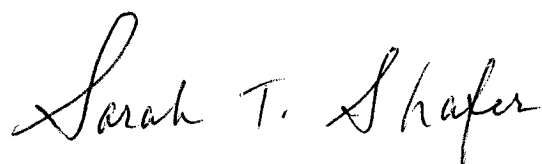
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A handwritten signature in black ink that reads "Sarah T. Shafer". The signature is written in a cursive style with a large initial 'S'.

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ABSTRACT

Title of Thesis: "Behavioral and biological effects of housing conditions and stress in male rats -- Relevance to heart disease"

Author: Sarah T. Shafer, Master of Science, 2006

Thesis directed by: Neil E. Grunberg, Ph.D., Professor

Department of Medical and Clinical Psychology

The present experiment examined the effects of environmental enrichment and stress on behavioral and biological measures relevant to cardiovascular disease risk (i.e., plasma corticosterone levels, elevated plus maze, locomotor activity in an open field chamber, body weight and food consumption, and heart morphology). Seventy-two Sprague-Dawley rats were raised in enriched environments (social or social and physical enrichment) or non-enriched environments for a total of 48 days. Half of the animals were placed in stress conditions in which they received 14 days (20 minutes/day) of restraint stress and the other half of the animals were placed in a no-stress condition.

Results revealed that : (1) rats in the stress condition had increased plasma corticosterone levels compared with non-stressed rats, (2) rats in the enriched group had decreased open-field locomotor activity and increased habituation to a novel environment compared with non-enriched rats, (3) rats in

the stress and enrichment groups had decreased body weight and food consumption compared with non-stressed and non-enriched rats, (4) rats in the stress with social enrichment conditions had heart dimensions that differed from rats in the other stress conditions without social enrichment. Social enrichment appeared to attenuate some effects of stress on the heart. These findings and future research are discussed with regard to risk for cardiovascular disease.

Behavioral and Biological Effects of Housing Conditions and Stress in Male Rats

-- Relevance to heart disease

by

Sarah T. Shafer

Master's Thesis submitted to the Faculty of the
Department of Medical and Clinical Psychology
Graduate Program of the Uniformed Services University
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INTRODUCTION

Overview

Heart disease is the leading cause of death in the United States and accounts for nearly 40% of all deaths (Centers for Disease Control, 2005). Heart disease also costs the United States approximately \$394 billion per year in health care (Centers for Disease Control, 2005). People living in disadvantaged neighborhoods are at greater risk for heart disease than are individuals living in advantaged neighborhoods (60% greater risk for Caucasians and 50% greater risk for African-Americans) (Roux, Merkin, Arnett, Chambless, Massing, & Nieto, 2001). These marked increases in heart disease risk may reflect several different causes, including effects of housing conditions.

The present research used an animal model to examine the effects of two environmental conditions (restraint stress and environmental enrichment) on biological and behavioral factors relevant to cardiovascular disease risk. The research addressed three specific aims: (1) to determine the extent to which restraint stress affects biological (plasma corticosterone levels, body weight, and heart morphology) and behavioral (elevated plus maze, open field locomotor activity, and food consumption) factors relevant to cardiovascular disease risk; (2) to determine the extent to which differential housing conditions affect these variables; and (3) to determine the extent to which housing condition attenuates the effects of stress.

As background for the research, this paper first reviews the literature on stress and environmental enrichment. Next, the rationale for each independent and dependent variable included in this research project is provided. Then, the hypotheses, methods, data analytic strategy, and results for the experiment are presented. A

discussion of the findings (including implications, limitations, and future research directions) follows.

Stress

Historical Context of Stress

Stress can be defined as the process by which environmental demands (i.e., stressors) tax or exceed the adaptive capacity of an organism, resulting in psychological and biological changes that may place a person at risk for disease (Cohen, Kessler, & Gordon, 1995, p. 3). This psychobiological definition of stress considers several different aspects of stress and the stress response. The disease risks of stress include heart disease, gastrointestinal diseases, immune-mediated conditions, and behavioral consequences that may lead to disease (e.g., cigarette smoking) (Baum, Gatchell, & Krantz, 1997).

Early conceptualizations of the stress response focused on biology. Walter Cannon (1935) suggested that organisms respond to events or challenges to an internal homeostasis with reactions that attempt to restore a balance within the body. Cannon (1935) indicated that illness results when an organism is chronically activated in maintaining homeostasis in response to an imbalance caused by environmental events. Similarly, Hans Selye (1973) conceptualized the stress response from a biological perspective. According to Selye's (1973) General Adaptation Syndrome (GAS), stress is a non-specific response of the body to demands for adaptation, primarily involving the Hypothalamic-Pituitary-Adrenal (HPA) Axis. Specific events, positive or negative, activate the HPA Axis resulting in various biological responses.

Later stress theorists emphasized the mind-body interaction with regard to stress. John Mason (1974) suggested that the individual's experience of stress depends on one's appraisal of a situation or stimulus, personality factors, situation or environmental influences, and an integrated multi-hormonal response. Rahe and Arthur (1978) attempted to quantify stress-inducing events by examining an individual's level of stressful experiences. Richard Lazarus and colleagues emphasized the contribution of cognitive factors in the individual's response to a stressor (Lazarus, 1966; Lazarus & Folkman, 1990). Other investigators emphasized the role of perceived controllability and predictability (i.e., cognitive control) in determining a person's response to stress (Glass & Singer, 1972; Grunberg & Singer, 1990). It has become clear that biological, psychological, and environmental variables are relevant to stress responses and that these factors are important to include in research investigating the effects of stress.

Effects of Stress on Biological and Behavioral Variables

Stress can be experienced in different ways, such as negative emotions, behavioral disruptions, and physiological reactions (Grunberg & Singer, 1990; Baum, Gatchel, & Krantz, 1997; Park, Cambell, & Diamond, 2001; Bauer, Perks, Lightman, & Shanks, 2001). These categories of findings are consistent in both animal and human investigations. The present discussion focuses on animal research because the experimental work of this master's thesis used rats as subjects.

Biological Effects of Stress. Challenges to an organism's survival can produce biological responses that range from activation of biochemicals involved in the HPA axis to altering the physiology of internal organs and organ systems (Kvetnansky, Weise, & Kopin, 1971; Keim & Siggs, 1976; Martijena, Cavlo, Vosolin, & Monlina, 1997; Raygada,

Shaham, Nespor, Kant, & Grunberg, 1992; Pham, Soderstrom, Henriksson, & Mohammad, 1997; Bielajew, Konkle, & Merali 2002; Bauer, Perks, Lightman, & Shanks, 2001; Elliott, Faraday, & Grunberg, 2003). Activation of the HPA axis is one of the most recognized biological responses of stress (DeVries, Glasper, & Catillion, 2003). Measuring the stress hormones related to the HPA axis (e.g., corticosterone [CORT], adrenocorticotrophin hormone [ACTH], and corticotropin-releasing factor [CRF]) is a common way to measure biological effects of stress. In particular, plasma corticosterone levels have been reported to increase in response to stressors in different experimental stress models (Bhatnagar & Meaney, 1995; Meaney, Aiken, Sharma, & Viau, 1992; Larsson et al., 2002; Belz et al., 2003) which, in turn, can differentially affect the heart. In addition, stress results in an increased release of catecholamines that affect the heart and cardiovascular system (Baum, Grunberg, & Singer, 1982).

The effects of stress on heart morphology have been reported. In rats exposed to 20 minutes of restraint stress for 14 days had decreased heart lengths, decreased left ventricle cavity widths, and increased septal wall thickness compared to rats not exposed to restraint stress (Elliott, Faraday, & Grunberg, 2003). Dobutamine (a synthetic catecholamine that can be used to mimic some actions of stress) decreased left ventricular dimensions in rats (Plante, LaChance, Drolet, Roussel, Couet, & Arsenault, 2005). Changes in heart morphology also have been reported in hamsters exposed to various psychological stressors (e.g., shocks and restraint stress) (Tapp & Natelson, 1988).

Behavioral Effects of Stress. Behavioral responses to stress (in animal models) include interruption of learning and memory, an increase in anxiety-like behaviors, and changes in feeding and body weight. Animals exposed to stressors exhibit poorer performance on cognitive tasks compared with animals not exposed to stressors. Stress can interrupt attentional processing in rats as measured by pre-pulse inhibition of the acoustic startle reflex (Acri, 1994; Faraday, 2002). With regard to learning and memory, stressed rats display inferior spatial learning and memory in the radial arm maze compared to non-stressed rats (Park, Campbell, & Diamond, 2001).

Stress also increases anxiety-like behaviors. In response to inescapable foot-shocks or immobilization, rodents decreased overall activity and increased defecation in an open field arena (Gamallo et al., 1988; van Dijken, Mos, van der Heyden, & Tilders, 1992; Faraday, 2002). Predator stress (i.e., exposure of rats to a cat) impaired habituation to a novel environment by increasing activity within the open field (i.e., Open-Field) (Park, Campbell, & Diamond, 2001). In studies using the elevated plus maze (EPM), exposure to an inescapable shock decreased time in the open arms, which suggests an anxiogenic response (Steenbergen, Heinsbroek, Van Hest, & Van de Poll, 1990; Martijena et al., 1997; Kalinchev et al., 2002).

Food consumption and body weight also can be affected by stress. Rats that are crowded or experience changes in their housing environment decrease food consumption (Brown & Grunberg, 1995; O'Conner & Eikelboom, 2000). Electric shock and restraint stress decrease food consumption (Rickards, Job, & Boakes, 1997; Marti, Marti, & Armario, 1994; Zylan & Brown, 1996); exposure to repeated cold stress increases feeding (Kawanishi, Fukuda, Tamura, Nishijo, & Ono, 1997); noise stressors

increase (Rasbury & Shemberg, 1971; Wilson & Cantor, 1986) and decrease feeding (Krebs, Macht, Weyers, Weijers, & Janke, 1996). Pijlman, Wolterink, and Van Ree (2003) suggest that stress may influence the sensitivity of subjects to rewarding stimuli. They report that physical stress induces a long-term decrease in preference for saccharine and open field activity compared to control treatment. Further, emotionally stressed animals increase open field behavior activity and saccharine preference.

Enriched Environments

Historical Context of Enriched Environments

Charles Darwin (1875) was the first person credited with observing that animals from different environments had different brain sizes. He reported that the brains of domestic rabbits were considerably smaller compared to the brains of wild rabbits and argued that the reduced brain size of the domestic animals was a consequence of a deprived environment because domesticated animals did not exert their intellects, instincts, or senses as much as animals did in the wild.

It was not until 1947 that Donald Hebb observed similar phenomena. He noted that the laboratory rats that he had taken home for his children to play with exhibited superior performance on maze learning when compared to rats kept in the laboratory environment. Hebb concluded that nerve cells in the brains of the rats had changed in response to the enriched and varied experiences outside the laboratory. He hypothesized that the number of synaptic connections increased and that these structural changes resulted in functional (i.e., behavioral) modifications. Hebb believed that these changes reflected new learning. This particular report of Hebb was remarkably consistent with Darwin's (1875) observation.

More than 20 years later, Mark Rosenzweig (1966) introduced what became the classic paradigm for studying the impact of enriched environments on rats. Animals are housed in groups to provide opportunities for social interaction (i.e., social enrichment). Physical stimulation (i.e., physical enrichment) involves providing objects in the cages to allow tactile stimulation and physical activity (Rosenzweig & Bennett, 1996; Woodcock & Richardson, 2000). Most subsequent environmental enrichment studies (c.f., Mohammad et al., 1993; Pham et al., 1999) have included social and physical enrichment components. Enriched environments are distinguished from non-enriched environments by the amount of stimulation and activity available in the environment. The standard non-enriched environment limits the physical and social enrichment by housing the animals individually without objects (Varty, Paulus, Braff, & Geyer, 2000). Commonly, across human and animal research, environmental enrichment refers to physical and social stimulation provided in the environment.

Effects of Enriched Environments

Enriched versus non-enriched housing environments have different biological and behavioral consequences. This section briefly reviews biological and behavioral consequences of environmental enrichment.

Biological Effects of Enrichment. Animal experiments reveal that enriched experiences alter neurotransmitters in the cerebral cortex (Rosenzweig & Bennett, 1996). Stimulating environmental conditions (i.e., enriched environments) significantly influence brain development and functioning including: increased size and weight of the cortex, increased neuron sizes and dendritic branching, increased synapse formation, and elevated protein levels (Rosenzweig, Bennett, & Diamond, 1972; Mohammed et al.,

2002). Diamond (1991) reported that laboratory rats housed in enriched environments could have up to 25 percent more neurons in their brains when compared to non-enriched rats.

Another biological effect of enrichment is the amount of food consumed. Food consumption has been found to be altered in enriched animals compared with non-enriched animals. Tomchesson (2004) reported decreased food consumption of regular rat chow in enriched rats compared with non-enriched rats. In a follow-up study, rats in enriched cages also consumed less high fat foods (e.g., Oreo cookies and potato chips) than non-enriched rats (Tomchesson, 2006).

Similarly, body weight also is affected by environmental enrichment. Animals raised in enriched environments have decreased body weights compared with animals raised in isolated environments (Tomchesson, 2004; 2006). This effect was found in mice as well (Van de Weerd, Aarsen, Mulder, Kruitwagen, Hendriksen, & Bauman, 2002). In addition to body weight, organ weights (including the heart) from animals raised in enrichment also show more variability than organ weights from animals raised in isolation (Tsai, Pachowsky, Stelzer, & Hackbarth, 2002).

Behavioral Effects of Enrichment. In addition to the biological changes of rats reared in an enriched environment, enriched rats exhibit more complex behaviors than rats reared in non-enriched environments (Mohammad et al., 1993; Pham et al., 1999; Kobayashi, Ohashi, Ando, 2002). Environmental enrichment can alter performance on learning and memory tasks, behavioral assessments of anxiety, and food consumption.

Enrichment has been found to improve the cognitive functioning of animals on behavioral tasks of attention, memory, and learning compared to animals reared in

standard non-enriched environments. For example, early social isolation leads to an interruption of attentional processing in rats as measured by acoustic startle reflex (Robbins, 1996). Differential performances also have been found on the Morris water maze and the radial arm maze, widely used measures of rodent learning and spatial memory. When compared to non-environmentally enriched rats, the enriched rats perform significantly better in the Morris water maze task (Daniel, Roberts, & Dohanich, 1999; Williams, Luo, Ward, Redd, & Gibson, 2001; Elliott & Grunberg, 2005) and the radial arm maze (Juraska, Einon, 1980).

In addition to the previously mentioned tasks of cognitive functioning, rats raised in enriched environments habituate (a form of simple learning) to novel environments faster than rats raised in isolation (Elliott & Grunberg, 2005; Grunberg et al., under review; Tomchesson, 2004; 2006; Schrijver, Bahr, Weiss, Wurbel, 2002; Zimmermann, Stauffacher, Langhans, Wurbel, 2001; Pham, Ickes, Albeck, Soderstrom, Granholm, Mohammed, 1999). Similar results have been reported for mice (Pietropaolo, Branchi, Cirulli, Chiarotti, Aloe, & Alleva, 2004; Van de Weerd, 2002).

Behavioral assessments of anxiety also have yielded differential responses in rats raised in environmental enrichment compared to rats raised in isolation. Rats from enriched environments spend more time in the open arms and have more entries into the open arms of the elevated plus maze (a behavioral index of anxiety) than rats from isolated environments (Friske & Gammie, 2005). Similar results have been reported for mice (Benaroya-Milshtein, Hollander, Apter, Kukulansky, Raz, Wilf, et al., 2004; Chapillon, Manneche, Belzung, & Caston, 1999).

It is well established that enriched environments, characterized by the presence of physical objects and the opportunity for social interaction, have marked biological and behavioral effects in developing organisms. The research literature has focused on effects of environmental enrichment on learning and on the brain. In light of the interplay of biological and behavioral variables in health, including heart function and disease, it is important to determine the extent to which environmental enrichment alter factors relevant to heart disease risk (i.e., stress responses and heart morphology).

Environmental Enrichment and Stress

A limited number of rodent studies have considered environmental enrichment and stress together. These experiments mostly have studied rats and emphasized biological responses to stress. These experiments have typically included behavioral response to stress, but none of these studies have examined heart morphology.

A majority of the studies examining the biological effects of environmental enrichment and stress report a decrease in the stress response for environmentally-enriched animals. Gadek-Michalska and Bugajski (2003) found that rats in enriched environments (seven rats in a large cage without toys) had corticosterone responses that were reduced by 41.5% in response to handling compared with isolated rats. Schrijver et al. (2002) reported that enriched rats (physical enrichment [isolated with toys], or combined enrichment [social and physical enrichment]) had attenuated ACTH and plasma corticosterone responses to stress compared to non-enriched rats. Belz et al. (2003) reported that rats of both sexes raised with physical enrichment (i.e., toys and no other rats) had significantly lower baseline ACTH and CORT concentrations compared to rats housed without enrichment. Also, ACTH responses to a mild stress of

saline injection were significantly lower in the female rats housed with enrichment. Other research has reported that environmental enrichment reverses the effects of prenatal and early childhood stress (e.g., maternal separation) (Morley-Fletcher, Rea, Maccari, and Laviola, 2003; Francis, Diorio, Plotsky, Meaney, 2002).

There also are some reports of environmental enrichment and stress resulting in an increase in biological responses to stress. For example, Moncek, Dunko, Johanson, & Jezova (2004) reported that rats raised in environmental enrichment (10 rats per one large cage with toys) had pronounced changes in neuroendocrine regulation, including larger adrenals and increased adrenocortical function - an indication of chronic stress. However, rats in enriched environments also showed less of a stress response to an acute stressor (handling) than isolated rats. According to this study, it appears that in the long-term environmental enrichment may increase stress, however, enriched rats are better able to handle acute stressors than non-enriched rats. Another example of mixed findings has been documented by Marashi, Barnekow, Ossendorf, Sachser (2003) who reported elevated stress hormone levels for enriched animals, but stress hormones for male mice raised in super enrichment (mice in a large cage with toys) were not as elevated. These researchers suggest that environmental enrichment is beneficial as long as there is enough space to accommodate all of the animals.

Only a handful of studies have examined the behavioral effects of environmental enrichment and stress. Morey-Fletcher et al. (2003) reported that the reduced expression of social play in rats that were prenatally stressed was reversed by environmental enrichment. Widman, Abrahamsen, and Rosellini (1992) reported that

environmental enrichment did not attenuate the stress response to an uncontrollable stressor, as assessed by an appetitive-noncontingent test (an index of learning).

Tomchesson (2004) found that environmental enrichment did attenuate stress on a measure of simple learning (open-field horizontal activity habituation).

It appears that enrichment may have beneficial effects with regard to attenuation of the biological stress response as assessed by HPA axis reactivity as well as some behavioral responses. However, several questions remain. First, it is unclear how environmental enrichment affects HPA reactivity to a *chronic* stressor. The studies reviewed here either used an acute stressor (e.g., handling), used a chronic stressor that was pre or post-natal (e.g., maternal separation), or measured baseline levels of CORT or ACTH without assessing the response to a stressor. Second, the effect of different types of enrichment needs to be researched further. Specifically, social enrichment appears to be the type of enrichment responsible for the mixed results. The only study to investigate a small number of rats together without toys was Schrijver et al. (2002) who found that rats housed in social enrichment (4 per cage) had a delayed recovery after exposure to a stressor. More research is needed to differentiate if the stress response is differentially affected by the type of enrichment. Third, no reported studies have investigated other biological indices of stress besides measures of HPA activity.

Another biological variable that may be affected by environment is the heart *per se* which is important to examine when considering heart disease risk. In particular, the size of the left ventricle (also termed left ventricular mass) is related to heart disease risk (Wong, Black, & Gardin, 2000). Left ventricular mass is associated with body mass

index, male gender, blood pressure, present smoking, major and minor electrocardiographic abnormalities, high-density-lipoprotein cholesterol, and pulse pressure (Wong et al., 2000; Gardin, Arnold, Gottdiener, Wong, Fried, Klopfenstein, et al., 1997). Increased, left ventricular mass also has been shown to lead to an increased incidence of heart disease events and mortality (Levy, Garrison, Savage, Kannel, & Castelli, 1990). Elliott et al. (2003) found that heart morphology was affected in rats as a result of exposure to a chronic stressor. The finding that stress can lead to changes in the heart is particularly important because these changes may be indicative of heart disease risk. It would be valuable to determine if heart morphology also may be affected by environmental conditions, such as enrichment. If environmental enrichment alters heart morphology, then environmental factors might be manipulated to help prevent or treat heart disease. This possibility remains speculative until experimental evidence indicates whether manipulation of environmental conditions alters heart morphology. Further mechanisms for this possible link might involve biochemical aspects of stress responses (e.g., HPA axis), so this possibility also deserves research attention.

The Present Research

The present research examined the effects of stress and environmental enrichment (as manipulated by rats in isolation, rats in social enrichment [3 rats per cage with no objects] and rats in combined enrichment [physical and social enrichment]) on biological and behavioral factors related to heart disease risk. The specific dependent variables were: plasma corticosterone levels (to reflect HPA axis activity), behavioral indices of stress (elevated plus maze and locomotor center time), measures

relevant to body weight (food consumption and body weight), and heart morphology. There were three specific aims of the present research: (1) to replicate the effects of restraint stress on the biological and behavioral factors relevant to cardiovascular disease (e.g., plasma corticosterone levels, behavioral index of stress, general activity, food consumption and body weight, and heart morphology), (2) to replicate and extend what is known about the effects of environmental enrichment on these biological and behavioral variables, and (3) to determine if environmental enrichment attenuates the stress response as measured by these biological and behavioral variables.

Hypotheses

Specific Aim #1: Restraint stress and biological and behavioral measures relevant to cardiovascular disease risk

Hypothesis 1a. Restraint stress will result in increased plasma corticosterone.

The first hypothesis is a manipulation check to determine if restraint stress is effective. Previous research indicates that restraint stress results in elevated stress hormones including plasma corticosterone (Kant, 1983; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992; Acri, 1994; Faraday 2002). Bauer, Lightman, and Shanks (2001) reported that one 30-minute session of restraint significantly increased plasma corticosterone in male, Sprague-Dawley rats. Increased plasma corticosterone levels also were evident after repeated sessions (i.e., 30 minutes of restraint daily for 13 days). Additionally, increased plasma corticosterone levels were found after 14 days of restraint stress for 20 minutes (Faraday, 2002).

Hypothesis 1b. Restraint stress will increase behavioral measure of stress (specifically decreased time spent in the open arms of the elevated plus maze and decreased time in the center of a locomotor chamber).

This hypothesis is based on previous research reports that stress increases anxiety behaviors in the elevated plus maze in rodents (Wigger & Neumann, 1999; McIntosh et al., 1999; Kalinichev et al., 2002). Marinjena, Calvo, Volosin, and Molina (1997) restrained rats for 15 minutes, tested them 24 hours later on the elevated plus maze, and reported an anxiogenic profile (i.e., less time in the open arms of the maze). Similar results were reported following a 2-hour restraint stressor with a 24-hour delay (Padovan, Del-Bel & Guimaraes, 1996; Mendonca & Guimaraes, 1998). Restraint stress also has been reported to decrease open-field activity in rats (Galea, Wide, & Barr, 2001; Faraday, 2002). After 20 minutes of restraint, open-field activity was decreased in male Sprague-Dawley and male Long-Evans rats (Faraday, 2002).

Hypothesis 1c. Rats in the stress group will have decreased food consumption and body weight compared with rats in the non-stress condition.

This hypothesis is based on previous research reporting that both food consumption and body weight are affected by stress (Faraday, 2002; Penke, Felszeghy, Fernette, Sage, Nyakas, Bulet, 2001; Krahn, Gosnell, Grace, & Levine, 1986).

Hypothesis 1d. Rats in the stress group will have different heart measurements than rats in the non-stress group. Specifically, rats in the stress condition will have decreased heart lengths and left ventricle cavity widths and increased septal wall thickness as a result of exposure to restraint stress.

This hypothesis is based on previous research by Elliott and colleagues that found these results in rats exposed to restraint stress (Elliott et al., 2003).

Specific Aim #2: Examine the effects of environmental conditions on biological and behavioral measures relevant to cardiovascular disease

Hypothesis 2a. Rats in the enriched conditions will have lower plasma corticosterone levels than rats in the isolated conditions.

This hypothesis is based on previous research reporting differences in plasma corticosterone between enriched and non-enriched subjects (Van de Weerd et al., 1997; Pham et al., 1999; Larsson et al., 2002). Belz et al. (2003) reported that rats reared with toys had significantly lower levels of corticosterone.

Hypothesis 2b. Rats in the enriched environments will have decreased behavioral indices of stress (specifically more time in the open arms of the EPM and more time in the center of the locomotor chamber).

This hypothesis is based on previous research by Schmitt and Heimke (1998) who reported that handling (a simple form of enrichment) resulted in subjects spending more time in the open arms of the maze, interpreted as a reduction in anxiety. This hypothesis is based on previous research reporting that animals raised in enriched environments exhibit reduced locomotor activity and reduced exploration over time (Varty et al., 2000; Bowling *et al.*, 1993; Van Wass & Soffie, 1996; Paulus, Bakshi, & Geyer, 1998; Zimmerman, Stauffacher, Langhans, & Wurbel, 2001; Tomchesson, 2004; Grunberg, Shafer, Elliott, & Grunberg, in preparation).

Hypothesis 2c. Rats in the enriched environments will eat less and weigh less than rats raised in the isolated environments.

This hypothesis is based on previous research reporting that food consumption and body weight are affected by environmental manipulations (Brown & Grunberg, 1995; Tomchesson, 2004).

Hypothesis 2d. Rats in the enriched environments will have heart dimensions that resemble the non-stressed isolated condition more than the stressed isolated animals will.

This hypothesis is based on the report that plasma corticosterone levels are lower in enriched animals compared with isolated animals (Belz et al, 2003). Therefore, enriched animals may be less stressed. If stress indeed alters the heart, then animals that are enriched (and therefore hypothesized to be less stressed) should have lower stress levels as indicated by decreased corticosterone levels and should have hearts that differ from animals that are not enriched. Also, environmental enrichment affected heart weights in mice compared with isolated mice (Tsai, Pachowsky, Stelzer, Hackbarth, 2002).

Specific Aim #3: Environmental conditions and attenuation of stress

Hypothesis 3a. There will be a stress x enrichment interaction, such that rats in the enriched conditions will have less of an increase in plasma corticosterone under stress than will rats in the isolated condition.

This hypothesis is based on previous research reporting differences in plasma corticosterone between enriched and non-enriched subjects (Van de Weerd et al., 1997; Pham et al., 1999; Larsson et al., 2002). Belz et al. (2003) reported that rats reared in isolation with toys had significantly lower levels of corticosterone.

Hypothesis 3b. There will be a stress x enrichment interaction, such that rats in the enriched conditions will have less of an increase in time spent in the open arms under stress than will rats in the isolated condition.

This hypothesis is based on previous research by Schmitt and Heimke (1998) who reported that handling (a simple form of enrichment) resulted in subjects spending more time in the open arms of the maze, interpreted as a reduction in anxiety. Also, animals raised in enriched environments exhibit reduced locomotor activity and reduced exploration over time (Varty et al., 2000; Bowling *et al.*, 1993; Van Wass & Soffie, 1996; Paulus, Bakshi, & Geyer, 1998; Zimmerman, Stauffacher, Langhans, & Wurbel, 2001; Tomchesson, 2004; Grunberg, et al., in preparation).

Hypothesis 3c. There will be a stress x enrichment interaction for food consumption and body weight such that rats in the enriched and stress conditions will have decreased food consumption and body weights under stress, but not to the same extent that isolated animals will experience under stress.

This hypothesis is based on previous research reporting that stress and enrichment both decrease body weight and food consumption (Faraday 2002; Tomchesson, 2004).

Hypothesis 3d. There will be a stress x enrichment interaction for heart dimensions such that the hearts of enriched rats will be less affected by stress than the hearts from the isolated rats. This hypothesis is based on reports finding that biochemical markers of stress are lower in enriched animals compared with isolated animals (Belz et al, 2003). Therefore, enriched animals may be less stressed. If stress indeed alters the heart, then animals that are less stressed and animals that are

enriched should have lower stress levels as indicated by decreased corticosterone levels.

METHODS

Overview

The present experiment was designed to determine the extent to which differing environments affect biological and behavioral risk factors for heart disease. This experiment was inspired by Tomchesson (2004), but included several differences. The present experiment studied a different group of rats; included more housing conditions (three rather than two); and included dependent variables relevant to cardiovascular disease risk (i.e., heart morphology). The design was a 2 (Stress or No Stress) x 3 (Isolated, Social, or Combined housing condition) design. The experiment was run in two cohorts of 36 animals per cohort for a total of 72 animals.

Independent Variable

Stress Manipulation: Immobilization

Stress manipulation in animal experiments varies greatly (e.g., electric shock, crowding, cold water immersion, predator, intruder, or immobilization). The present experiment used short-term restraint (e.g., 15 – 30 minutes in a Fisher Scientific Centrap Cage). This widely used stress manipulation does not appear to be painful and elicits behavioral and biological stress responses in rodents, including elevations in hypothalamic-pituitary-adrenocortical (HPA) hormones (Kant, Leu, Andersen, & Mougey, 1987; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992; Plotsky & Meaney, 1993; Acri, 1994; Faraday, O'Donoghue, & Grunberg, 1999; Faraday, 2002). Animals in this experiment received 20 minutes of restraint stress for 14 days.

Environmental Enrichment

Environmental enrichment is defined as the presence of physical objects and opportunities for social interaction. There are several different ways to manipulate and conceptualize environmental enrichment in animal models. Some examples include neonatal handling (Meaney, Aitken, Sharma, & Viau, 1992), social enrichment (Renner & Rosenzweig, 1986; Varty et al., 2000), physical enrichment (Renner & Rosenzweig, 1986; Varty et al., 2000), and incorporation of natural environmental objects (Schrijver et al., 2002). Enriched environmental exposure can vary from 12 days (Passineau, Green & Detrich, 2001; Elliott & Grunberg, 2005) to a year (Ickes et al., 2000). The most common enrichment paradigms in animal research house 3 to 12 rats in cages filled with toys and objects (e.g., pieces of wood, plastic bones, exercise wheels, balls, tunnels), allowing opportunities for social interaction and physical stimulation (Rosenzweig & Bennett, 1996; Woodcock & Richardson, 2000). Enriched environments differ from isolated environments in the number of animals per cage and the number of objects per cage (Rosenzweig & Bennett, 1996; Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998; Van Praag, Kempermann, & Gage, 1999; Varty, et al., 2000; Schrijver et al., 2002).

The present experiment housed animals in isolation, social enrichment (3 animals per cage with no toys), and combined enrichment (3 animals per cage with toys) for a total of 48 days. Detailed descriptions of housing conditions are provided in the Methods section.

Dependent Variables

The dependent variables were plasma corticosterone, elevated plus maze, locomotor center time, food consumption, body weight, and heart morphology. This section provides a description of each dependent variable. Details describing the equipment and exact procedures are presented in the Methods section of this paper.

Plasma Corticosterone (CORT)

The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to a stressor. HPA activity is reflected by plasma concentrations of several biochemicals, including corticosterone (CORT) (Selye, 1973; Hennessy, 1997; Pham et al., 1999; Belz, Kennell, Czambel, Rubin, & Rhodes, 2003). Investigations that examine biological markers of stress routinely examine levels of plasma corticosterone (Brown & Grunberg, 1995; Faraday, 2002; Larsson et al., 2002; Belz et al., 2003).

Plasma Corticosterone and Restraint Stress. Restraint results in elevated stress hormones including plasma corticosterone (Kant, 1987; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992, Acri, 1994; Faraday 2002). Bauer, Lightman, and Shanks (2001) reported that one 30-minute session of restraint significantly increased plasma corticosterone in male, Sprague-Dawley, rats. These investigators reported that increased plasma corticosterone levels also were evident after repeated sessions (i.e., 30 minutes of restraint daily for 13 days). Additionally, increased plasma corticosterone levels were found after 14 days of restraint stress for 20 minutes (Faraday, 2005). Specifically, nonstressed rats had plasma corticosterone concentrations at 215 ± 10 ng/ml and stressed rats had mean plasma corticosterone concentrations at 585 ± 20 ng/ml (Faraday, 2005).

Plasma Corticosterone and Enrichment. Several studies report differences in plasma corticosterone between enriched and non-enriched subjects, with a majority of the studies reporting a decrease in plasma corticosterone between enriched and non-enriched subjects (Van de Weerd et al., 1997; Pham et al., 1999; Larsson et al., 2002). However, environmental conditions differ among these studies. The present study included corticosterone as a manipulation check of stress and to determine how the enrichment conditions in this experiment affected corticosterone.

Elevated Plus Maze (EPM)

Elevated Plus Maze is commonly used to index anxiety in rodent research (Elliott, Faraday, Phillips, & Grunberg, 2004; Pellow, Chopin, File, & Briley, 1985; Hogg, 1996; Kalinichev et al., 2002). The apparatus consists of four radiating platforms that are at right angles to each other. Two of the arms have high walls that enclose the platforms; two of the arms have no walls. Each subject is initially placed on an open-arm platform and time and entries into the open and closed platform arms are observed and recorded. This task does not require training, food or water deprivation, or aversive stimuli. The task is easy to conduct and typically takes 5 minutes to complete. A variety of species have been used in the elevated plus maze, including rats (Pellow, Chopin, File, & Briley, 1985), mice (Lister, 1987), guinea pigs (Rex, Fink, & Marsden, 1994), and wild voles (Hendrie, Eilam, & Weiss, 1974). The EPM is bidirectionally sensitive to anxiety manipulations and anxiety-like responses. Therefore, EPM is sensitive enough to detect both increases and decreases in anxiety. The two primary indices of anxiety in the EPM are the percentage of time spent on the open arms and the percentage of entries into open arms.

Elevated Plus Maze and Restraint Stress. Stress has been reported to increase anxiety behaviors in the EPM in rodents (Wigger & Neumann, 1999; McIntosh et al., 1999; Kalinichev et al., 2002). Martijena, Calvo, Volosin, and Molina (1997) restrained rats for 15 minutes, tested them 24 hours later on the EPM, and reported an anxiogenic profile (i.e., less time in the open arms of the maze). Similar results were reported following a 2-hour restraint stressor with a 24-hour delay (Padovan, Del-Bel & Guimaraes, 1996; Mendonca & Guimaraes, 1998).

Elevated Plus Maze and Enrichment. Few studies have examined enrichment and EPM performance. One study reported that rats raised in enrichment spent the same amount of time in the open arms as rats raised in isolation (Tomchesson, 2004); however, it is not clear if a longer enrichment and stress phase would change these results. Schmitt and Heimke (1998) reported that handling (a simple form of enrichment) resulted in subjects spending more time in the open arms of the maze, interpreted as a reduction in anxiety. Handling decreased overall activity but did not significantly affect the number of transitions from the open to closed arms of the EPM. Santucci et al. (1994) reported that handling neonatal rats decreased anxiety according to EPM performance indexed by more time spent in the open arms of the elevated plus maze and more transitions between open and closed arms of the maze. This experiment used a longer enrichment period followed by a longer stress period to determine if there are any effects on EPM as an index of anxiety.

Open-Field Activity (OF)

Open-Field locomotion refers to an animal's behavior when placed in a non-home cage arena. The apparatus is an empty box with clear sides and a clear top that

is used to measure the animals' activity in a novel environment. Animal behaviors in the Open-Field have been used as measures of general locomotion, exploration, and anxiety or stress responses. For the present experiment, the amount of activity spent in the center of the open-field arena is analyzed and discussed. Open field activity provides a useful way to examine effects of enrichment and stress on anxiety-like behavior.

Open-Field and Restraint Stress. Restraint stress has been reported to decrease open-field activity in rats (Galea, Wide, & Barr, 2001; Faraday, 2002). After 20 minutes of restraint, open-field activity was decreased in male Sprague-Dawley and male Long-Evans rats, but only on the first day of stress. Increased center time has been interpreted as decreased anxiety and decreased center time is interpreted as increased anxiety (Gamallo et al., 1986; Lee, Tsai, & Chai, 1986; Beck & Luine, 2002). Variations in the amount of restraint and the type of subjects used to investigate stress responses have provided different results. In addition, it is not clear if the behaviors of adult subjects also occur in adolescent rats. The effects of environmental enrichment are primarily studied using adolescent subjects. Therefore, to investigate the effects of environmental enrichment and stress, it is important to examine the effect of stress on the adolescent subject.

Open-Field and Enrichment. Animals raised in enriched environments exhibit reduced locomotor activity and reduced exploration over time (Varty et al., 2000; Bowling et al., 1993; Van Wass & Soffie, 1996; Paulus, Bakshi, & Geyer, 1998; Zimmerman, Stauffacher, Langhans, & Wurbel, 2001; Tomchesson, 2004; Grunberg et al., under review). Environmental enrichment appears to improve information

processing and adaptation to novel environments, but there are few reports of enrichment's effects on center time.

Food Consumption (FC) and Body Weight (BW)

Food consumption and body weight are relevant to many physical and mental health conditions (e.g., anxiety, depression, eating disorders, obesity) and are used in many rodent experiments as a measure of general health or to determine the effect of various manipulations on the animal. Further, food consumption and body weight are face-valid measures of food consumed and body weight that are used with humans and animals (Brown & Grunberg, 1995; O'Conner & Eikelboom, 1999; Faraday, 2002). Food consumption and body weight were included in this experiment to provide an index of the animal's health and because stress (Faraday, 2002) and environmental changes (Tomchesson, 2004) are known to affect food consumption and body weight.

Heart Morphology

Heart morphology is the assessment of the heart's dimensions (e.g., left ventricular size, length, width, weight, etc). Diet has been reported to affect heart morphology in rats (Rossi, Carillo, & Oliveira, 1981). Specifically, rats fed a cholesterol-enriched diet developed lesions in the interlamellar spaces in the aorta, fibrosis of coronary arterial wall, and myocardial fibrosis, putting them at risk for advanced atherosclerotic lesions (Bennani-Kabchi, Kehel, El Bouayadi, Fdhil, Amarti, & Saidi, et al., 2000). Stress also has been reported to affect heart morphology in rats (Elliott, Faraday, & Grunberg, 2003).

Heart Morphology and Restraint Stress. The effect of restraint stress on heart morphology needs to be further examined. Male rats have decreased heart length and

left ventricle cavity width and increased septal wall thickness as a result of exposure to restraint stress. Specifically, non-stressed rats had a mean left ventricle cavity of 4.9 ± 0.4 mm and a mean septal wall width of 2.4 ± 0.2 mm and stressed rats had a mean left ventricle cavity of 3.8 ± 0.5 mm and a mean septal wall width of 3.2 ± 0.2 mm.

However, no significant differences were reported for female rats (Elliott, Faraday, & Grunberg, 2003). Another study reported a significant increase in the heart weight of rats receiving various types of stress (including restraint stress) (Nagaraja & Jeganathan, 1999).

Heart Morphology and Environmental Enrichment. The effect of environmental enrichment on heart morphology is not widely studied. One experiment reported that mice raised in environmental enrichment did not differ in mean heart weight from isolated animals, but they had more had more variability in heart weights than animals raised in isolation (Tsai, Pachowsky, Stelzer, Hackbarth, 2002). This finding suggests that environmental conditions may affect the heart *per se*. Environmental conditions may indirectly affect the heart through its affect on diet, activity, or stress (e.g., Tomchesson 2006; Belz et al., 2003). If the environment affects heart health (directly or indirectly), then steps can be taken to intervene on an environmental level. However, the effects of environmental enrichment on specific heart morphology have not been examined.

Experimental Design and Determination of Sample Size

This experiment examined the effects of stress and environmental enrichment on male adolescent Sprague-Dawley rats. The experiment was conducted as a 2 (Stress

or No Stress) x 3 (Isolated, Social, or Combined Enrichment) full factorial design with 12 subjects per cell.

The sample size (cell size of $n = 12$) was determined in two ways: (1) based on previous reports using similar dependent measures and responses to environmental enrichment and stress (e.g., Tomchesson 2004; Elliott et al., 2003), and (2) a power analysis based on previous research using the independent variables of stress and enrichment.

Studies in the research literature reported statistically significant effects from cell sizes of 7 – 12 animals for enrichment (e.g., Van Praag et al., 1999; Passineau et al., 2001; Elliott & Grunberg, 2003; Tomchesson, 2004) and 9 - 11 animals for stress effects (Schrijver et al., 2002; Faraday, 2002). Mering, Kaliste-Korhonen, and Nevalainen (2000) determined that 5 - 10 animals were needed to find statistically significant effects for enrichment on various biological measures (e.g., Body weight, adrenal gland weights, fat adipose tissue). Studies of stress and enrichment report statistically significant effects for food consumption, body weight, and locomotor activity with a cell size of 12 (Tomchesson, 2004).

Sample size was determined by using the procedures of Keppel (1991); Keppel, Saufley, and Tokunaga (1992); and Cohen (1988). Estimates of effect size in the population were determined to provide at least 0.80 power by calculating an estimated omega squared (ω^2). Keppel (1991) uses the formula:

$$\omega^2_A = \frac{\sigma^2_{S/A}}{(\sigma^2_A + \sigma^2_{S/A})}$$

where σ^2_A refers to the estimated population treatment effects, $\sigma^2_{S/A}$ refers to the estimated population error variance, and ω^2_A provides a measure of effect size that is relatively independent of sample size and is expressed as a proportion of the total variability (σ^2_A) associated with the treatment or manipulation (σ^2_A) (Keppel et al., 1992). Alternatively, $\omega^2 = (SS_{\text{effect}} - (df_{\text{effect}})(MS_{\text{error}})) / MS_{\text{error}} + SS_{\text{total}}$, where SS = sum of squares, df = degrees of freedom, and MS = mean square. Using values reported by Tomchesson (2004) for the enrichment x stress interaction on food consumption, $\omega^2 = (13609.32 - (1)(473.004)) / 473.004 + 36379.502 = 13136.316 / 36852.506 = 0.356$. According to Keppel (1991), a large effect size is produced by the sample size of this experiment because ω^2 exceeds 0.15.

Research Design and Methods

Subjects

The subjects were 72 male, Sprague-Dawley rats from Charles River Laboratories. The rats were 21 days upon arrival. Other investigators have defined adolescence in the rat as 21-42 days old and up to day 55 for male rats (Spear & Brake, 1983; Ojeda & Urbanski, 1994; Faraday, Elliott & Grunberg, 2001). Male subjects were used in the present experiment as a first step to determine if a relationship between stress, enrichment, and heart disease risk exists. Adolescent animals were used to maximize the developmental impact of environmental environment and because of the investigator's interest in child/adolescent development. Sprague-Dawley rats were used because they are the most commonly used strain of outbred albino rats.

Before arrival animals were housed with their mothers and shipped in groups of 12. Immediately upon arrival, the animals were randomly placed in a housing condition.

Housing

All animals were housed on hardwood chip bedding (Pine-Dri) with continuous access to food (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. The housing room was maintained at 23⁰ C and 50% relative humidity on a 12-hour reversed light/dark cycle (lights off at 0500 hours). The reversed light cycle was maintained so that behavioral measures could be accomplished during the animals' normal activity period. Animals were assigned to one of six housing conditions in a full factorial design that manipulated two levels of stress and three levels of housing conditions (Isolated/Not Stressed [INS], Isolated/Stressed [IS], Social Enrichment/Not Stressed [SNS], Social Enrichment/Stressed [SS], Combined Enriched/ Not Stressed [CNS] and Combined Enrichment/Stressed [CS]). In housing conditions INS and IS, animals were single-housed in standard polycarbonate rat cages (40 cm x 20 cm x 20 cm) with no additional objects (see Figure 1a). In conditions SNS, SS, CNS, and CS, animals were housed in groups of three in large polycarbonate cages (46 cm x 36 cm x 20 cm). For the SNS and SS conditions three animals were housed per cage with no additional objects (see Figure 1b). For the CNS and CS animals, a variety of objects (durable dog and cat toys including colored textured balls, rings, and bones) were placed in the cage to provide physical and tactile stimulation (see Figure 1c). Objects were removed 2-3x / week (or sooner if damaged) and were replaced with new objects. The objects used, changing schedule, and cage dimensions were based on methods described in previous studies (Gardner et al., 1975; Varty et al., 2000; Elliott, 2004; Elliott & Grunberg, 2005). This

experimental protocol was approved by the USUHS Institutional Animal Care and Use Committee and was conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub, 82-23, rev. 1985).

Procedure

The experiment was conducted in two phases: Pre-stress (enrichment only), and Enrichment with or without Stress (also called Stress Phase). The Pre-stress phase consisted of 34 days of exposure to environmental enrichment. On day 1, animals arrived at the facility and were put into a housing condition. On days 2-3, animals were handled once a day for 5 minutes. Handling reduces the stress associated with repeated handling that is necessary to conduct behavioral measures (Meaney et al., 1998). All animals then were acclimated to the open-field chambers (Day 4) to minimize contamination of responses by any stressful effects of exposure to a novel situation (Faraday & Grunberg, 2000). Acclimation procedures do not affect later measurement of Open-Field habituation. The experimental time line was based on previous studies in this laboratory in which these behavioral measures were used (Faraday et al., 1999; Faraday & Grunberg, 2000; Elliott & Grunberg, 2003; Elliott & Grunberg, 2005). Animals remained in the assigned housing conditions for the remainder of the experiment (i.e., a total of 48 days).

The Stress phase was a 14-day Housing Condition with or without stress in which animals in the stress condition were individually immobilized in a non-painful plastic restrainer (Fisher Scientific Centrap Cage) (see Figure 4) for 20 minutes each day outside of their home cages. The animals remained in their assigned housing condition throughout both phases.

Animals were tested individually for all behavioral measures. Behavioral measures also were conducted between 0530 and 0900 hours (at the beginning of the active/dark cycle). This period of time was used to maximize behavioral performance and activity. Behavioral testing also was done individually for all rats.

Dependent Variables

Plasma Corticosterone (CORT)

Sample Collection. On Day 48, animals were taken to another laboratory and decapitated rapidly using a standard rodent guillotine (4.5 inch blade) and blood was immediately taken from the remaining trunk. The blood was placed in microcollection tubes and placed on ice for 20 minutes. The plasma was separated by centrifugation (3000 RPM for 14 minutes) and immediately placed into a - 80 °C freezer for later assay.

Plasma Corticosterone Extraction Process. Plasma corticosterone was assayed by an ImmuChem Double-Antibody radioimmunoassay (RIA) kit using ¹²⁵I-labeled corticosterone (ICN Biomedicals, Costa Mesa, CA). A limited amount of specific antibody is reacted with a fixed quantity of ¹²⁵I-labeled corticosterone. The concentration of unlabeled corticosterone in samples increased as a function of the decreasing percentages of bound radioisotope-labeled corticosterone. A second antibody precipitates antibody bound to antigen. The quantity of endogenous corticosterone was determined by measuring the radioactivity of the precipitate with known standards from the same assay in a gamma counter and converting DPM into concentrations. All samples and standards were run in duplicate. The sensitivity of the assay is 8 ng/ml (Faraday, 2000) and the coefficient of variation is 6.93%. This

measure was included to verify that restraint stress was indeed a stressor (as assessed by HPA axis activity) and to determine if there were any enrichment effects on HPA axis activity.

Elevated Plus Maze (EPM)

Elevated Plus Maze was measured on Day 35. The EPM apparatus was built following the basic plus maze design of Pellow (1985). It has four arms radiating out from a central square platform and is looks like a large plus sign (also referred to as an x shaped). It is elevated 60 cm above the floor. Two of the four arms have opaque sidewalls (50 cm in height), while the remaining two arms have no walls or ledges (see Figure 2a). These two types of arms (enclosed and non-enclosed) are placed on opposing sides of the central platform, and are generally referred to as closed and open arms, respectively. Animals were placed in the center of the maze and allowed to explore the maze for 5 minutes. EPM was conducted in a dedicated room (made with cinder blocks) where outside sound was kept to a minimum and environmental lighting was provided by a six-foot floor lamp with a 40-watt light bulb placed approximately 15-feet from the EPM and pointed away from the apparatus. Elevated plus maze activity was recorded using a video camera and a commercially available software tracking system acquired from Actimetrics Corporation, Wilmetta, Illinois.

Open Field (OF)

Open-field activity was measured on Days 4, 16, 28, 36, 40, 44, and 47. Open-field activity was measured using an Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM [16 TAO]; Omnitech Electronics, Columbus, OH) in a dedicated room (made with cinder blocks) where outside sound was kept to a minimum.

Animals were placed singly in a 40 x 40 x 30 cm clear Plexiglas arena and a Plexiglas lid with multiple 3.5 cm diameter holes was placed on top of the arena (see Figure 2b). The lid ensures that subjects have adequate ventilation but cannot escape during data collection. A photocell array measured horizontal activity using 16 pairs of infrared photocells located every 2.5 cm from side-to-side and 16 pairs of infrared photocells located front-to-back in a plane 2 cm above the arena floor. A second side-to-side array of 16 pairs of additional photocells located 10.5 cm above the arena floor measured vertical activity. Data were transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. Once subjects were placed in the test arenas, the experimenter turned off the lights and left the room. The apparatus monitored animal activity continuously for a total testing period of 1 hour.

The interfaced software generates 21 sub-variables. Center time was the variable measured because it is considered to be a behavioral assessment of stress.

Food Consumption (FC) and Body Weight (BW)

The rats were given continuous access to food (Teklad 4% Mouse/Rat Diet 7001) and food consumption and body weight was measured every other day, except during the first four days of restraint stress when it was measured daily. A total of twenty-seven food consumption and body weight measurements were obtained during phases I and II of the experiment. Food pellets were placed on the top of each cage and animals had continuous access to food. Food consumption was determined by subtracting new food weights from previous food weights (e.g., subtracting Day 16 food weights from Day 14). When food was added, the new weight was recorded and this new weight was used in the next calculation.

Body weight was measured at the same time as food consumption. Animals were removed from their cages and gently placed in a weighing pan on an electronic scale. To ensure accurate weight measurements (i.e., reduce measurement error) the electronic scale automatically obtained multiple weight readings and provided an average of these readings.

Heart Morphology

Immediately after decapitation and draining any remaining blood, the heart was removed from the chest cavity using a scalpel and immediately placed in a vial containing 10% buffered formalin phosphate for later analyses. The analysis procedure was based on Elliott et al. (2003) as recommended by R. Virmani (1999). Calipers (10mm) were used to measure the length of each heart from base to apex. Cross-sectional slices of the heart were made through the ventricles (midway between the apex and base of the heart) (see Figure 7). Hearts were weighed using an electronic analytic balance. Next, measurements were made of the left ventricle cavity width, right ventricle cavity width, anterior wall thickness, posterior wall thickness, lateral wall thickness, and septal wall thickness. Three observers measured each heart independently. The two measurements most highly correlated were used in the analyses. This measure was included to determine the effects of stress on heart dimensions.

Data Analytic Strategy

Subjects were randomly assigned to experimental conditions. Different data analytic strategies were employed depending on the dependent variable.

Plasma corticosterone and elevated plus maze data were analyzed with separate analyses of variance (ANOVA). Open-Field activity also was analyzed using separate analyses of variance to examine the effects of stress and enrichment on locomotor activity (i.e., horizontal, vertical, center time, and center time ratios) during each phase of the study (i.e., prestress and stress). In addition within-session Open-Field activity was analyzed using repeated-measures ANOVA with stress and enrichment as the between-subjects factors and time as the within-subject factor. If the initial analyses indicated significant between-subjects effects, then repeated-measures ANOVAs were performed separately for each of the Open-Field trials.

Body weight and food consumption were analyzed using repeated-measures ANOVAs to assess over time (both over the course of the whole experiment as well as during each phase). Body weight and food consumption also were analyzed at various time points using separate analyses of variances. Any significant main effects or interactions were examined using separate ANOVAs following the procedures of Keppel (1991). Heart morphology was analyzed by MANOVA. Based on previous studies (e.g., Elliott et al., 2003), heart morphology also was analyzed using separate ANOVA's for each dependent measure. If there was a significant effect, then Tukey HSD post-hoc analyses were performed.

Eta-squared values were used to determine the relative magnitude of enrichment effects for each group. Eta-squared is a measure of effect size that indicates the proportion of variance in a dependent variable explained by a given independent variable (Cohen & Cohen, 2003).

Several strategies were used to minimize the probability of Type I error. First, the experiment was designed to provide adequate power (0.80). Type I error is minimized when sample size supports adequate power (Keppel, 1991). In addition, only if overall analyses revealed a significant main effect or interaction were subsequent analyses performed. This strategy reduces the number of statistical tests performed (Keppel, 1991; Cohen & Cohen, 1983). All tests were two-tailed with significance determined by $p \leq 0.05$.

Results

Plasma Corticosterone Levels

Plasma corticosterone results revealed that the restraint stress manipulation was effective in inducing an HPA axis response. There was a significant main effect for stress, such that animals in the stress condition had significantly higher plasma corticosterone levels ($\bar{x} = 447.00 \pm 159.53$ ng/ml) than animals in the non-stress condition ($\bar{x} = 263.67 \pm 90.15$ ng/ml) ($F [1, 65] = 35.76, p < 0.001$) across all housing conditions (see Figure 4). There was no main effect for enrichment (i.e., housing condition) and no stress by enrichment interaction. One-way ANOVAs revealed significant effects for stress within each housing condition. Isolated/Stressed (IS) animals had higher corticosterone than Isolated/Not Stressed (INS) animals ($F [1, 21] = 11.37, p < 0.01$), Social Enrichment/Stressed (SS) animals had higher corticosterone than Social Enrichment/Not Stressed (SNS) animals ($F [1, 22] = 25.13, p < 0.001$), and Combined Enrichment/Stressed (CS) animals had higher corticosterone than Combined Enrichment/Not Stressed (CNS) animals ($F [1, 22] = 6.78, p < 0.01$). The greatest effects were in the social enrichment condition in which SNS animals displayed less

plasma corticosterone ($\bar{x} = 256.48 \pm 107.15$ ng/ml) than the SS animals ($\bar{x} = 499.50 \pm 129.30$ ng/ml). These findings suggest either that non-stressed socially housed animals experienced the least amount of stress when not challenged by a stressor, or that they were the most responsive to stress compared with the isolated and combined enrichment housing conditions.

Elevated Plus Maze (EPM)

The percent of time in the open arms and the number of entries into the open arms of the EPM provide indices of anxiety or stress (Santucci et al., 1994; Hogg, 1996). Specifically, decreased time spent in the open arms and fewer entries into the open arms provide indices of anxiety. Increased time spent in open arms and higher numbers of entries into the open arms indicate a decrease in anxiety. There were no significant main effects of enrichment or stress on open arms or the number of entries and there were no significant interactions. However, the INS rats were more likely to fall off than any other group (and therefore were not able to complete the trial) compared with animals that stayed on and whose data were included in the analyses ($\chi^2(5) = 17.83, p < 0.01$). Also, it is noteworthy that within the non-stress condition, animals in the combined enrichment group spent significantly more time in the open arms compared with isolated animals (112 seconds [± 77.04] vs. 89 seconds [± 16.06], respectively) and the social group spent the most time in the open arms (139 seconds [± 66.07]). These results suggest that rats in the SNS condition were the least stressed. The corticosterone data support this interpretation; that is, the group with the lowest corticosterone level was the social, non-stress condition.

Open Field

Locomotor activity was measured in the open field chambers for 60 minutes, three times during the pre-stress phase, and four times during the stress phase. Changes in center time provide an index of changes in anxiety.

Regarding center time findings, as hypothesized, stress decreased center time in all stress sessions except the first session. Figures 5a-c show the center time results for each of the conditions during the pre-stress and stress phase. Figure 5a presents the change scores between the first and last pre-stress measure of center time. A higher score is reflected of a larger change from the first pre-stress assessment of center time to the last pre-stress measure of center time. In other words, the higher scores are more indicative of less anxiety. For center time in the first pre-stress period (not shown), there was a trend of the enrichment (social and combined group) being significantly more anxious than isolated rats (i.e., enriched rats as evidenced by spending less time in the center of the chambers than isolated rats) ($F [2, 69] = 2.75, p < 0.07$). By the end of the stress phase, the social rats appeared least anxious followed by the isolated and then the combined animals ($F [2, 68] = 8.01, p < 0.001$). This finding can be seen by looking at the graph of the changes scores in Figure 5a.

During the stress phase, it does appear that stress decreases center time differentially based on housing. Figures 5b and 5c present total center time for the second and third locomotor measurements during the stress phase. Animals in the IS condition appeared to be more anxious than the SS and CS animals. In other words, enriched stressed (social and combined) animals appear to be less affected by the stress than the IS animals. One-way ANOVA's comparing isolated animals in the stress

and non-stress conditions revealed significant effect for the second session of locomotor during the stress phase ($F [1, 22] = 5.66, p = 0.03$), a trend during the third session of locomotor during the stress phase ($F [1, 22] = 3.22, p = 0.08$), and no differences for the first and final session of locomotor (these results are not shown) during the stress phase. There were no significant effects between social and combined enrichment for any of the stress sessions.

Center time also may be affected by overall moving time because there were significant differences in horizontal activity. Therefore, analyses were conducted using a ratio of total center time divided by total movement time to account for any affects that overall movement time might have on center time. For example, two animals may have the same amount of center time (e.g., 300 seconds), but the first animal had an overall movement time of 1000 seconds whereas the second animal had an overall movement time of 2000 seconds. In this case, the first animal's center time indicates less anxiety because the ratio is larger. For the pre-stress phase, there were no remarkable results. For the stress phase, there is a consistent pattern of combined enrichment reversing the effect of stress. For the isolated and social rats, center time ratios decreased for stress in all four sessions. However, for the combined group, the ratios increased for the stress condition in all four sessions suggesting that only the combined housing condition attenuated the behavioral responses to stress.

Food Consumption & Body Weight

Food consumption and body weight were measured every other day during the experiment. There were clear enrichment and stress effects on both variables.

During the prestress phase, there were clear enrichment effects on food consumption. Figures 6a and 6b illustrate a significant main effect for enrichment ($F [2, 67] = 3.91, p = 0.03$), with rats in the isolated condition eating the most ($\bar{M} = 55.59 \pm 5.75$ g) per day compared with the social group ($\bar{M} = 52.01 \pm 3.55$ g) and the combined group ($\bar{M} = 52.24 \pm 4.94$ g).

During the stress phase there were effects of stress on food consumption. An ANOVA with the last day of the experiment revealed significant differences between experimental groups. Figure 7c shows a main effect for stress ($F [1, 64] = 34.62, p < 0.001$), with the non-stress group consuming more food than the stress group. There was no main effect of enrichment and no stress by enrichment interaction.

For body weight, there were effects of enrichment and stress, but these effects differed based on the time of the measurement. See Table 1 for body weights on day 1 of the experiment.

During the pre-stress phase there were enrichment effects. Figure 7a shows the body weights of each group over the entire experiment with a line indicating when the stress period started. Figure 7b shows the body weights of each group at the end of the experiment which also was the last day of the stress period. Analyses revealed no significant differences at day 1 of enrichment, confirming that there were no initial differences in body weight. Repeated-measures ANOVA revealed a main effect for time ($F [17, 493] = 1114.96, p < 0.001$) and no time by enrichment interaction, indicating that enriched and non-enriched animals both gained weight over the course of the experiment as expected given their young age at the start of the experiment. A two-way ANOVA on the last day of the pre-stress phase revealed a trend toward a main effect

for enrichment ($F [2, 64] = 2.46, p < 0.09$). Specifically, enriched (social and combined) weighed less than isolated animals. There was no interaction or main effect for stress on body weight.

During the stress phase, there were effects of stress and enrichment on body weight, but no significant interaction. Figure 7b shows the mean body weights of each group on the last day of the stress phase (which also was the last day of the experiment). A two-way ANOVA examining body weight on the last day of the experiment revealed a significant main effect for stress ($F [1, 29] = 12.85, p < 0.001$) (see Table 2a) such that animals in the stress condition ($\bar{x} = 371.10 \pm 25.43$ grams) weighed less than non-stressed animals ($\bar{x} = 399.4 \pm 40.19$ grams), and for enrichment ($F [2, 64] = 3.94, p < 0.001$), such that animals in the isolated condition weighed more ($\bar{x} = 401.10 \pm 42.37$ grams) than animals in the combined group ($\bar{x} = 376.15 \pm 34.08$ grams), with no significant interaction.

Heart Morphology

Several measurements make up the variable of heart morphology: heart weight, length, width, circumference, left ventricle cavity size, right ventricle cavity size, septal wall, lateral wall, anterior wall and posterior wall. Two-way ANOVAs and a MANCOVA revealed no significant main effects of stress or enrichment or any significant interactions on the dependent variables of circumference, lateral wall, or anterior wall. However, there were stress effects on the other dependent variables as well as an interesting pattern of housing, suggesting that enrichment may buffer the effects of stress on the heart.

A MANCOVA was the first analysis run to determine any effects of stress and environmental enrichment on the related dependent variables of left ventricle, right ventricle, septal wall, posterior wall, lateral wall, and anterior wall. There was a significant main effect for environmental enrichment on posterior wall ($F[2, 21] = 3.84, p = 0.04$) with the social and combined groups differing from each other. There also was a significant stress x enrichment interaction for septal wall ($F[2, 21] = 7.68, p < 0.01$), such that stressed and socially enriched rats had smaller septal walls compared with all other groups.

Heart weight also was affected by both stress and enrichment. An ANCOVA with body weight at the end of the experiment as the covariate, revealed a main effect of stress ($F [1, 52] = 4.24, p = 0.04$) (see Figure 8a and Table 3a), such that hearts from the stressed animals weighed less than hearts from the non-stressed animals. One-way ANOVA's comparing stress versus no-stress within housing conditions revealed an effect for the social ($F [1, 17] = 21.65, p < 0.001$) housing condition and a trend for the isolated housing condition ($F [1, 17] = 8.28, p = 0.10$). The fact that there were not significant differences of heart weight in the combined condition may be due to low power or, alternatively, it could suggest a buffering of stress for animals in the combined condition because they were not as responsive to the stress as were the isolated and social animals.

There also were stress and housing effects on heart length (see Figure 8b). There was a significant main effect for stress ($F [1, 53] = 4.53, p = 0.04$) such that the hearts from animals in the non-stress group were longer (= 17.90 mm) than hearts from the stressed animals (= 17.60 mm) (see Table 3b). Again, there were

differences within the isolated housing condition across stress levels ($F [1, 17] = 4.31, p = 0.03$), suggesting a buffering effect for the social and combined enrichment groups. The social group is particularly striking for the heart length measure because there was only a 0.02 mm difference between the stressed and non-stressed rats' heart lengths compared with a 0.44 mm difference for isolated rats and a 0.34 mm difference for combined enriched rats.

Regarding left ventricle cavity size, there were no significant main effects, but there was a significant stress x housing interaction ($F [2, 53] = 3.64, p = 0.03$) (see Table 3c). The CS group ($\bar{x} = 4.70$ mm) had larger left ventricle cavities than did the CNS group ($\bar{x} = 3.80$ mm) ($F [1, 18] = 6.96, p < 0.01$) (See Figure 9c). There were no significant differences between stress conditions and within the isolated or social housing conditions. In the case of the left ventricle measurement, stress appears to decrease the size (Elliott et al., 2003), yet in the current experiment the social condition seems resistant to this decrease because the animals in the stressed and social condition had larger left ventricles than animals in the SNS condition.

This phenomenon of decreased ventricle size is reversed for the right ventricle, perhaps because the right and left ventricles are likely dependent on each other given their neighboring locations in the heart's anatomy. Figure 8d shows the mean widths of the right ventricle for each group. There was a significant stress x housing interaction ($F [2, 53] = 3.73, p < 0.03$). The right ventricle was affected by stress as evidenced by a significant main effect for stress ($F [1, 53] = 5.12, p = 0.03$) (see Table 3d), with the stressed rats having larger right ventricles ($\bar{x} = 1.75 \pm 0.30$ mm) than non-stressed rats ($\bar{x} = 1.46 \pm 0.56$ mm). The right ventricle also appears to be affected by housing

because there was a trend for an enrichment main effect ($F [2, 53] = 2.71, p = .076$) with hearts from the isolated and combined conditions ($\bar{x} = 1.65 \pm 0.36$ mm and $\bar{x} = 1.73 \pm 0.34$ mm, respectively) being larger than hearts from the social condition ($\bar{x} = 1.3$ mm). The right ventricle results in the SNS condition are particularly noteworthy. Rats' hearts from that condition are smaller than every other condition ($\bar{x} = 1.1 \pm 0.67$ mm) compared with all other conditions (mean's range from 1.65 – 1.77 mm [standard deviations from 0.41 -0.67 mm] for all other conditions). This finding may suggest that the SNS group is the only group resistant to stress, be it stress of an external manipulation (e.g., restraint stress) or the stress of a housing condition.

Regarding septal wall measurements, there was a significant stress x housing interaction ($F [2, 53] = 4.26, p = 0.02$) (see Table 3e) with particular differences within the social condition ($F [1, 18] = 4.39, p = .05$). The SS condition had the narrowest septal wall compared with all the other conditions ($\bar{x} = 2.95 \pm 0.27$ mm) (see Figure 8e). This is noteworthy given that stress appears to increase septal wall thickness in previous studies (Elliott et al., 2003) and in the current research. As a result, the smaller septal wall thickness in the SS group may be further support for a buffering effect in which the social condition is less responsive to an external stressor.

Social and combined enrichment appear to buffer the effects of stress on posterior wall measurements as well. There were significant main effects for stress and enrichment on the posterior wall measurement ($F [1, 53] = 5.55, p = 0.02$ and $F [2, 53] = 6.43, p < 0.01$, respectively) (see Table 3f). The stressed rats had thicker posterior walls ($\bar{x} = 4.10 \pm 0.44$ mm) than the non-stressed ($\bar{x} = 3.83 \pm 0.43$ mm) and the rats in the isolated condition ($\bar{x} = 4.00$ mm) had thicker walls than the rats in the enriched

condition ($\mu = 3.60$ mm) (See Figure 8f). There was a trend within the combined condition and across stress conditions ($F [6, 10] = 3.51, p = 0.07$). Specifically, the CS group had thinner posterior walls than the CNS group (see Figure 8f). This finding is in contrast to the within group analyses for the isolated condition in which the thicknesses for the IS and INS rats are approximately equal. As a result, a decrease in the combined enrichment and a trend for the social enrichment suggests that enrichment buffers the effects of stress on the heart.

Additional Analyses

Additional analyses were conducted to explore whether HPA axis markers (corticosterone) and body weight affect heart morphology and to what extent these factors were related to each other. The data suggest that corticosterone may be involved in the cardiac effects of stress exposure. Pearson correlations were performed to explore possible relationships between corticosterone and the other dependent variables. There was a significant positive relationship between body weight and heart weight ($r = 0.56, p < 0.001$). Furthermore, corticosterone was inversely related to food consumption ($r = -0.57, p < 0.001$), body weight ($r = -0.36, p = 0.002$), and heart weight ($r = -0.29, p = 0.03$). These findings indicate that relationships among these variables indeed exist, and that corticosterone may play a role in the effects of stress on the heart in the present study.

ASSESSMENT & DISCUSSION

Assessment of Study Hypotheses

Specific Aim #1: Restraint stress and biological measures relevant to cardiovascular disease

The restraint stress manipulation was effective in producing a corticosterone response (as assessed by increased plasma corticosterone). As a result, this study provides further support for the use of a restraint stress model in rats.

Hypothesis 1a. Restraint stress will result in increased blood markers of stress (i.e., plasma corticosterone).

This hypothesis was supported. There was a significant main effect for stress, such that the plasma corticosterone levels of animals in the stress group were higher than those levels of animals in the non-stress group.

Hypothesis 1b Restraint stress will increase behavioral measure of stress (specifically decreased time spent in the open arms of the elevated plus maze and decreased time in the center of a locomotor chamber).

This hypothesis was partially supported. There was no main effect for stress with time spent in the open arms as the dependent variable. However, it is worth noting that the no-stress social group appeared to be the least stressed based on the increased time spent in the open arms compared with all other groups. For center time, the hypothesis of rats in the stress group spending less time in the center of the locomotor activity held true, but only for the isolated rats and the last three stress phase sessions.

Hypothesis 1c Rats in the stress group will have decreased food consumption and body weight compared with rats in the non-stress condition. **This hypothesis was fully supported.**

Hypothesis 1d. Rats in the stress group will have different heart morphologies than rats in the non-stress group. Specifically, rats in the stress condition will have decreased heart lengths and left ventricle cavity widths and increased septal wall thickness as a result of exposure to restraint stress.

This hypothesis was supported. There were significant stress differences for the heart measurements of heart length, weight, width, right ventricle width, and posterior wall.

Specific Aim #2: Effects of environmental conditions on biological and behavioral measures relevant to cardiovascular disease

Hypothesis 2a. Rats in the enriched conditions will have lower plasma corticosterone levels than rats in the isolated conditions.

This hypothesis was not supported. There were no significant main effects. However, it is worth noting that the non-stress social group appeared to be the least stressed as assessed by plasma corticosterone levels.

Hypothesis 2b Rats in the enriched environments will have decreased behavioral indices of stress (specifically more time in the open arms of the EPM and more time in the center of the locomotor chamber).

This hypothesis was partially supported. The non-stress social condition animals spent the most time in the open arms, followed by the combined group. The isolated group spent significantly less time in the open arms compared with the social

group. Again, there appears to be a pattern of the no-stress social group being the least stressed. For center time, the results were opposite from the hypothesis. There were main effects of enrichment, but the social and combined groups spent significantly less time in the center than isolated animals.

Hypothesis 2c. Rats in the enriched environments will eat less and weigh less than rats raised in the isolated environments. **This hypothesis was supported.**

Hypothesis 2d. Hearts from the rats in enriched conditions will have heart dimensions that resemble the non-stressed isolated condition more than the stressed isolated animals will.

This hypothesis was partially supported. There was a trend of enrichment for right ventricle width and a significant main effect of enrichment on posterior wall measurements. There were other housing effects that differed by stress condition. These effects are addressed under Hypothesis 3d.

Specific Aim #3: Environmental conditions and attenuation of stress

Hypothesis 3a. There will be a stress x enrichment interaction, such that rats in the non-stressed and enriched conditions will have lower plasma corticosterone levels than rats in the stressed and isolated condition.

This hypothesis was not supported. Interestingly, it may actually be that enrichment increases plasma corticosterone levels, especially when a stress manipulation is present. This finding should be investigated further.

Hypothesis 3b. There will be a stress x enrichment interaction for behavioral indices of stress such that rats in the non-stressed and enrichment conditions will have

increased time in the open arms of the EPM and center time in the open field chamber than rats in the stressed and isolated condition.

This hypothesis was partially supported. However, the social group's responses differed from the isolated and combined groups. Specifically, the SNS rats spent significantly more time in the open arms than the CS rats. This is in contrast to the isolated and combined groups in which the stressed and nonstressed rats spent approximately the same amount of times in the open arms. It appears that for indices of anxiety measured by EPM, the CNS group is the least stressed, or the stress and combined group may be the most reactive to a stressor compared with the other housing groups. For center time, enriched animals were less responsive to stress than the isolated and stressed animals with the exception of the first locomotor session in the stress phase.

Hypothesis 3c. There will be a stress x enrichment interaction for food consumption and body weight such that rats in the enriched and stress conditions will have decreased food consumption and body weights compared with rats in the isolated and stress condition.

This hypothesis was not supported. Body weight and food consumption decreased with stress regardless of housing condition

Hypothesis 3d. There will be a stress x enrichment interaction for heart dimensions such that the hearts of enriched rats will be less affected by stress than the hearts from the isolated animals.

This hypothesis was partially supported. For heart weight, the hypothesis was supported but for the combined enrichment group only. For heart length, this

hypothesis was supported, but for the social enrichment group only. For the left ventricle, this hypothesis was not supported; however, the rats from the SS group most closely resembled the hearts from the INS group. For right ventricle, the hypothesis was supported for the combined group, and again, the social group is particularly noteworthy because the right ventricles of the hearts from the non-stressed social group are significantly smaller than any other group. For septal wall, the hypothesis was supported for the combined group, and again the social group has a different pattern. Specifically, stress decreased the septal wall, whereas stress increased the length or remained the same for the isolated and combined groups, respectively. For posterior wall, the hypothesis was not supported, but measurements from the stressed social and combined groups decreased compared with the measurements for the stressed isolated group which increased compared with non-stressed.

Discussion

The purpose of this study was to examine if stress, environmental enrichment, and/or the combination of the two variables alter biological and behavioral variables relevant to cardiovascular disease. It is known that stress affects biological (Kvetnansky, Weise, & Kopin, 1971; Keim & Siggs, 1976; Grunberg & Singer, 1990; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992; Martijena, Cavlo, Vosolin, & Monlina, 1997; Pham, Soderstrom, Henriksson, & Mohammad, 1997; Baum, Gatchel, & Krantz, 1997; Park, Cambell, & Diamond, 2001; Bauer, Perks, Lightman, & Shanks, 2001; Bielajew, Konkle, & Merali, 2002; Elliott, Faraday, & Grunberg, 2003) and behavioral (Acri, 1994; Park, Campbell, & Diamond, 2001; Faraday, 2002) variables relevant to cardiovascular disease in animals and humans. It also is known that

environmental enrichment affects behavioral variables such as learning and memory (Elliott & Grunberg, 2005; Daniel, Roberts, & Dohanich, 1999; Williams, Luo, Ward, Redd, & Gibson, 2001; Robbins, 1996) as well as biological variables including body weight and food consumption (Tomchesson, 2004; 2006) and nicotine responsivity (Green et al., 2003, Grunberg et al., under review) that are relevant to cardiovascular disease. However, whether environmental enrichment affects and possibly even attenuates important biological variables relevant to cardiovascular disease risk, including biochemical markers of stress (e.g., corticosterone) or heart morphology, has not been reported previous to the present investigation. Whether environmental enrichment can buffer the effects of a stress manipulation also has not been previously reported.

The present experiment examined the dependent variables of plasma corticosterone, behavioral indices of stress (EPM and center time in an open field chamber), food consumption, body weight, and heart morphology to study the effects of stress and various housing conditions. Experimental results partially supported the proposed hypotheses. There are several overall conclusions that can be reached from the current study with regard to stress effects, enrichment effects, and the buffering effect of enrichment on stress.

First, stress (as manipulated by restraint) had biological and behavioral effects. Stress increased plasma corticosterone levels, increased behavioral indices of anxiety (operationalized as decreased time spent in the center of the open-field chamber), and decreased body weight and food consumption. Restraint stress also altered heart morphology, including right ventricle width, posterior wall width, hearth length, and heart

weight. The implications of this finding could affect heart disease events and mortality because changes in heart morphology have been associated with changes in heart function (Levy, Garrison, Savage, Kannel, & Castelli, 1990). These stress effects were consistent with findings from previous studies (Martijena, Cavlo, Vosolin, & Monlina, 1997; Faraday, 2002; Elliott, Faraday, & Grunberg, 2003; Tomchesson, 2004), thereby lending additional support to the value of restraint stress as a stress manipulation for rats.

Second, environmental enrichment also has behavioral and biological effects. Enriched housing conditions decreased food consumptions and body weight, consistent with previous reports (Tomchesson, 2004; 2006). There also were effects of environmental enrichment on the heart, especially for the right ventricle and posterior wall measurements. These heart effects are interesting as this is one of the only experiments, to date, that shows that something as simple as changes in the housing environment can affect the heart *per se*. In contrast, the results for housing and plasma corticosterone were not as hypothesized because enrichment did not decrease corticosterone levels. In fact, corticosterone increased some in the social condition, consistent with recent reports that corticosterone levels rise in response to enriched environments, (Moncek, Dunko, Johanson, & Jezova, 2004; Marashi, Barnekow, Ossendorf, Sachser, 2003) and suggesting that environmental enrichment does not biochemically buffer stress. The effects of environmental enrichment illustrate the powerful effect on behavior and biology of changing something as simple as housing environment.

Third, there were stress buffering effects of housing on the heart. Interestingly, the social housing condition had the most marked effect to attenuate (or buffer) the effects of stress on the heart. This buffering effect, however, was not evident in the behavioral variables to the extent hypothesized. Environmental enrichment did not buffer the effects of stress on EPM, center time, or food consumption. Perhaps, the behavioral measures used in the present study were not sensitive enough to reflect buffering effects. Yet, the biological buffering effects that did occur were on the heart but not in the biochemical markers of stress. Therefore, the results are not consistent with a broad social buffering explanation. Instead, the social variables manipulated in the present experiment had little effect on behaviors, seemed stressful with regard to biochemical arousal, but still attenuated stress effects on the heart.

The buffering hypothesis states that social support “buffers” (i.e., protects) individuals from the potentially pathogenic influence of stressful events and that this happens through one of two ways: (1) support may intervene between the stressful event, thereby attenuating or preventing a stress appraisal response (Cohen & McKay, 1984; House 1981), or (2) adequate support may alleviate the impact of stress appraisal by providing a solution to the problem, by reducing the perceived importance, or by tranquilizing the neuroendocrine system so people are less reactive to stress (House, 1981). The current study does not support either mechanism. The first mechanism suggests a change in appraisal of the situation and, while this may be a factor for humans, it is likely not a factor in an animal experiment given the limited higher-order cognitive functioning of animals. The second mechanism suggests a tranquilization of

the neuroendocrine system, which the plasma corticosterone results do not support and, if anything, contradict.

The findings from the current study indicate that further research is needed. First, it would be valuable to replicate the current findings and to conduct a similar experiment examining stress and enrichment on heart disease risk factors in female rats. Heart disease is the leading cause of death of American women (National Heart, Lung and Blood Institute, 2002; American Heart Association, 2002) as well as men. Therefore, identification of variables (e.g., enrichment) that might be worth manipulating to prevent heart disease in females is worthwhile. Estrogen has been reported to be protective against heart disease (Sullivan, Vander Zwaag, Hughes, Maddock, Kroetz, & Ramanathan, 1990; Wong et al., 2000), but recent clinical trials have reported little benefit and maybe even some risk of hormone replacement therapy (AHA, 2005). Therefore, different results may be found in female rats than male rats as a result of estrogen. In addition, sex differences in stress responses have been reported (Frankenhaeuser, von Wright, Collins, von Wright, Sedvall, Swahn., 1978; Taylor, Klein, Lewis, Gruenewald, Gurung, Updegraff, 2000; Palanza, 2001) and sex differences in coping responses have been reported to stress (Cohen & Wills, 1985). There may be sex differences in responses to the same stressors that may alter effects of enrichment on the heart in males versus females as a result of different behavioral responses to stress (Cohen & Wills, 1985). Future studies need to include females as well as males.

In addition to studying female rats, future studies should focus on heart function as well as more detailed analyses of heart morphology. Studies of function *per se* would add to the knowledge about the impact of environment and stress on

cardiovascular function relevant to health, including blood pressure, ejection fraction, and cardiac output.

If the current preclinical findings are, indeed, confirmed, then human research should follow. Human research could involve laboratory experiments or epidemiological research using data sets. A recent study in humans found that social isolation (assessed as being single or widowed) was associated with elevated risk for the presence of coronary calcification, even after adjusting for age and other coronary risk factors (Kop, Berman, Gransar, Wong, Miranda-Peats, White, et al., 2005). There also may be differential effects in populations known to have healthy hearts, such as athletes. Perhaps athletes on a team (such as soccer or football players) would have better heart health than athletes who compete individually (such as tennis players or swimmers). The answers to these questions are worth investigating to determine the extent to which environmental conditions, especially social interaction, affect heart health.

Mechanisms of action of social interaction and support also merit further investigation. In light of the present findings, social interaction does not decrease biochemicals associated with stress responses, and may even increase these responses. Yet, social interaction seems to buffer the effects of stress on the heart. Perhaps there is a Yerkes-Dodson effect in that social support is most effective because there is a moderate amount of arousal, but too much arousal (e.g., in the combined enrichment condition) may be detrimental. Alternatively, social interaction may act as a positive stressor (“eustress”) and effects of eustress may differ from other negative

stressors (“distress”) despite Selye’s notion that eustress and distress have similar biological effects (Selye, 1973).

More information about the degree and mechanisms of how social support affects health outcomes will help researchers and clinicians understand why low social support is highly associated with heart disease in human populations (Lett, Blumenthal, Babyak, Stauman, Robins, & Sherwood, 2005) and how best to help individuals with low social support. If indeed social support is helpful, not because it decreases biological stress hormones, but because it helps an individual perceive a situation as less stressful, then patients who have low social support can be taught cognitive techniques to help decrease their appraisal of certain situations. If arousal is the mechanism underlying social support’s effect, then patients should be encouraged to reach for an attainable goal, meet new people, or engage in other activities that increase arousal to a healthy extent. These interventions could have a profound impact in preventing cardiovascular disease.

Limitations

There are some limitations of the present experiment. First, the heart measures included in the experiment were simple and were made with 10 mm calipers. Electronic digital calipers would have decreased the probability of measurement error. Moreover, histological and molecular analyses of the heart may have revealed more subtle effects of the housing condition. These additional histological and molecular analyses of the heart in future studies (especially animal studies) would be valuable given that no strong and consistent pattern was obvious in the present heart findings. In other words, the current heart data show that the stressed social rats’ hearts are different from the rats’

hearts from other conditions, but there is no pattern for the heart measurements being consistently smaller or larger. For example, it would be expected that rats with heavier hearts would have bigger septal walls, but such a relationship is not obvious from the present data. Additional studies are needed to determine morphological, histological, and molecular responses to environmental enrichment and various stress paradigms.

The second major limitation was that all of the subjects were male rats. Future studies should include males and females, rats and humans. Research with females would provide additional knowledge about possible different stress and social responses between males and females as well as the potential role of estrogen in moderating the effects of stress or the environmental on heart disease risk.

The third major limitation of the study is that the pre-shipment stress and housing conditions of the rats were not under the experimenter's control and details are not provided by the dealer. Usually rats are housed with their mothers until post-natal day 21. The rats are then shipped in cartons of 12 rats per carton. Upon arrival for the experiment, the rats were then randomly assigned to a housing condition. However, it is unknown what the housing conditions of the rats were prenatally. In other words, were the rats' mothers housed socially or in an isolated condition and whether or not that affected the pups. Also, all rats experienced some stress during shipment to laboratory.

The fourth limitation is that the assessment of the rats was measured and analyzed separately, however rats in groups may tend to have similar data given the similar environment they share. This is often the case in twin or sibling studies. As a

result, analyses that capture the similarity of groups (e.g., hierarchical linear models) in responding may produce different results.

CONCLUSIONS

The purpose of this study was to determine if rearing rats in stressed or non-stressed and enriched or non-enriched environments altered biological and behavioral factors relevant to cardiovascular disease risk. It was hypothesized that environmental enrichment would attenuate the behavioral and biological effects of stress. The results indicate that stress does affect corticosterone, center time, body weight, food consumption, and heart morphology and that environmental enrichment affects open center time, body weight, food consumption, and heart morphology. Three conclusions can be drawn from the present experiment: (1) restraint stress is a valid model of stress manipulation in an animal model, (2) environmental enrichment has marked effects on body weight, food consumption, and simple learning (as measured by habituation in the open field chamber), and (3) environmental enrichment buffers the effects of stress on the heart, but this buffering is not the result of environmental enrichment decreasing plasma corticosterone as originally hypothesized. In particular, social enrichment had the most marked buffering effect.

The fact that environmental enrichment interacted with stress to affect plasma corticosterone levels, activity, food consumption, body weight, and heart morphology are interesting findings with potential clinical relevance. It is known that stress, lack of activity, increased food consumption, and increased body weight pose health hazards to the heart. It is known that environmental enrichment improves learning and alters the brain. Now it appears that environmental enrichment alters behavioral and biological

variables that are relevant to heart health. These findings are consistent with reports that social support is good for heart health. The present findings suggest that rat models are meaningful to study the effects of environmental enrichment on behavioral and biological variables relevant to heart health. Further, the present findings set a solid foundation for future studies of environmental enrichment and heart health.

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APPENDIX A: TABLES

Table 1. Demographics: Body weight on Day 1 of Experiment

	No Stress			Stress		
	Isolated	Social	Combined	Isolated	Social	Combined
Body Weight (g)	46.72 ± 5.83	44.88 ± 6.02	46.45 ± 4.88	47.67 ± 4.96	45.07 ± 6.75	46.47 ± 5.98

Table 2. Body Weight: Last Measurement

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Stress	13910.812	1	13910.812	12.851	.001	.167	.942
Enrichment	8520.667	2	4260.334	3.936	.024	.110	.688
stress * enrichment	102.934	2	51.467	.048	.954	.001	.057
Error	69276.532	64	1082.446				
Total	10475985.213	70					

R Squared = .254 (Adjusted R Squared = .195)

Table 3a. Heart Weight

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Body weight as covariate	.146	1	.146	14.334	.000	.216	.960
Stress	.043	1	.043	4.238	.045	.075	.524
Enrich	.006	2	.003	.287	.752	.011	.093
stress * enrich	.018	2	.009	.885	.419	.033	.194
Error	.530	52	.010				
Total	102.967	59					

R Squared = .413 (Adjusted R Squared = .345)

Table 3b. Heart Length

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Enrich	1.883	2	.942	2.063	.137	.072	.406
Stress	2.067	1	2.067	4.528	.038	.079	.551
enrich * stress	1.453	2	.727	1.592	.213	.057	.322
Error	24.190	53	.456				
Total	18653.089	59					

R Squared = .183 (Adjusted R Squared = .106)

Table 3c. Left Ventricle Cavity Width

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Enrich	2.031	2	1.015	1.954	.152	.069	.387
Stress	.096	1	.096	.186	.668	.003	.071
enrich * stress	3.786	2	1.893	3.642	.033	.121	.647
Error	27.544	53	.520				
Total	979.850	59					

R Squared = .177 (Adjusted R Squared = .099)

Table 3d. Right Ventricle Cavity Width

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Enrich	.955	2	.478	2.709	.076	.093	.514
Stress	.915	1	.915	5.189	.027	.089	.609
enrich * stress	1.316	2	.658	3.731	.030	.123	.659
Error	9.346	53	.176				
Total	165.205	59					

R Squared = .256 (Adjusted R Squared = .186)

Table 3e. Septal Wall Length

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Stress	.090	1	.090	.744	.392	.014	.135
Enrich	.176	2	.088	.730	.487	.027	.167
stress * enrich	1.026	2	.513	4.264	.019	.139	.721
Error	6.377	53	.120				
Total	584.625	59					

R Squared = .169 (Adjusted R Squared = .090)

Table 3f. Posterior Wall Length

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Stress	.906	1	.906	5.549	.022	.095	.638
Enrich	2.097	2	1.049	6.427	.003	.195	.887
stress * enrich	.073	2	.036	.222	.801	.008	.083
Error	8.648	53	.163				
Total	937.843	59					

R Squared = .264 (Adjusted R Squared = .195)

Table 4. F Values for Biological Measures

VARIABLE	STRESS VS. NONSTRESS CONDITIONS		
	Isolated	Social	Combined
Corticosterone	11.37 (< 0.01)*	25.13 (< 0.01)*	6.78 (< 0.01)*
Body weight	2.94 (0.10)	9.20 (< 0.01)*	3.69 (0.06)
Heart weight	8.28 (0.01)*	21.65 (<0.01)*	1.04 (0.32)
Heart length	4.31 (0.05)*	-----	1.81 (0.19)
Left ventricle	-----	-----	6.96 (0.02)*
Right ventricle	-----	8.30 (0.01)*	-----
Septal Wall	-----	4.39 (0.05)*	-----
Posterior Wall	-----	-----	8.65 (< 0.01)*

* = Significant; p < 0.05 (p value in parentheses)

APPENDIX B: FIGURES

Figure 1a. Isolated Housing Condition**Figure 1b. Social Housing Condition****Figure 1c. Combined Housing Condition**

Figure 2a. Animal Restrainer

Figure 2b. Elevated Plus Maze



Figure 2c. Open Field Arena

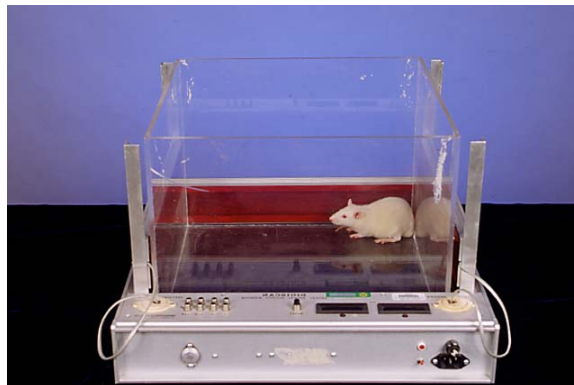


Figure 3. Rat Heart



Figure 4. Plasma Corticosterone Concentration

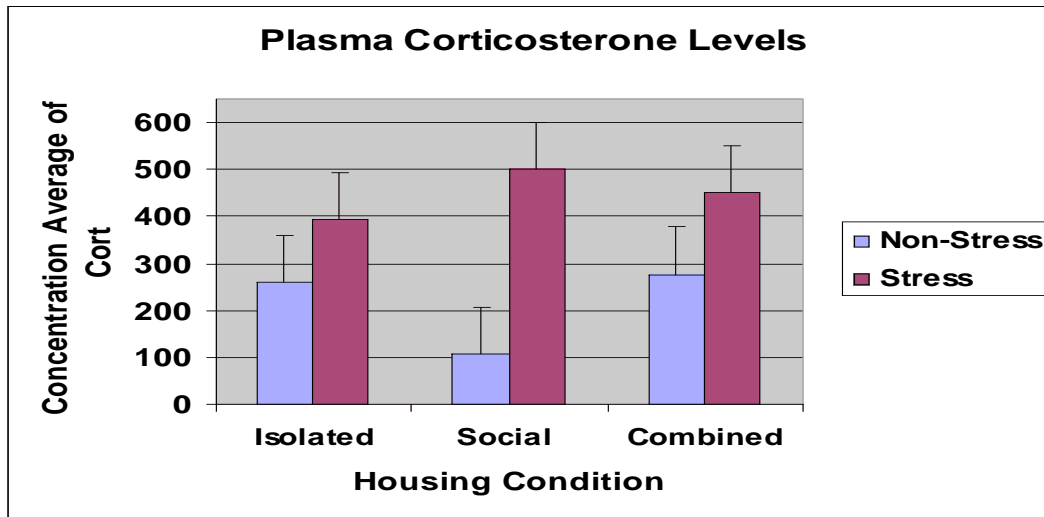


Figure 5a. Total Center Time: Change Scores During Pre-Stress Phase

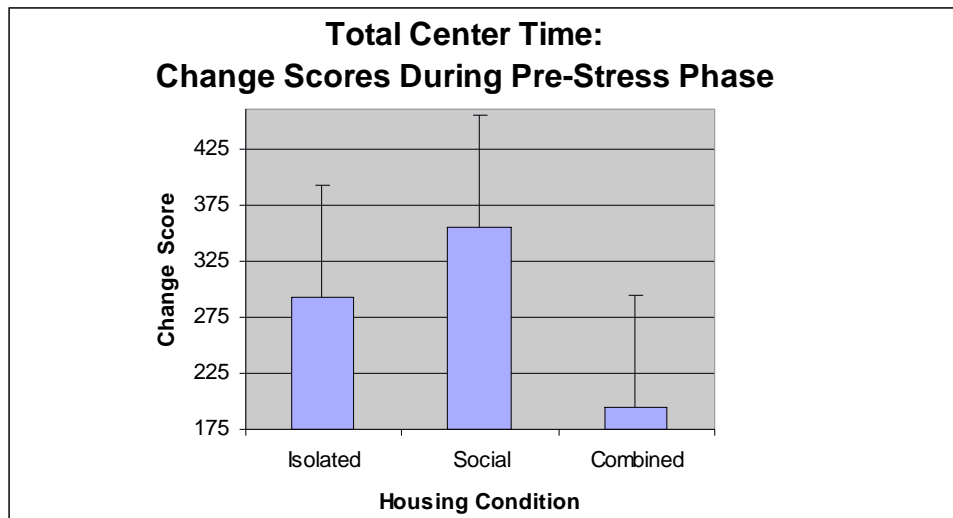


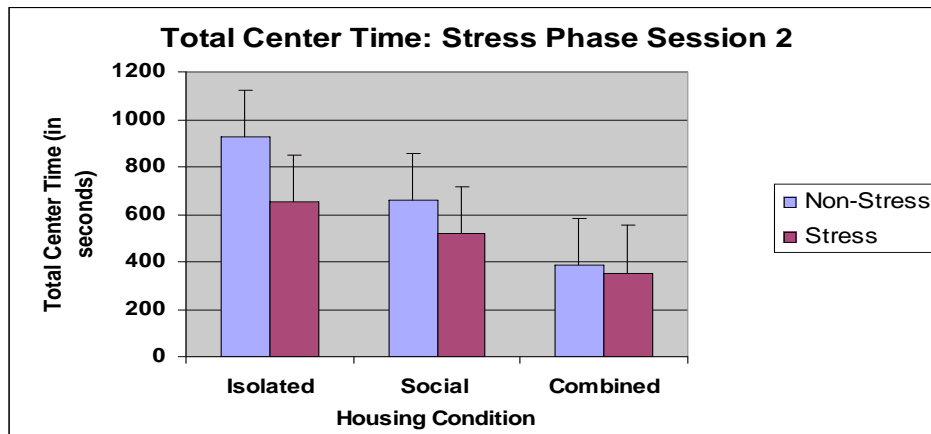
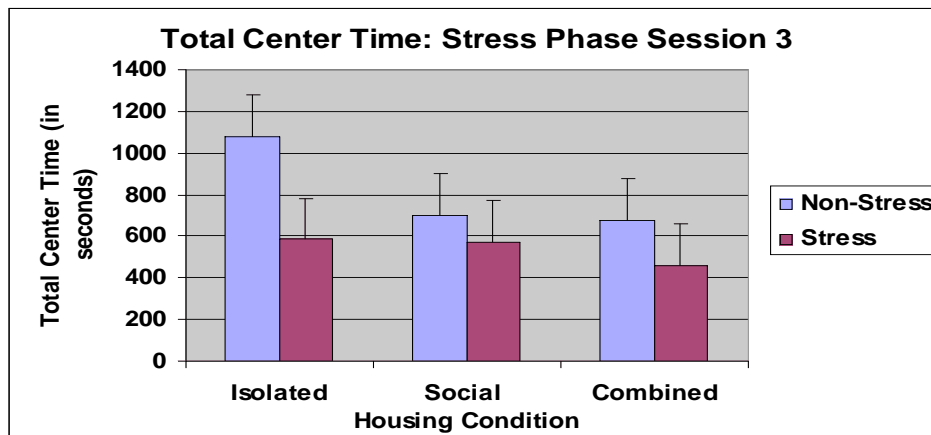
Figure 5b. Total Center Time: Stress Phase Session 2**Figure 5c. Total Center Time: Stress Phase Session 3**

Figure 6a. All Food Consumption Measurements

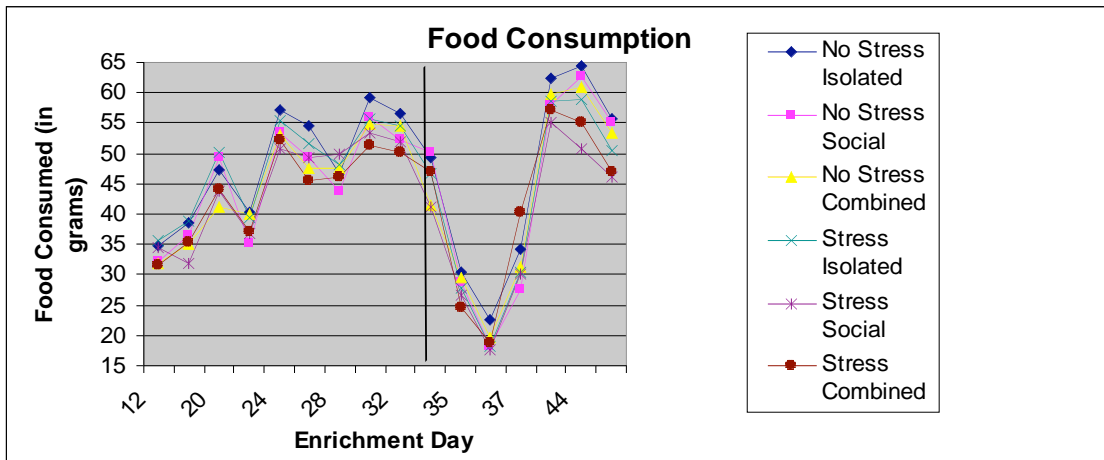


Figure 6b. Food Consumption: Last Day of the Stress Phase

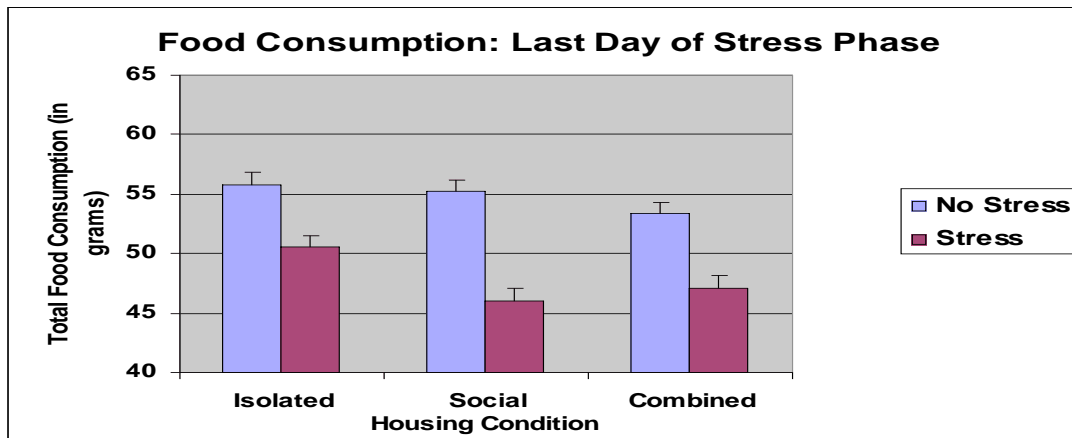


Figure 7a. All Body Weight Measurements

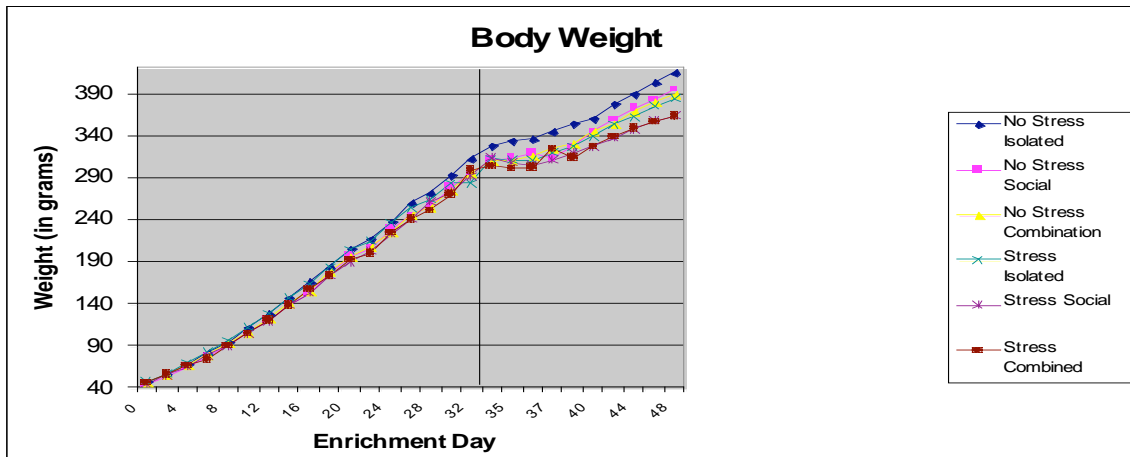


Figure 7b. Body Weight: Last Day of Stress Phase

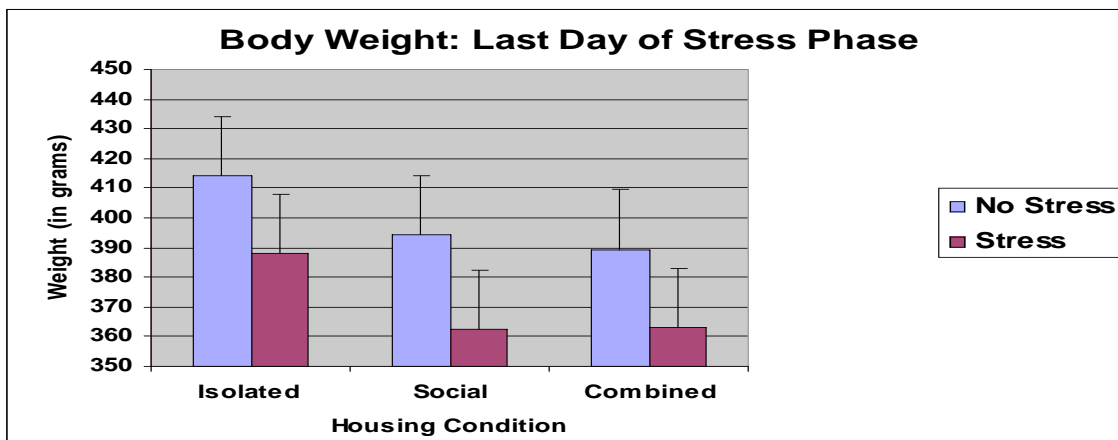


Figure 8a. Heart Weight

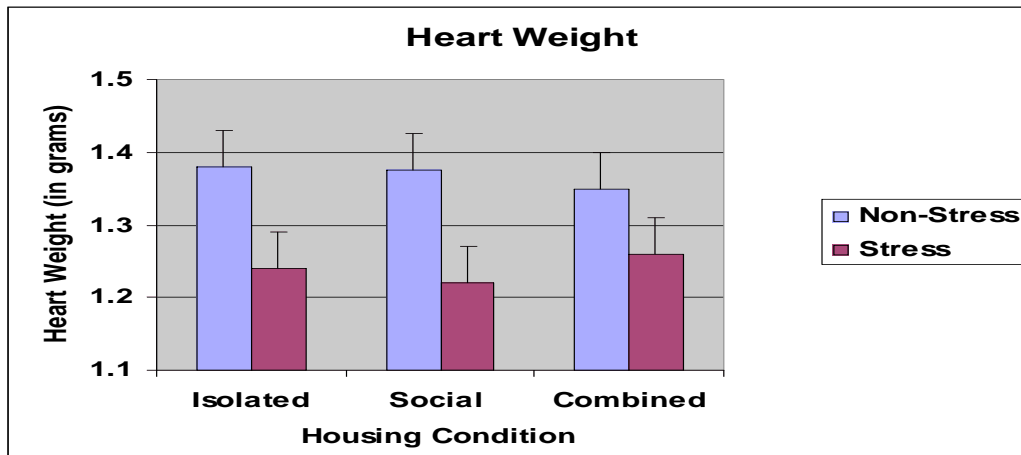


Figure 8b. Heart Length

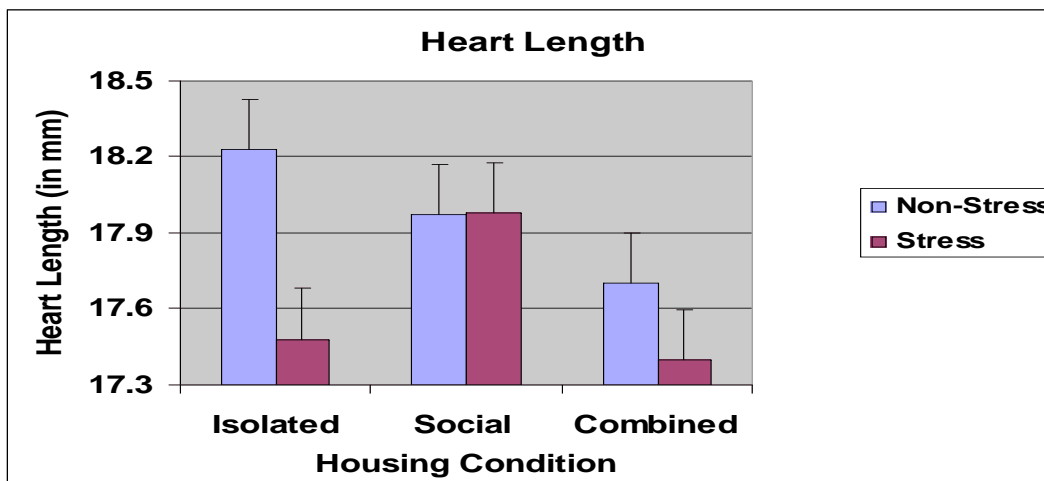


Figure 8c. Left Ventricle Cavity Size

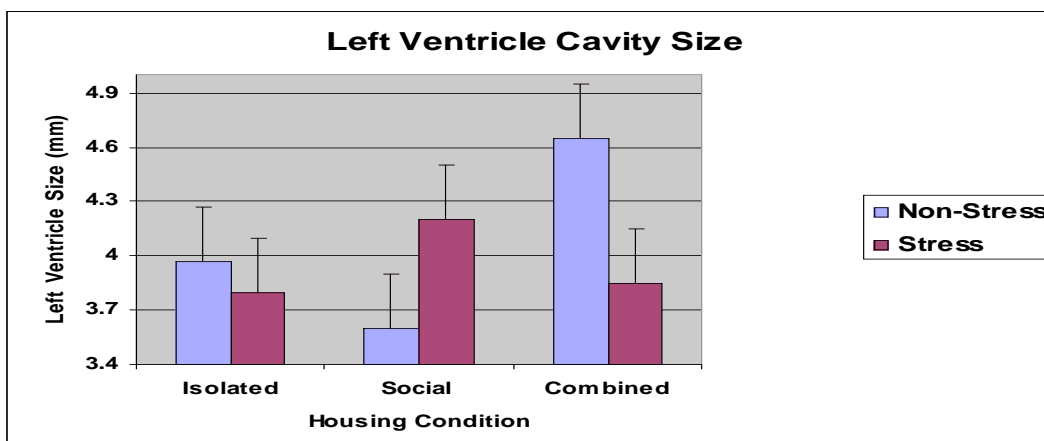


Figure 8d. Right Ventricle Cavity Size

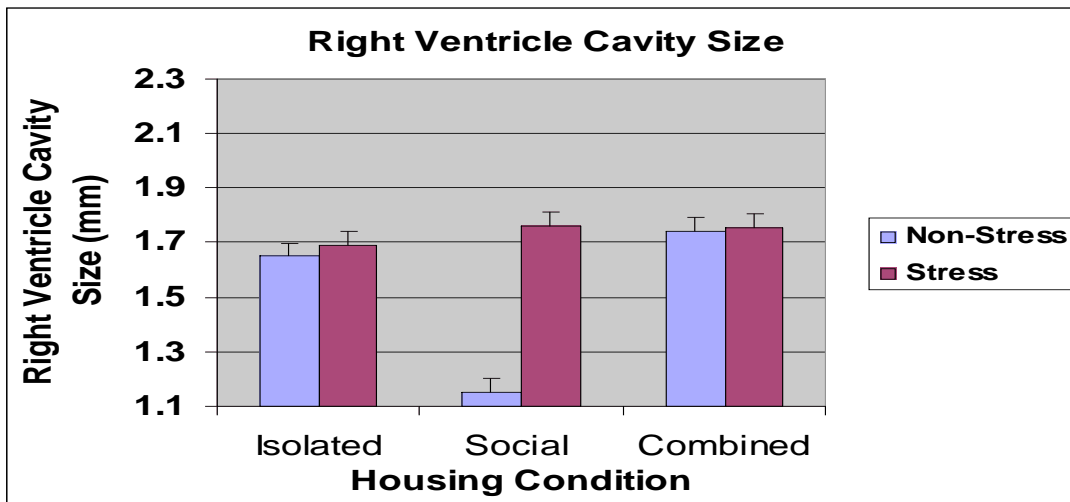


Figure 8e. Septal Wall Measurements

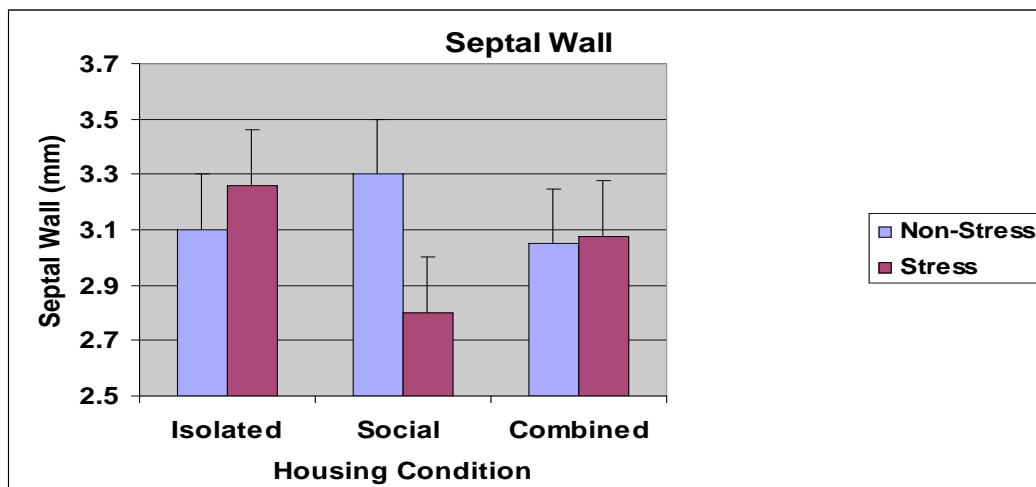


Figure 8f. Posterior Wall Measurements

