THE EFFECTS OF PRENATAL ETHANOL EXPOSURE AND STRESS IN ADULTHOOD ON THE ENDOCRINE AND IMMUNE SYSTEMS IN RATS.

by

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ABSTRACT

The two studies in this thesis were undertaken to examine the possible interactive effects of prenatal ethanol exposure and exposure to chronic stress in adulthood on hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadal (HPG) and immune measures. Male and female offspring from prenatal ethanol-exposed (E), pair-fed (PF) and ad libitum-fed control (C) conditions were exposed to varying lengths of a chronic intermittent stress regimen in adulthood. Animals were exposed daily to 2 of 6 different stressors, one each at random times in the morning and afternoon, with the same pair of stressors being repeated every 4 days. In the first study, following either 6 d or 18 d of chronic stress, CORT and ACTH levels, body weights, adrenal weights and gonadal weights as well as thymus and spleen cell counts were measured. In the second study, following 21 d of chronic stress, body weights and adrenal weights as well as splenic lymphocyte proliferative responses to three different mitogens (Con A, PWM, LPS) were assessed. The data demonstrate that although E, PF and C animals did not significantly differ in pituitary-adrenal activity, body and organ weights, or immune cell counts, there were effects of prenatal ethanol exposure of lymphocyte proliferative responses to Con A and PWM in males and to PWM in females. In addition, there were nutritionally-mediated effects of ethanol and effects of pair-feeding on lymphocyte proliferative responses in females

The chronic stress regimen used in these studies produced effects on CORT and ACTH levels, body and organ weights as well as immune cell counts in males. For females, chronic stress affected immune cell counts in the first study and lymphocyte proliferative responses to mitogens in the second study.

Together, these findings indicate that, although exposure to chronic intermittent stress in adulthood may have marked effects on the endocrine and immune systems in adulthood, specific effects on the immune system of E animals may only become apparent when these animals are exposed to chronic stress. In addition, male and female offspring are differentially affected by prenatal ethanol exposure and exposure to chronic stress in adulthood.

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INTRODUCTION

Fetal Alcohol Syndrome.

More than two decades ago, the independent identification of fetal alcohol syndrome (FAS) by Lemoine, Harousseau and Borteyru (1968) in France, and Jones, Smith, Ulleland and Streissguth (1973) in Seattle underscored the teratogenic effects of ethanol consumption during pregnancy. Although ethanol readily crosses the placenta, controversy remains concerning what level of ethanol use may alter development in the fetus. FAS has been most frequently reported in the offspring of women who consumed high doses of ethanol during pregnancy but the full syndrome does not consistently occur in all infants born to women with chronic alcoholism. FAS is diagnosed by the identification of impairments in three categories: (1) intrauterine and/or postnatal growth retardation, (2) characteristic craniofacial abnormalities and (3) central nervous system (CNS) dysfunctions such as intellectual impairment and developmental delays (Clarren & Smith, 1978).

Intrauterine growth retardation appears to be directly proportional to the degree of maternal ethanol intake (Streissguth, Landsman-Dwyer, Martin & Smith, 1980) and children with FAS are usually below the third percentile in weight, height and head circumference (Streissguth, Clarren & Jones, 1985). Craniofacial dysmorphology used in the diagnosis of FAS includes such characteristics as midface hypoplasia, thin upper lip, epicanthal folds, low set ears, short palpebral fissures and a long flat philtrum. Brain anomalies such as the failure or interruption of neuronal and glial migration and cerebral nuclear dysgenesis are common (Clarren, Alvord, Sumi, Streissguth & Smith, 1978). In addition, prenatal ethanol exposure may result in malformations in almost every system of the body. Cardiac, genital and renal

malformations all occur with high incidence in children with FAS (Steeg & Woolf, 1979; Qazi, Masakawa, Milman, McGann, Chua & Haller, 1981).

Prenatal ethanol exposure is also associated with numerous behavioral and cognitive deficits. These include mental retardation, motor abnormalities, perceptual deficits, hyperactivity, poor attention span, and impaired habituation (Streissguth, 1986). Children with FAS display higher rates of speech and language problems (Greene, Ernhart, & Martier, 1990), auditory disorders (Church & Gerkin, 1988), visual disorders (Stromland, 1990) and visual perceptual problems (Aronson, Kyllerman, Sabel, Sandin & Olegard, 1985). In addition, many of the behavioral problems seen in children with FAS persist through adolescence and into adulthood, the most common being attention deficits, poor social adaptation and problems with comprehension and abstraction (Streissguth, Aase, & Clarren, 1991). Children with FAS also reportedly show marked decrements in IQ when assessed as adolescents and adults. Furthermore, the persistence of these deficits into adulthood may hinder the ability of these individuals to establish independence in either housing or income (Streissguth, Aase, & Clarren, 1991).

It appears that the harmful effects of ethanol exposure *in utero* on the development of the CNS and other organ systems may vary according to several factors. Ethanol exposure in the first trimester is more often associated with organ and musculoskeletal anomalies while exposure in the second and third trimesters is linked to growth, intellectual and behavioral deficits (Aronson & Olegard, 1987). In addition, both animal and human studies have demonstrated that binge drinking at high levels may result in more devastating effects on the developing fetus than intake of the same dose of ethanol over a longer period of time (Pierce & West, 1986).

Since ethanol easily crosses the placenta, fetus and mother are exposed to nearly comparable blood alcohol concentrations (BACs) (Brien, Loomis, Tranmer & McGath, 1983). However, the fetus is unable to metabolize the ethanol because it lacks the necessary enzymes and is dependent upon maternal elimination of ethanol following its passive diffusion across the placenta into the maternal circulation (Guerri & Sanchis, 1985). In addition, the rate of ethanol elimination from amniotic fluid is approximately two times slower than that from maternal blood, resulting in high ethanol concentrations in the amniotic fluid even when ethanol in the blood has been eliminated (Brien, Loomis, Tranmer & McGath, 1983). Thus, the period of development during which ethanol is present, the length of time ethanol exposure occurs, and the peak BACs may all influence the nature and extent of dysfunction observed in children with FAS.

It has been estimated that the incidence of FAS worldwide ranges from 0.43 to 3.1 cases for every 1000 live births and FAS is recognized as one of the major causes of mental deficiency originating before birth, ranking third behind Down's syndrome and neural tube defects (Abel, 1984). FAS represents an extreme end of mental and physical deficits resulting from prenatal exposure to high doses of ethanol. The terms fetal alcohol effects (FAE) and alcohol-related birth defects (ARBD) are both used to describe situations in which children meet one or more of the three diagnostic criteria for FAS and ethanol is considered the most likely teratogen. Children with ARBD may exhibit more subtle forms of dysfunction than children with FAS, but their symptoms can be just as disabling (Sokol & Clarren, 1989).

Animal Models of FAS.

Many of the physical and behavioral features reported in children with FAS have been successfully reproduced in rodent models. Retarded prenatal and postnatal growth and development, malformations and skeletal anomalies, and increased fetal deaths and/or resorptions have all been reported in rodents prenatally exposed to ethanol (Abel & Dintcheff, 1978; Chernoff, 1977; Leichter & Lee, 1980; Randall, Taylor & Walker, 1977). Rodents exposed to ethanol during the time equivalent to the period of most rapid brain development in humans exhibit significant cognitive and behavioral abnormalities, and prenatal ethanol exposure has been shown to affect both the timing and rate of neurogenesis (Miller, 1986) and neuronal migration (Clarren, Alvord, Sumi, Streissguth, & Smith, 1978). Rodents exposed to ethanol *in utero* display reductions in neuronal cell numbers and decreases in the mean size of the neuronal cell bodies in most cortical layers (Barnes & Walker, 1981). Ethanol has also been demonstrated to impair astrocyte growth and differentiation even at low doses, possibly hindering the production of trophic factors by astrocytes which are essential for normal growth and differentiation of neurons (Lokhorst & Druse, 1991).

These aberrations in glial and neuronal formation, differentiation and migration may result in the delayed development of functional neuronal connections which could be responsible for behavioral abnormalities documented in offspring prenatally exposed to ethanol (Pratt, 1984). This possibility may explain why many of the behavioral changes documented in rodents exposed to ethanol *in utero* mirror dysfunctions reported in children with FAS. In rodents, behavioral hyperactivity and hyperresponsiveness such as increased exploratory behavior (Riley, Shapiro, & Lochry, 1979) and increased startle reactivity (Anandam, Felegi, & Stern, 1980) have been reported. In addition, ethanol exposure *in utero*

has been shown to produce deficits in passive avoidance learning (Riley, Lochry & Shapiro, 1979), taste aversion (Riley, Baron, & Hannigan, 1986) and reversal learning (Lochry & Riley, 1980), reflecting possible impairments in response inhibition. Lastly, rodents prenatally exposed to ethanol have demonstrated an inability to use environmental cues (Abel, 1979; Weinberg, 1992a). Consequently, it appears that rodents exposed to ethanol *in utero* may provide an valuable model for further investigations into the causes of the behavioral and cognitive deficits documented in children with FAS.

Animal models of prenatal ethanol exposure also provide an opportunity to isolate ethanol effects from environmental effects such as pattern and level of exposure, exposure to other substances of abuse and, most notably, nutritional status. Primary malnutrition is a significant consideration in studies involving ethanol use. When consuming ethanol, humans will lose weight even if caloric intake is sufficient to maintain body weight (Pirola & Lieber, 1972), and ethanol administration decreases food consumption in animals. The inclusion of a pairfed group (animals whose caloric intake is matched to ethanol consuming animals) in animal models of prenatal ethanol exposure makes it possible to isolate the teratogenic effect of ethanol from the effects of primary malnutrition. However, ethanol can also cause secondary malnutrition by impairing the ability of an organism to digest or absorb nutrients from the gastrointestinal tract or utilize those nutrients once they have been absorbed (Lieber, 1988; Weinberg, 1984). In addition, ethanol can produce vascular changes in the placenta which may hinder the transport of nutrients and oxygen from mother to fetus (Gordon, Durandin, Rosso & Winick, 1982). Although animal models provide a larger degree of control over many variables, they are not perfectly able to isolate the teratogenic effects of ethanol.

The Hypothalamic-Pituitary-Adrenal (HPA) Axis.

The hypothalamic-pituitary axis controls the function of most of the endocrine glands in the body including the adrenals, gonads, and thyroid. Regulation of the HPA axis occurs at several levels. In the CNS, environmental stimuli act on the paraventricular nuclei (PVN) of the hypothalamus where the cell bodies of neurons that synthesize and secrete corticotropin-releasing factor (CRF) are located. In addition to responding to a wide variety of stimuli, CRF release exhibits circadian rhythms, pulsatile bursts, and feedback control. CRF stimulates the release of proopiomelanocortin (POMC)-derived peptides from the corticotrophs of the anterior pituitary. Adrenocorticotropic hormone (ACTH) is a peptide hormone coreleased with β-endorphin (β-EP) and melanocyte-stimulating hormone (MSH) by the processing of POMC. ACTH shows both a circadian rhythm and cyclic bursts regulated by the release of CRF from the hypothalamus. ACTH is the primary regulator of glucocorticoid secretion (cortisol in humans and corticosterone in animals) from the adrenal cortex and the amount of ACTH release can be altered by negative feedback control at the level of the hypothalamus (ACTH and glucocorticoids) and pituitary (glucocorticoids).

Glucocorticoids are catabolic hormones that circulate free or bound and act in direct opposition to the major anabolic hormone, insulin, at target sites. At physiological concentrations, glucocorticoids promote protein mobilization and fat metabolism, facilitate vascular responsiveness and upregulate enzyme activity in metabolic pathways in the CNS and peripheral tissues. With adequate supplies of glucose, glucocorticoids promote triglyceride uptake into abdominal and facial adipose tissue while decreasing the sensitivity of target tissue to insulin. During fasting, glucocorticoids are essential because they increase protein mobilization while inhibiting its synthesis in muscle, thus sparing stores of glycogen

which are necessary to supply obligate glycogen users such as the CNS. In humans, symptoms of excessive amounts of cortisol include obesity caused by deposition of facial and abdominal fat, increased protein catabolism, loss of skeletal muscle mass, and increased susceptibility to infection (Berne & Levy, 1993) .

The HPA Axis and Stress.

Stress has historically been used to describe any disturbance of homeostatis by an event or stimulus to which an organism has been exposed . In order to survive, an organism must adjust to the physical and psychological stressors present in the external environment. It accomplishes this through the actions of several systems, including the HPA axis. A variety of stimuli can activate the HPA axis and an organism may respond to the stimuli differently depending on the influence of several factors including control, predictability, and feedback which may either attenuate or amplify the pituitary-adrenal response to stress.

Control or the ability to make active coping responses in order to avoid or escape from aversive stimuli appears to be both psychologically and physiologically important to the organism. When given a choice, animals prefer control over environmental events to its absence and the loss of control over noxious stimuli may be more stressful than never having control at all (Weinberg & Levine, 1980). The effects of stressor predictability on an organism may be dependent upon the presence or absence of other factors. In studies using shock as an aversive stimuli, it appears that when animals have control over the shock, signaled presentations of the stressor may be less aversive. However, when animals cannot make an active response to avoid or escape from the shock, signals of stressor exposure may be more aversive (Bassett, Cairncross & King, 1973).

Feedback is information which the organism receives following an aversive stimulus or after a response has been made to the stimulus indicating that the animal is safe for a given length of time. Lack of feedback can increase stress responses such as pituitary-adrenal activity and ulceration while increased feedback may reduce these stress responses. In addition, feedback may influence how the organism responds to the presence of predictability or control. For example, predictability may be less aversive if the organism either has control over the stimulus or has information about safety from the stimulus (Weinberg & Levine, 1980). Consequently, it appears that control, predictability and feedback are potent variables that may alter psychological and physiological responses to stress.

The duration of the exposure to the aversive stimulus may also influence the activation of the HPA axis. When compared to nonstressed animals, the exposure of adult rats to aversive stimuli results in a marked increase in plasma ACTH which may decrease during continuous stimulation (Rivier & Vale, 1987). A return toward pre-stress levels of circulating ACTH and corticosteroids may be attributed either to such physiological mechanisms as the inhibition of HPA activity by negative feedback or to such psychological alterations as a reduction in arousal levels to repeated presentations of the aversive stimuli. Therefore, the novelty of the aversive stimuli should also be considered since rodents repeatedly exposed to one type of stressor have been reported to respond to a novel, acute stimulus with elevated plasma ACTH levels compared to levels observed after exposure to the repeated stressor (Sakellaris & Vernikos-Danellis, 1975; Armario, Hidalgo & Giralt, 1988).

The ability to respond to stress has been acknowledged as an important basic adaptive mechanism and HPA activation is a central feature of this response. Increases in glucocorticoids may protect the body against its own natural defenses following exposure to

stressful stimuli by preventing excess endocrine, metabolic and immune reactions during the recovery process when these systems could potentially overrespond and cause damage (Munck, Guyre, & Holbrook, 1984). The overall protective mechanism provided by high levels of glucocorticoids is normally self-limiting since elevations in glucocorticoids negatively feedback on the hypothalamus and pituitary, suppressing HPA activity. Consequently, an inability to habituate or adapt to an aversive stimulus may not only adversely affect an organism's ability to respond to new environmental challenges but prolonged increases in pituitary-adrenal activity may seriously impair the well-being of an organism by disrupting homeostatis of physiological systems.

The HPA Axis and Prenatal Ethanol Exposure.

Ethanol exerts a potent influence on the maternal HPA axis and, consequently, may disrupt hormonal interactions between the maternal and fetal systems, producing long-term disturbances in fetal metabolism and physiology which may significantly alter offspring endocrine function into adulthood. During ethanol consumption in rats, maternal adrenal weights, basal corticosterone levels, and the adrenocorticol response to stress have all been demonstrated to be elevated compared to pairfed and ad libitum-fed control females (Weinberg & Bezio, 1987; Weinberg & Gallo, 1982). These changes in maternal endocrine function persist throughout gestation and the increased corticosterone released by the activated maternal endocrine system may cross the placenta and suppress fetal HPA activity (Weinberg & Bezio, 1987). However, the passage of ethanol across the placenta may simultaneously stimulate the fetal HPA axis. Consequently, the opposing physiological

responses to both elevated maternal corticosteriods and ethanol *in utero* may permanently affect the development and organization of the fetal neural structures.

At birth, neonates exposed to ethanol *in utero* (E) have increased adrenal weights, and raised plasma and brain concentrations of corticosterone, as well as elevated plasma levels and reduced pituitary content of β-EP compared to pairfed (PF) and ad libitum-fed control (C) offspring (Taylor, Branch, Kokka & Poland, 1983; Weinberg, 1989). In addition, although the endocrine response to stressors such as ether and novel environment is decreased in all offspring during the preweaning period, E offspring reportedly have greater reductions than PF and C offspring (Taylor, Branch, Nelson, Lane & Poland, 1986; Weinberg, 1989). This effect is transitory, however, and, after weaning, E animals display hormonal hyperresponsive in the form of enhanced pituitary-adrenal activation to stressors (Taylor, Branch, Kokka & Poland, 1983; Weinberg, 1988; Weinberg, 1992b). Significantly, this difference is only apparent following stress. E offspring do not appear to differ from PF or C animals when tested in adulthood under basal conditions.

The hormonal hyperresponsiveness demonstrated by E offspring following exposure to stressors may be limited to specific types of stimuli. Neurogenically-mediated stressors such as cardiac puncture, noise and shake, and morphine or ethanol challenge elicit greater plasma corticosterone responses in E offspring than in PF and C animals whereas metabolic stressors such as exposure to cold (4°C for 1 hr) or prolonged fasting (72 hr) do not always clearly differentiate between E, PF or C offspring (Taylor, Nelson, Branch, Kokka & Poland, 1984). In addition, male and female offspring may exhibit hormonal differences in response to aversive stimuli. Following repeated exposures to stress, E males reportedly show an increased β-EP release to stressor presentations compared to PF and C males. In contrast, E

females show elevated corticosterone and ACTH levels in response to stress compared to PF and C females (Weinberg & Gianoulakis, 1991). These findings suggest that the stressors used to test hormonal hyperresponsiveness in E animals may impact significantly on the type and degree of differences among E, PF and C offspring.

The hyperresponsiveness of the endocrine system in E offspring may contribute to the behavioral deficits exhibited in animals prenatally exposed to ethanol. Central administration of CRF produces behavioral responses resembling stress-induced behaviors such as decreases in feeding in familiar and novel environments, decreases in sexual behavior, increases in acoustic startle and increases in defensive withdrawal (Koob, Heinrichs, Pich, Menzaghi, Baldwin, Miczek & Britton, 1993). These stress-induced behaviors are reportedly attenuated or reversed following the administration of CRF antagonists (Berridge & Dunn, 1987). The effects of CRF may partially explain the behavioral changes documented in E animals. During the stress of a testing situation, elevated HPA activity in E animals may lead to increased CRF levels compared to PF and C animals, resulting in behavioral hyperactivity and deficits in response inhibition.

The Immune System.

Cells of the immune system mediate multiple processes including the elimination of foreign pathogens, the neutralization of toxins and the lysing of tumor cells. Immune cells arise from pluripotent hematopoietic stem cells in the bone marrow and fetal liver. The common lymphoid progenitor gives rise to B and T lymphocytes, while the myeloid progenitor gives rise to monocytes, macrophages, neutrophils, eosinophils, basophils and mast cells. Cooperation between the cells of the two lineages is required for the initiation,

continuation, and ultimate downregulation of many immune responses. One of the hallmarks of an immune response is antigen specificity which is conferred by the two major classes of lymphocytes, T cells and B cells, which develop in the primary lymphoid organs (thymus and bone marrow, respectively)

Mature B and T lymphocytes possess surface proteins that enable them to recognize and bind to foreign antigens and to interact with other lymphoid cells and products of lymphoid cells. During their development and maturation in the adult bone marrow, B lymphocytes undergo immunoglobulin gene rearrangement resulting in the assembly and expression on the cell surface of the immunoglobulin antigen receptor. Pre-T lymphocytes migrate from the bone marrow to the thymus where they differentiate and mature. During the process of maturation, T lymphocytes migrate from the cortex of the thymus to the inner medulla and develop into functionally distinct populations, the best defined of which are helper T cells and cytolytic T lymphocytes (CTLs). These two cell types can be differentiated by the membrane proteins they express. Most helper T cells express a surface protein termed CD4 and most cytolytic T lymphocytes express a protein known as CD8.

Once they express a specific complement of surface markers, the mature T lymphocytes are released from the thymus and travel through the blood to the secondary lymphoid organs (spleen, lymph nodes) where they participate in immune responses. Mature, antigen-specific T lymphocytes do not recognize antigens in free or soluble form. They only respond to peptide antigens that are attached to proteins encoded in the major histocompatibility complex (MHC) which is expressed on the surfaces of antigen presenting cells (APCs). There are two different types of MHC gene products, termed class I and class II MHC molecules. T cell activation depends upon the interaction of the CD3/CD4 and

CD3/CD8 receptor complex on helper T cells and CTLs, respectively, with an antigen/MHC complex on the APC. The CD3/CD4 receptor complex on helper T cells recognizes antigen bound to class II MHC molecules on the APC and the CD3/CD8 receptor complex of CTLs recognizes antigen bound to class I MHC molecules on the APC.

T and B lymphocytes confer acquired immunity which displays antigen specificity and memory. This memory allows the speed, magnitude and defensive capabilities of the immune response to increase with each successive exposure to a particular antigen. Acquired immunity contrasts with innate immunity in that the components of innate immunity do not discriminate among most foreign substances and are not enhanced by prior exposures. Both acquired and innate immunity are in large part mediated by protein molecules called cytokines that are produced by cells of the immune system. Acquired immune responses can be categorized as either humoral or cell-mediated and are distinguished by the activities of B and T lymphocytes, respectively. The induction of both humoral and cell-mediated immune responses requires antigen recognition by helper T cells which divide and differentiate into cytokine-secreting cells following recognition of antigen in association with class II MHC proteins. Cytokines perform a variety of functions, ranging from inhibiting viral replication by interferon (IFN), to enhancing the proliferation of helper T cells and the growth and differentiation of B cells by interleukin-1 (IL-1).

Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. Resting B cells, which have not been previously exposed to antigen, require two distinct types of signals for their proliferation and differentiation into antibody-secreting cells. One type of signal is provided by the antigen, which interacts specifically with immunoglobulin molecules expressed on the surfaces of B cells. This interaction stimulates the differentiation and

proliferation of the B cell, while at the same time increasing the membrane receptors for helper T cell-derived cytokines. The protein antigens are internalized and processed by the B cells which present the antigen in association with class II MHC to helper T cells. This leads to the second type of signal which is provided by helper T lymphocyte-derived cytokines. In response to these cytokines, antigen-recognizing B lymphocytes proliferate and differentiate into antibody-secreting B cells and the secreted antibody molecules bind to the antigen, initiating mechanisms that will ultimately eliminate it.

Cell-mediated immune responses are physiologically most important for eradicting microbes or viruses that live intracellularly or for eliminating cells the body recognizes as foreign. Cell-mediated immune responses can be defined as those immune responses in which antibody plays a subordinate role. There are several forms of cell-mediated immune reactions which are generally initiated by activation of helper T cells which secrete cytokines that, in turn, activate various cell populations. In CTL reactions to viral infections or organ transplants, antigen-activated CD8+ T cells differentiate in response to signals from a target cell expressing specific antigen-MHC complexes or a target expressing foreign class I MHC molecules and from a combination of helper T cell-derived cytokines. Upon differentiation, CTLs acquire the ability to lyse target cells. Natural killer (NK) cells, stimulated by cytokines from antigen-activated CD4+ T cells, differentiate into lymphokine-activated killer (LAK) cells which nonspecifically lyse target cells.

Clearly, an immune response requires highly-ordered, cooperative interactions among multiple types of immune cells. The diverse cells that comprise the immune system function intimately within an immune response and are dependent upon the actions of other immune cells. Consequently, aberrations in any type of cell may impair the response of the system as

a whole. In addition, cytokines play a major role in the initiation, propagation and regulation of immune and inflammatory responses. They are a diverse group of proteins with a wide range of functions and target cells, but they share a number of properties. Cytokines initiate their action by binding to specific cell surface receptors on target cells. These receptors show high affinities for their ligands, with dissociation constants in the range of 10^{-10}-10^{-12} M. This suggests that only very small amounts of a cytokine need to be produced in order to elicit a biological response (Benveniste, 1992).

Most of the current knowledge of the cellular events in immune cell activation is based on *in vitro* experiments in which immune cells are stimulated in a controlled manner, and their responses are measured. One type of procedure uses polymeric plant proteins called lectins that bind in a nonspecific manner to certain sugar residues on T cell surface glycoproteins. Lectins most commonly used include phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM). These proteins, termed mitogens, are polyclonal activators of T cells in that they can stimulate populations of T cells irrespective of T cell specificity. Con A and PHA selectively stimulate T cells by binding to surface molecules involved in cell activation such as the T cell receptor (TCR).

PWM is both a T and B cell mitogen and is able to stimulate antibody secretion by B cells only in the presence of T lymphocytes which supply cytokines for the B cell response. The helper T cell-production of interleukin-2 (IL-2), a cytokine that is the major autocrine growth factor for T lymphocytes, leads to further proliferation of T cells following stimulation by PWM. B cell proliferation and differentiation can also be studied using T cell-independent antigens such as lipopolysaccharide (LPS) which possess the ability to directly stimulate B cells, thereby, bypassing the requirement for helper T cells and their cytokines. LPS is a

component of cell walls of gram-negative bacteria. It is a polyclonal B cell activator, although at low concentrations, B cells that bind to LPS via antigen receptors may be favored, resulting in stimulation of specific B cell subsets. The proliferation of immune cells in these procedures is measured by determining the amount of radioactive thymidine (tritiated thymidine) incorporated into the replicating DNA of cultured cells as a quantitative measure of the rate of DNA synthesis.

Immune function may also be assessed by determining the levels of cytokines such as IL-2 following lymphocyte stimulation. Furthermore, the application of IL-2 to T lymphocytes or T blasts that have entered the cell cycle following stimulation by antigen or mitogen is another method of measuring T cell function. T blast proliferation in response to IL-2 *in vitro* may provide an indication of T cell responsiveness to IL-2 *in vivo*. In addition, NK cell function can be assessed. NK cells do not express T cell antigen receptors and they kill targets in an MHC-unrestricted manner. NK cells lyse both virally infected cells and certain tumor cell lines, especially hematopoietic tumors *in vitro*, and the lysis of such lines serves as a bioassay for NK activity. The target cells are labeled with chromium which is released into the surrounding fluid when the target cells are lysed and can then be measured. All of these quantitative *in vitro* tests are assumed to mimic activities that occur *in vivo* (reviewed in Abbas, Lichtman & Pober, 1991; Paul, 1993).

Interactions Between the Immune System and HPA Axis.

The existence of bidirectional pathways of communication between the endocrine and immune systems provides a foundation for the observation that stress may influence immune function and, reciprocally, that the immune system may exert control over neuroendocrine

function (Blalock, 1989). Hormone receptors on cells of the immune system as well as neuroendocrine peptide hormone production by such cells provides a molecular basis for these interactions. In addition, cytokine production during an immune response may not only be influenced by neuroendocrine processes but may also directly affect the levels of circulating hormones.

In general, glucocorticoids appear to be primarily suppressive on immune function. Among their effects, glucocorticoids inhibit virtually all functions of monocytes and macrophages including chemotaxis, phagocytosis and the capacity to present antigen to T lymphocytes (Guyre & Munck, 1989). In addition, glucocorticoids may cause an acute redistribution of circulating leukocytes into various lymphoid compartments, limiting the ability of the immune system to respond to stimulation by decreasing the circulating numbers of lymphocytes, eosinophils, basophils and monocytes in the peripheral blood (Claman, 1989). Glucocorticoids have also been reported to decrease NK cell function (Gatti et al, 1987).

Inhibition of the production of immune cytokines is probably a major constituent of the immunosuppressive effects of glucocorticoids and the cellular mechanisms of inhibition of cytokine production vary from one cytokine to another. Interleukin-1 (IL-1) production, which enhances the proliferation of helper T cells and the growth and differentiation of B cells, appears to be blocked at the levels of transcription, translation and secretion by glucocorticoids (Knudsen, Dinarello & Strom, 1987). For IL-2, decreased production is accounted for by lowered mRNA levels (Grabstein, Dower, Gillis, Urdal & Larsen, 1986). In addition, glucocorticoid administration posttranscriptionally blocks the production of cytokines such as tumor necrosis factor (TNF) that protects against viral infection and

interferon-β (IFN-β) that initiates inflammatory reactions that protect against bacteria (Beutler & Cerami, 1988; Gessani, McCandless & Baglioni, 1988, respectively). Considering the numerous functions of cytokines, the inhibitory effects of glucocorticoids may produce wide-ranging impairments in the immune response.

Recent evidence suggests that peptide hormones of the HPA axis may also influence immune functioning. *In vitro* administration of CRF into cultures of human lymphocytes has been shown to augment IL-2 receptor expression on activated T cells and increase proliferation in response to T cell mitogens such as Con A and PHA (Singh, 1989). In addition, B lymphocytes from rodents display an increased proliferative response to mitogen stimulation following treatment with CRF *in vitro* (McGillis, Park, Rubin-Fletter, Turck, Dallman & Payan, 1989). These findings indicate that, in contrast to the suppressive effects of glucocorticoids, CRF may actually enhance the functioning of certain immune cells. However, central administration of CRF in rats reportedly produces reductions in splenic and peripheral blood NK cell activity, lymphocyte responses to mitogenic stimulation and percentages of splenic NK cell numbers (Strausbaugh & Irwin, 1992; Irwin, Hauger, Brown & Britton, 1988), suggesting that cytokines may have a modulating influence on the effects of CRF on immune cells *in vivo*.

ACTH receptors have been demonstrated on T and B lymphocytes, and mitogenic stimulation of these cells increases the number of high affinity ACTH binding sites (Clarke & Bost, 1989). Like glucocorticoids, ACTH may also influence cytokine activity. The production by T lymphocytes of interferon-γ (IFN-γ), which increases the lytic potential of NK cells and CTL, is inhibited by ACTH administration *in vitro* (Johnson, Torres, Smith, Dion & Blalock, 1984). In addition, ACTH reportedly enhances the proliferation of B cells to

mitogen in the presence of IL-4 and IL-2, but this stimulatory effect is lost in the absence of interleukins (Alvarez-Mon, Kehrl & Fauci, 1985). Clearly, the hormones of the HPA axis can influence immune functions, and therefore they play an important role in the interactions between the immune and endocrine systems.

Significantly, immune cells are capable of producing certain hormones normally associated with the endocrine system. Adrenocorticotropin, β-endorphin, and their parent POMC have been shown to be produced by activated lymphocytes and certain macrophages (Blalock, 1988). Specific viral infections increase the level of POMC transcripts in murine splenocytes (Westly, Kleiss, Kelley, Wong & Yuen, 1986) and human lymphocytes (Oates, Allaway, Armstrong, Boyajian, Kehrl & Prabhakar, 1988). Hormone synthesis can also apparently be stimulated by CRF and, in turn, inhibited by glucocorticoids, in the same classic feedback regulation that takes place in the pituitary (Smith & Blalock, 1981). In fact, the amino acid and nucleotide sequence identity between pituitary and leukocyte ACTH has been established (Galin, LeBoeuf & Blalock, 1990).

An irCRF in human lymphocytes and neutrophils has also been demonstrated (Stephanou, Jessop, Knight & Lightman, 1990), indicating that immune cells may be capable of producing hypothalamic-releasing hormones. Although cells of the immune system respond to hypothalamic releasing-hormones in a manner similar to pituitary target cells (Smith, Full, Morrill, Meyer & Blalock, 1986), their production of both hypothalamic and pituitary analogs suggests that they may be able to control the production of hormones independently of the HPA axis. These leukocyte-derived neurohormones may mainly act locally as paracrine or autocrine secretions or they may reach threshold levels in the serum for

communication with distant organs, providing a bidirectional pathway of communication between the immune and endocrine systems.

Another method of communication between the immune system and endocrine system may be in the form of cytokines produced by activated immune cells. IL-1β has been shown to stimulate the release of CRF from the hypothalamus and increase plasma levels of ACTH and corticosterone (Sapolsky, Rivier, Yamamoto, Plotsky & Vale, 1987). IL-2 and IL-6 can stimulate the production of ACTH from pituitary cells in a dose-dependent manner (Karanth & McCann, 1991; Naitoh, Fukata, Tominaga, Nakaai, Tamai, Mori & Imura, 1988). In addition, IFNs-α/β can both induce corticosteroid production in adrenal cells (Blalock & Harp, 1981). These results indicate that cytokine production during an immune response provides a ready means of conveying information on immune activation to the endocrine system. In response, the endocrine system may produce hormones which will ultimately suppress immune activity, demonstrating the dynamic interactions that exist between the immune system and the endocrine system.

The Immune System and Stress.

Research on the influence of acute stress on the immune system in humans has produced varied results, presumably through differential activation of physiological systems in response to distinct types of stressors. Even though such studies involve exposure to a single event, the duration of anticipatory or consequent distress may vary. The degree of perceived self-efficacy in managing the stressor and the controllability of the stressor may also affect alterations in immune function with increases in lymphocyte numbers and NK cell activity during acute stress correlated with reports of either high self-efficacy (Wiedenfeld,

O'Leary, Bandura, Brown, Levine & Raska, 1990) or control (Schedlowski et al, 1993). In addition, a frequent criticism of acute stress research employing healthy subjects is that any observed alterations in immunity may be of little clinical significance.

In general, exposure to acute stressors in animals has been associated with suppression of immune function. Reductions in NK cell activity (Irwin & Livnat, 1987; Pollack, Lotzova & Stanford, 1989) and numbers of splenic lymphocytes have been reported (Kugler, Kalveram & Lange, 1990; Esterling & Rabin, 1987) following acute stress. In addition, splenic lymphocytes from animals exposed to an acute rotational stress and a carcinogen have significantly lower levels of an enzyme important in DNA repair. In this way, stress may potentiate the carcinogenic effects of certain compounds (Glaser, Thorn, Tarr, Kiecolt-Glaser & D'Ambrosio, 1985).

Significantly, Esterling and Rabin (1987) found that adrenalectomy did not eliminate decreases in lymphocyte populations induced by stress, suggesting that the reductions were glucocorticoid-independent. In addition, Cunnick, Lysle, Kucinski and Rabin (1990) reported that adrenalectomy did not attenuate the footshock-induced suppression of mitogenic proliferation of splenic T cells, although it did prevent reductions in mitogenic proliferation of peripheral blood T cell. These findings suggest that distinct immune compartments may be differentially affected by stressors that activate the HPA axis. In addition, the observation that acute stress may enhance murine lymphocyte responsiveness to Con A and PHA under specific intensities and durations of electric footshock (Lysle, Cunnick & Rabin, 1990) emphasizes the important influence different elements of the stressor may have on immune function and underscores the necessity of evaluating the subjective or emotional nature of the stimuli.

Unlike acute stress, chronic stress in humans has been associated with the suppression of immune function which may not adapt over time. Several studies have reported that individuals experiencing stressful life events show decreased immune function and an increased susceptibility to a variety of illnesses. Stress associated with life difficulties such as caregiving for a family member with Alzheimer's disease (Kiecolt-Glaser, Glaser, Shuttleworth, Dyer, Ogrocki & Speicher, 1987), bereavement (Bartrop, Lazarus, Luckhurst, Kiloh & Penny, 1977), and divorce and/or separation (Kiecolt-Glaser, Fisher, Ogrocki, Stout, Speicher & Glaser, 1987) have been correlated with reductions in either function of or numbers of immune cells. However, these results may be confounded by depression in these subjects; individuals with major depression reportedly have decreased B and T cell numbers and reduced lymphocyte responses to mitogen stimulation compared to controls (Schleifer, Keller, Meyerson, Raskin, Davis & Stein, 1984).

Decreases in the number and function of NK cells, reductions in the percentage of helper T lymphocytes and a lowering of lymphocyte proliferation in response to mitogens have also been observed following more moderate stressors such as academic examinations in medical students (Glaser, Kiecolt-Glaser, Stout, Tarr, Speicher & Holliday, 1985). In addition, Glaser et al (1987) found increases in antibody titres to Epstein-Barr virus and herpes virus, indicating poorer cellular immune control over latent viruses in students preparing for academic examinations. Poor eating habits, and sleep deprivation may increase during chronic stress and may produce direct effects on the immune system. Therefore, an important consideration in this research concerns the role of behavioral factors in the mediation of the influence of these stressors on immunity.

Although stress in animals may be initially associated with immunosuppression, chronic stress may eventually result in a return of immune function to pre-stress levels. Monjan and Collector (1977) reported that sound stress initially suppressed lymphocyte proliferation to LPS and Con A in male rats, but that more extended exposure to the stress resulted in enhanced responses to these same mitogens. In addition, the suppressed reactivity of splenic lymphocytes to Con A was shown to diminish with repeated sessions of frequent footshocks (Lysle, Cunnick & Rabin, 1990; Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988). In contrast, Batuman et al (1990) found decreased numbers of splenic and whole-blood $CD8^+$ T cells as well as reduced IL-2 production in response to stimulation by PHA in animals exposed to a combined chronic stress regimen of restraint and mild electric footshock. These findings suggest that compensation of immune function following chronic stress may be dependent upon the type, duration and intensity of the stressor.

The return of immune function to pre-stress levels may only occur under specific circumstances due to the influence of the HPA axis. It has been found that daily exposure of animals to an identical stressor reduces the pituitary-adrenal response to that stressor (Kugler, Kalveram & Lange, 1990; Armario, Lopez-Calderon, Jolin & Balasch, 1986). Thus, reductions in HPA activation, termed adaptation, following chronic exposure to the same stressor may result in decreased inhibition of the immune response. However, the pituitary-adrenal system may be hypersensitive to additional, novel stimuli. Plasma ACTH and corticosterone responses to novel acute stressor superimposed on chronic stress were reportedly faster, greater and more sustained than those seen in nonstressed rats (Sakellaris & Vernikos-Danellis, 1975; Vernikos et al, 1982). Thus, compound stressors (Batuman et al,

1990) may not result in adaptation of the HPA response as rapidly or extensively as a single stressor, and immune function may continue to be impaired.

These examples underscore the importance of examining different components of the physiological response to stress individually and at the same time. It is overly simplistic to discuss immune enhancement or immune suppression in global terms when studying complex interactions involving emotional or subjective states as well as multiple physiological systems. Furthermore, it is important to remain aware of the bidirectional nature of immune and endocrine communication when assessing the effects of stress on immune function.

The Immune System and Prenatal Ethanol Exposure.

Clinical studies have demonstrated numerous immune deficiencies in children with FAS including an increased incidence of minor and life-threatening illnesses such as pneumonia, meningitis, and urinary tract and upper respiratory tract infections (Johnson, Knight, Marmer & Steele, 1981). These problems have been documented in children with FAS up to the age of ten, suggesting that immune deficiencies associated with FAS are persistent and may have serious health consequences well into the child's life. Johnson and colleagues (1981) reported that children with FAS had increased rates of bacterial infections, decreased erythrocyte-antibody complement (EAC) rosette forming lymphocytes and diminished mitogen-induced lymphocyte proliferation. In addition, several malignancies including rhabdomyosarcoma, Wilms-tumor, acute lymphocytic leukemia and adrenal carcinoma have been reported to occur with increased frequency in children with FAS (Kinney, Faix & Brazy, 1980).

Evidence of immune deficits following prenatal ethanol exposure has also been demonstrated in animals. Prenatal ethanol exposure has been shown to reduce thymus weight (Ewald & Walden, 1988), diminish T lymphocyte responsiveness to Con A (Monjan & Mandell, 1980; Norman, Chang, Wong, Branch, Castle & Taylor, 1991) and alter the development of T lymphocyte populations, resulting in a decrease in thymus cell number (Ewald, 1989; Ewald & Walden, 1988). In addition, splenocytes obtained from adult animals prenatally exposed to ethanol and preactivated with Con A were less responsive to stimulation with IL-2 than were splenocytes from controls (Norman, Chang, Castle, Van Zuylen & Taylor, 1989). This reduced responsiveness to IL-2 may have a profound effect on the immunocompetence of an organism since IL-2 plays a key role in stimulating and promoting a variety of immune functions (Abbas, Lichtman & Pober, 1991).

Past findings suggest that males exposed to ethanol *in utero* are more susceptible to ethanol-induced immune deficits than female offspring (Weinberg & Jerrells, 1991). E males reportedly had decreased splenic lymphocyte proliferative responses to IL-2 (Norman et al, 1989) even though endogenous levels of IL-2 and expression of IL-2 receptors were normal, suggesting that the decreased responsiveness to IL-2 was due to an inability of lymphocytes to utilize IL-2 (Weinberg & Jerrells, 1991). In addition, E males exhibit decreases in thymocyte number and reductions in splenic lymphocyte proliferation in response to Con A (Weinberg & Jerrells, 1991). It has been hypothesized that these findings may be partly attributable to the presence of estrogen which may attentuate the adverse impact of ethanol on the immune system in females (Weinberg & Jerrells, 1991).

In the first study to investigate the interactive effects of prenatal ethanol exposure and exposure to stress in adulthood, specific deficits were apparent only when fetal ethanol-

exposed males were exposed to stressors (Giberson & Weinberg, in press). This finding corresponds to the reports in hormonal studies that E animals only differ from PF and C animals when challenged with stressors (Taylor, Branch, Van Zuylen & Redei, 1988). In E males, exposure to stress resulted in a reduction in the number of pan T cells in the thymus and whole blood (Giberson & Weinberg, in press). Irrespective of stress condition, both E and PF male offspring had reduced numbers of thymic pan T, $CD4^+$ and $CD8^+$ T lymphocytes compared to C males whereas E and PF females had decreased numbers of lymph node $CD4^+$ T cells and increased numbers of thymic $CD8^+$ T lymphocytes compared to C females, demonstrating the nutritionally mediated effects of ethanol. (Giberson & Weinberg, in press).

In summary, prenatal exposure to ethanol leads to many alterations in immune competence in adulthood, some aspects of which may only become apparent following the additional challenge of stress. It is possible that the effects of ethanol on absorption and/or utilization of nutrients as well as the nutritional effects of mildly reduced food intake may impact on the fetal immune system. There may also be critical periods in the development of the fetal immune system during which exposure to ethanol may differentially affect immune function. In addition to these possibilities, the complexity of interactions between the immune, endocrine and nervous systems, as well as the effects of ethanol and stress on these systems, highlights the ease with which these systems may be disrupted and the necessity of considering these interactions when studying prenatal ethanol exposure.

The Hypothalamic-Pituitary-Gonadal (HPG) Axis.

Hypothalamic neurons release gonadotropin releasing hormone (GnRH) in pulses to gonadotrophes in the anterior pituitary where GnRH stimulates the secretion of two structurally similar glycoproteins: follicle stimulating hormone (FSH) and luteinizing hormone (LH). GnRH is, therefore, known by its actions as a follicle stimulating hormone releasing hormone (FSHRH) and a luteinizing hormone releasing hormone (LHRH). Among its many functions, FSH stimulates a variety of protein products essential to spermatogenesis. In the female, FSH recruits follicles in the ovaries, initiates the production of estrogen precursors in theca cells, and increases LH receptors on granulosa cells. LH stimulates Leydig cells in the testis to produce and release testosterone in the male. Testosterone has a pulsatile release from the Leydig cells in response to LH stimulation, and it also has negative feedback mechanisms at the hypothalamic and pituitary levels. In the female, LH causes granulosa cells in the ovary to produce estradiol (E_2), the most potent and major form of estrogen in the mature female which has negative feedback mechanisms at the hypothalamic and pituitary levels.

During early fetal life, the placenta produces and releases a wide range of hormones which mimic the actions of the hypothalamus and pituitary until the eventual maturation of the fetal hypothalamic/pituitary structures. Under their influence, fetal Leydig cells produce and release testosterone in the male. Therefore, steroidogenesis (testosterone production) is active in the male fetus even though gametogenesis (sperm production) is absent until puberty. Neonatal levels of testosterone surge shortly prior to and immediately following parturition. The activity of the hypothalamic pituitary axis then decreases in response to

feedback inhibition generated by high levels of testosterone and remains low until puberty when phasic secretion of GnRH, LH and testosterone begins.

In the female, development of the HPG axis in the fetus initiates germ cell division and ovarian development. Gametogenesis (oocyte production) but not steroidogenesis (E_2 and progesterone) occurs during prenatal development. As a consequence, teratogens consumed by the mother can affect the formation of oocytes in her female offspring, ultimately impacting her grandchildren. Similarly to the male, female neonatal hypothalamic activity is depressed and puberty is marked by the gradual increase in levels of GnRH, LH, and FSH as well as the production and release of E_2 (Berne & Levy, 1993).

Interactions Between the HPG Axis and the Immune System.

The interaction of the immune system and the HPG axis were demonstrated almost one hundred years ago with the finding that thymic mass increased following castration in adult male rabbits (Calzolari, 1898 as cited in Grossman, 1989). Recently, a number of investigations have identified sex steroid receptors in human lymphoid tissues. High affinity, low capacity steroid receptors for estrogen and androgen have been found in the thymus of rodents (McCruden & Stimson, 1985). LHRH receptors have also been identified on thymocytes (Marchetti, Guarcello, Morale, Bartoloni, Farinella, Cordaro & Scapagnini, 1989). In addition, lymphocytes reportedly synthesize and release irLH in response to LHRH (Costa, Mulchahey & Blalock, 1990). Similar to the production of HPA hormones by lymphocytes, the lymphocyte-derived LH shares molecular mass and subunit structure with the hormone produced in the pituitary. Cultured rat lymphocytes also produce irFSH which may increase following exposure to Con A (Gorospe & Kasson, 1989).

Females display enhanced humoral and cell-mediated immune responses compared to males and the effects of gonadal steroids may account for this sexual dimorphism (Grossman, 1989). Females tend to have elevated serum immunoglobulin concentrations (Butterworth, McClellan & Alansmith, 1967) and, therefore, an increased humoral immune response compared to males. In addition, the majority of autoimmune diseases in humans occurs more frequently in females than males, indicating a higher reactivity in the female immune system (Nelson & Steinberg, 1986). Females also display a longer primary and secondary immune response to challenges with antigens (Terres, Morrison & Habicht, 1968).

It appears that estrogen may be responsible for the documented enhancements of cell-mediated and humoral immunity in females while testosterone may suppress both types of immune response in males. B lymphocyte proliferation in response to PWM is reportedly inhibited by testosterone and stimulated by estrogen *in vitro* (Sthoeger, Chiorazzi & Lahita, 1988). IL-2 production by maturing thymocytes in culture can also be reduced by treatment with a converted form of testosterone, termed dihydrotestosterone (DHT) (Kovacs & Olsen, 1987). In addition, testosterone treatment during early embryonic life appears to produce abnormalities in the production of immunoglobin in chickens (Szenberg, 1970 as cited in Grossman, 1989). Castration and the subsequent decline in testosterone increases protection against viral, fungal, bacterial and parasitic infections (Grossman, 1984). Furthermore, orchiectomized males reject skin grafts more rapidly than sham operated controls (Graff, Lappe & Snell, 1969). Thus decreases in testosterone production can be correlated with increases in immune functioning. These findings of interactions between the immune and gonadal systems may explain the sexual dimorphism of the immune response.

The reciprocal nature of the interaction between the two systems is demonstrated by findings that the immune system can impact upon reproductive functions. In gonadectomized rats, administration of IL-1β causes a significant and prolonged decrease in plasma LH and can inhibit ovulation if it occurs on the afternoon of proestrus (Rivier & Vale, 1990). Acute, central administration of IL-β into intact females will also disrupt the estrous cycle and decrease the biosynthesis and release of LHRH (Rivest, Lee, Attardi & Rivier, 1993). In addition, application of IL-2 *in vitro* to pituitary cells has been shown to inhibit the basal release of LH and FSH (Karanth & McCann, 1991). The importance of cytokines in immune and reproductive interactions is reflected by differences in cytokine bioactivity throughout the estrous cycle. In ovariectomized mice, estrogen and progesterone administration produces significant amounts of IL-6 and TNFα mRNA while separate administration of estrogen or progesterone results in the induction of IL-1α and IL-1β mRNA expression. Although cytokine bioactivity was expressed throughout the estrous cycle, levels of each cytokine were highest during proestrus and/or estrus (De, Sanford & Wood, 1992). In summary, communication between the immune system and the HPG axis may occur at multiple levels, producing numerous functional effects, and may be partially cytokine-mediated.

The HPG Axis and Stress.

As previously discussed, stress is known to activate the hypothalamic-pituitary-adrenal axis. Therefore, it is not surprisingly that stress can affect reproductive function at all levels of the hypothalamic-pituitary-gonadal axis. In the rat, maternal stress during pregnancy has been shown to feminize male offspring and masculinize female offspring; effects that can be attributed to higher than normal secretion of CRF, ACTH and glucocorticoids which may

suppress HPG activity at critical times during fetal development (Pollard & Dyer, 1985). Glucocorticoids have also been shown to suppress the release of hypothalamic GnRH and pituitary LH as well as directly inhibiting the secretion of testosterone (Kamel & Kubajak, 1987).

Testosterone production has been shown to vary depending on the type, intensity and length of the stress exposure. Short-term surgical stressors produce increased levels of circulating testosterone (Gatenbeck, Eneroth, Johansson & Stromberg, 1987) and acute (10-30 mins) restraint produces LH increases in adult rats (Armario, Restrepo, Hidalgo & Lopez-Calderon, 1987). Prolonged stress stimulation including swimming (Bidzinska et al, 1993), immobilization (Demura, Suzuki, Nakamura, Komatsu, Odagiri & Demura, 1989; Srivastava, Taylor & Mann, 1993), and a combination of immobilization, starvation, and cold exposure (Gatenbeck, Eneroth, Johansson & Stromberg, 1987) have all resulted in reduced plasma testosterone levels.

Demura et al (1989) found that both acute and chronic stress decreased plasma LH concentrations, although corresponding decreases in plasma testosterone were only observed after chronic stress. Orr and Mann (1990) also reported dissimilar changes in LH and testosterone, finding lowered testosterone concentrations and unchanged LH levels following acute stress. Thus, it appears that stress may differentially affect LH and testosterone concentrations. The inhibition of testosterone during longer stressors may be due to decreased 17, 20 desmolase activity (Orr & Mann, 1990), the rate-limiting enzymatic step in steroid production. In addition, it is possible that increases in plasma glucocorticoids may suppress testicular responses to gonadotropins via glucocorticoid receptors on testicular interstitial cells (Orr & Mann, 1992).

The actions of the HPA axis may influence, either directly or indirectly, the changes in gonadal hormones observed during stress. However, the reproductive system may have a reciprocal influence on the activity of the HPA axis. In castrated male rats not treated with testosterone, acute immobilization elevated pituitary concentrations of β-EP whereas chronic immobilization did not. In contrast, castrasted male rats treated with testosterone showed increased levels of β-EP only during chronic immobilization, suggesting that gonadal steroids may differentially influence corticotrophs in the pituitary under acute and chronic stress conditions (Forman & Estilow, 1988). Furthermore, ovariectomized females without estrogen replacement display HPA responses to stressors at levels matching intact males (Lesniewska, Miskowiak, Nowak & Malendowicz, 1990). In addition, gonadal tissue can produce POMC peptides and, therefore, may be able to influence the level of POMC peptides independently of the HPA axis (Bardin et al, 1987).

The HPG Axis and Prenatal Ethanol Exposure.

Prenatal ethanol exposure may detrimentally affect the HPG axis at multiple levels. Males exposed to ethanol *in utero* display numerous reproductive deficiences beginning during gestation and continuing into adulthood. Male fetuses prenatally exposed to ethanol fail to display a testosterone surge on gestational days 18-19 and exhibit dramatically lower testosterone release in response to stimulation by LH compared to control males (McGivern, Roselli & Handa, 1988). Neonatal male E animals exhibit a suppression of the postnatal testosterone surge which is not due to a decrease in testicular sensitivity to LH (McGivern, Handa & Redei, 1993). Furthermore, male neonates display decreased levels of plasma testosterone, plasma LH (Parker, Udani, Gavaler & Van Thiek, 1984; Udani, Parker, Gavaler

& Van Thiel, 1985) and brain dihydrotestosterone (Kakihana, Butte & Moore, 1980) and these reductions may continue into adulthood.

The observed reductions in LH and testosterone may be responsible for physiological alterations in E males such as reductions in numbers of Leydig cells and in weights of testes, prostate and seminal vesicles (Parker et al, 1984; Udani et al, 1985), decreases in anogenital distance (Udani, Parker, Gavaler, & Van Thiel, 1985), and increases in the presence of vacuoles in the seminiferous tubules (McGivern, Raum, Salido & Redei, 1988). In addition, the disruption of the prenatal and postnatal surges of testosterone have been associated with feminization of sex-linked behaviors in adulthood, and adult E males demonstrate increases in latency to mount, decreases in intromission frequency (Parker et al, 1984; Udani et al, 1985) and reductions in the volume and average cell size of the sexually dimorphic nucleus of the preoptic area of the hypothalamus (Barron, Tieman & Riley, 1988).

In female rodents, vaginal opening, behavioral estrus and regular behavioural estrus cycling are all delayed by prenatal ethanol exposure (Creighton-Taylor & Rudeen, 1991; Hard, Dahlgren, Engel, Larsson, Lindh & Musi, 1985), mirroring the reports of delayed onset of menarche in human females whose mothers drank heavily during pregnancy (Robe, Robe & Wilson, 1980). E females exhibit maternal behavior deficiencies such as impaired nest building and pup retrieval following parturition (Hard et al, 1985; Barron & Riley, 1985). In addition, increased sensitivity to exogenous gonadotropins (Hard et al, 1985), elevated plasma prolactin levels, decreased plasma LH (Esquifino, Sanchis & Guerri, 1986), and reduced hypothalamic LHRH levels (Morris, Harms, Peterson & MacArthur, 1989) have been demonstrated in E females compared to control females. Clearly, prenatal ethanol exposure

exerts a detrimental effect on reproductive functioning in E offspring compared to controls that is evident even into adulthood.

GENERAL METHODS

Animals and Mating.

Sprague-Dawley rats were obtained viral-free (confirmed by a serology report) from Canadian Breeding Farms, St. Constant, Quebec. During a 1-2 week adaptation period, females were group housed in an isolated colony breeding room controlled for temperature (21-22°C) and lighting (lights on 0600-1800 hr standard time). Following the adaptation period, each female was placed with a Sprague-Dawley male in a stainless-steel hanging cage (25 x 18 x 18 cm) with a mesh front and floor. Cage papers were checked daily and the presence of vaginal plugs indicated d 1 of gestation. During both the adaptation period and mating, animals were maintained with *ad libitum* access to standard laboratory chow (Ralston Purina of Canada, Woodstock, Ontario) and water.

Diets and Feeding.

On d 1 of pregnancy, females were rehoused into polycarbonate, transparent cages (20 x 50 x 20 cm) and assigned to one of three treatment groups: 1) Ethanol (E), liquid ethanol diet (36% ethanol-derived and 25% protein-derived calories), *ad libitum*; 2) Pair-fed (PF), liquid control diet (with maltose-dextrin isocalorically substituted for ethanol) in the amount consumed by a randomly paired E partner (g/kg body weight/day of gestation); or 3) Control (C), lab chow and water, *ad libitum*. [A minimum of fifteen females were assigned to each

treatment group.] Ethanol was administered via liquid diet to alleviate the stress encountered during other methods of administration such as injection and intubation which require a great deal of animal handling. Diets were prepared by Bio-Serv, Inc., Frenchtown, NJ. and formulated to provide adequate nutrition at high levels of ethanol intake. Diets were made fresh every three days and kept refrigerated until feeding. Fresh diet was presented daily in glass bottles with ball point drinking tubes to eliminate spillage and evaporation.

Animals were fed between 1600-1700 hrs daily (1-2 hrs prior to lights off) to minimize shifts in the maternal corticoid circadian rhythm. Pair-feeding is a procedure designed to control for the reduced food intake that typically occurs with alcohol consumption. Thus, PF animals usually receive a reduced ration of food which they generally consume within a few hours of being fed, resulting in food deprivation until feeding the next day. Gallo and Weinberg (1981) have previously demonstrated that animals on a restricted feeding schedule (e.g. PF animals) show a corticoid rhythm synchronized to the daily feeding time rather than to the normal light-dark cycle. Therefore, feeding prior to lights off eliminates a shift in peak corticoids and maintains the typical diurnal rhythm.

Diets were continued through d 22 of gestation, at which time liquid diets were replaced with laboratory chow and water *ad libitum* in all animals in order to minimize the adverse effects of ethanol on parturition and lactation. No obvious signs of withdrawal were observed (e.g. tremors, hyperresponsive to stimuli) following removal of the ethanol diet. In general, females decrease their food intake approximately two days prior to parturition suggesting that ethanol intake would have been reduced even if the diet had been continued.

Females were weighed and cages were changed on d 1, 7, and 14 of gestation. On d 21of gestation, females were weighed but cages were not changed in order to minimize

disturbances prior to parturition. At birth, designated d 1 of lactation, dams and pups were weighed and litters were culled to approximately equal numbers of males and females (i.e. 6 females and 5 males in Experiment 1, and 5 females and 5 males in Experiment 2). For litters containing fewer than the required number of pups, pups from a dam of the same treatment condition, born on the same day, were fostered in in order to bring the litter size up to the required numbers. Dams and offspring were weighed and cages were changed on d 1, 8, 15 and 22 of lactation. Aside from this activity, animals remained undisturbed until weaning on d 22, after which offspring were ear marked and group housed by sex and treatment condition until testing in adulthood. Both males and females were tested in both experiments.

Blood Sampling.

Blood samples were collected using either cardiac puncture under ether anaesthesia or decapitation. Cardiac puncture was used to obtain a true measure of basal corticosterone (CORT) levels without sacrificing the animals. Previous investigations in this laboratory have indicated that if the sample is obtained within 2 min of touching the animal's cage, CORT levels are not artifically elevated due to the handling disturbances or responses to ether anaesthetic. The blood samples (0.5 cc) were collected in heparinized syringes, placed in glass test tubes (10 x 75 mm), and centrifuged at 3500 rotations per min (rpm) for 10 min at 4° C. Plasma was collected, transferred to plastic Eppendorf tubes and stored at -80°C until assayed.

Decapitation was used to collect plasma samples for determination of CORT and ACTH. If performed within 10-15 sec of touching the animal's cage, decapitation can provide true concentrations of basal ACTH levels in addition to basal CORT levels. Trunk

blood was collected on ice in plastic test tubes (12 x 75 mm) containing ethylenediamine tetraacetic acid (EDTA) and aprotinen (0.2 ml/5 ml blood of a mixture of 7.5 mg EDTA plus 1000 KIU aprotinen) (an anticoagulant and a protease inhibitor, respectively). Plastic tubes and equipment were used for all trunk blood collection because ACTH adheres to glass. Trunk blood was centrifuged at 3500 rpm for 10 min at 4°C. Plasma was then transferred with plastic pipettes to plastic Eppendorf tubes and stored at -80°C until assayed.

All blood sampling was conducted at consistent times within the trough of the corticoid circadian cycle between 0800 and 1100 hr (e.g. 2-3 hr of lights on). Since any disturbance prior to blood sampling can elevate plasma CORT and ACTH levels, the animal room was closed off at 1800 hr each night and no one entered the room prior to sampling. In the morning, each cage was rapidly and quietly removed from the colony room to an adjacent laboratory where the blood sampling was conducted, thereby minimizing the disturbance to animals awaiting sampling in the colony room. Two to three people were often involved in blood collection so speed and accuracy could be maintained.

Chronic Stress Regimen.

During the chronic stress regimens used in both studies, each animal received two different stressors daily from a set of six possible stressors: 1) restraint in PVC tubes, 19 x 7 cm (male) or 15 x 6 cm (female), which restricted movement and prevented the animal from turning around (45 min duration); 3 sessions of 4 taps and 2 rotations were applied randomly to the tubes throughout the 45 min; 2) novel cage without bedding, food or water (45 min duration); a loud startle noise was applied randomly 3 times during the session; 3) exposure to ether vapors (~1 min); 4) swim stress (15 min duration) in room temperature water (approx.

22°C) in a 40 x 30 cm bucket; 5) confinement in a novel container (45 min duration) with 3 sessions of 4 shakes and 2 flips of the container appplied randomly during the session; and 6) drop, in which the animal was held by the base of the tail and rapidly lifted and lowered 5 times. In addition, water deprivation (from ~1600 hr to ~ 0800 hr) occurred twice weekly but never on consecutive nights or prior to ether stress. At all other times, animals had *ad libitum* access to lab chow and water.

Apart from water deprivation, all stressors were presented during the light phase. Two different stressors were presented daily at random times to prevent the animals from predicting the occurrence of the stressors. In addition, presentation of the two daily stressors were separated by a minimum of 2 hr in order to allow CORT to return to or decrease toward basal levels between stressors. The stressors were designed to produce elevated plasma CORT levels and the application of one stressor superimposed onto another (e.g., taps and rotations superimposed onto restraint stress) was implemented to reduce the possibility of habituation and maintain maximally elevated CORT levels throughout the stress session. Animals were also singly housed during the chronic stress regimens because single housing is reportedly stressful to social animals such as rats (Weinberg & Emerman, 1989).

EXPERIMENT 1

The objective of the present study was to examine the effects of prenatal ethanol exposure and exposure to chronic stress in adulthood on pituitary-adrenal activity, body and organ weights, and immune cell counts. In particular, the study assessed whether measures of the HPA, HPG and immune systems were differentially affected in E, PF and C males and females by exposure to chronic stress.

Previous studies have shown that ethanol consumption by pregnant females alters stress responsiveness in the offspring. Specifically, E animals display hormonal hyperresponsiveness manifested as enhanced pituitary-adrenal activation to stressors as well as delayed or deficient recovery to basal levels when tested in adulthood (Taylor, Branch, Kokka & Poland, 1983; Weinberg, 1988; Weinberg, 1992b). This difference is only apparent following stress; E offspring do not appear to differ from PF or C animals when tested in adulthood under basal conditions. However, most previous studies have examined effects of prenatal ethanol exposure either on responses to an acute stressor or on responses to repeated exposure to one particular stressor (Taylor, Branch, Liu & Kokka, 1982; Weinberg, 1988; Weinberg, Taylor & Gianoulakis, in press).

Studies on HPA activation following chronic stress have found that continuous exposure of adult rats to aversive stimuli may result in decreased CORT or ACTH responses to presentation of the stressor (Rivier & Vale, 1987; Hashimoto, Suemaru, Takao, Sugawara, Makino & Ota, 1988; Culman, Kopin & Saavedra, 1991). The present study was undertaken to determine if basal or undisturbed CORT and ACTH levels are altered from pre-stress, basal measures after 6 d or 18 d of exposure to a chronic intermittent stress regimen and if E, PF and C animals are differentially affected by the chronic stress.

Previous findings have also indicated that rodents repeatedly exposed to one particular stressor may respond to a novel, acute stimulus with elevated pituitary-adrenal activity compared to levels observed after exposure to the repeated stressor (Sakellaris & Vernikos-Danellis, 1975; Armario, Hidalgo & Giralt, 1988). Therefore, the present study was also designed to assess whether CORT and ACTH levels following exposure to a novel, acute stressor differ depending upon prior exposure to the chronic intermittent stress regimen and if E, PF and C animals exposed to chronic stress are differentially affected by the acute stressor.

In addition to effects on offspring HPA activity, prenatal exposure to ethanol has been reported to produce alterations in HPG function such as reductions in the prenatal (McGivern, Roselli & Handa, 1988) and postnatal testosterone surges in E males (McGivern, Handa & Redei, 1993) and delays in vaginal opening and regular behavioural estrus cycling in E females (Creighton-Taylor & Rudeen, 1991; Hard et al, 1985) compared to controls. Furthermore, exposure to chronic stress has been shown to alter HPG activity, especially plasma testosterone levels (Bidzinska et al, 1993; Demura, Suzuki, Nakamura, Komatsu, Odagiri & Demura, 1989; Srivastava, Taylor & Mann, 1993; Gatenbeck, Eneroth, Johansson & Stromberg, 1987). Consequently, the current study determined if gonadal weights are altered by exposure to the chronic intermittent stress regimen and if gonadal weights were differentially affected in E, PF and C animals.

Lastly, animal models of prenatal exposure to ethanol have demonstrated deficits in immune function in E offspring compared to control offspring in such measures as reductions in the numbers of thymocytes (Ewald, 1989), altered T-cell responsiveness to mitogens (Weinberg & Jerrells, 1991; Wong, Chiappelli, Chang, Norman, Cooper, Branch & Taylor, 1991; Monjan & Mandell, 1980), and decreased responsiveness of T-lymphoblasts to

interleukin-2 (Norman, Change, Castle, Van Zuylen & Taylor, 1989). Chronic stress has also been shown to alter immune function, producing initially depressed lymphocyte proliferation to mitogens which may recover following prolonged exposure to stress (Monjan & Collector, 1977; Lysle, Cunnick & Rabin, 1990; Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988). Therefore, the current study assessed whether thymus and spleen cell counts are altered by exposure to chronic intermittent stress and are differentially affected in E, PF and C animals.

METHODS

Male and female offspring were randomly selected from each of the three prenatal treatment groups (i.e. E, PF, C) at approximately 70 to 110 d of age for assignment to 0 d, 6 d or 18 d of chronic intermittent stress. Twenty males and twenty females from each prenatal treatment group were assigned to each of the chronic stress conditions. Of the twenty males and twenty females, ten each were assigned to an acute stress condition and ten were assigned to a no acute stress condition. Since animals within a litter are not independent subjects, no more than one male and one female from any litter was assigned to a prenatal treatment x chronic stress condition x acute stress condition cell to control for litter effects. Although some attrition did occur, each cell always contained a minimum of nine animals.

Two days prior to beginning the 6 d or 18 d chronic stress regimens, animals were weighed and singly housed in 25 x 20 x 15 cm transluscent plastic cages. Between 0800 and 0900 hrs one day prior to beginning the stress regimen (i.e. d 0), basal samples were collected by cardiac puncture for analysis of CORT levels. Undisturbed or resting blood samples were also obtained by cardiac puncture the day after finishing the chronic stress regimens (i.e. d 7

or d 19). The following day (i.e. d 8 or d 20), animals were terminated. On all other days (i.e. d 1-6 and d 1-18), animals were subjected to the chronic stress regimen described in the General Methods.

Aside from routine cage cleaning, animals in the 0 d chronic stress condition remained group housed and undisturbed until three days prior to termination. At that time, these animals were singly housed in 25 x 20 x 15 cm translucent plastic cages in order to allow for the collection of basal blood samples. One day prior to termination, blood samples were obtained from animals in the 0 d chronic stress condition by cardiac puncture to determine basal CORT levels, and animals were weighed following blood sampling. Animals in the 0 d chronic stress condition were isolated from animals in the 6 d and 18 d chronic stress conditions at all times during the study to avoid disturbance from vocalizations and odor cues (Pitman, Ottenweller & Natelson, 1988). In order to maintain isolation, all blood sampling and termination procedures for animals in the 0 d chronic stress condition were conducted on different days than animals in the 6 d and 18 d chronic stress conditions. For animals in the 0 d chronic stress condition, the order of sampling and termination was counterbalanced for prenatal treatment. Animals in the 6 d and 18 d chronic stress conditions were sampled and terminated together. Therefore, for these animals, the order of sampling and termination was counterbalanced for prenatal treatment and chronic stress condition. The same procedures and personnel were used on all sampling and termination days.

An intraperitoneal (i.p.) injection of saline (0.5 cc in males and 0.3 cc in females) was used to compare HPA responses to an acute, novel stressor in E, PF and C males and females in the 0 d, 6 d and 18 d chronic stress conditions. On the day of sacrifice, half of the animals from each of the prenatal treatment groups (E, PF and C) and chronic stress conditions (0 d, 6

d, and 18 d) were subjected to an i.p. injection (i.e. the acute stress condition), returned to their home cages and terminated 20 min later. The remaining animals were not subjected to an i.p. injection in order to obtain measures of undisturbed (i.e. the no acute stress condition) hormone levels in animals in the 0 d, 6 d and 18 d chronic stress conditions.

Following termination and trunk blood collection, the right adrenals and gonads were removed, cleaned and weighed. In addition, spleens and thymuses were immediately removed and weighed from animals in the no acute stress condition and cell counts were obtained.

Radioimmunoassay (RIA).

Corticosterone. Samples were thawed, vortexed and centrifuged at 3500 rpm for 10 min at 4°C. CORT was then measured by RIA in plasma extracted in absolute ethanol (1:10 vol/vol for basal samples or for samples from the no acute stress condition; 1:20, 1:50, and 1:100 vol/vol for samples from the acute stress condition). Each sample in duplicate was incubated overnight with 100 µl (1,2,6,7,-^{3}H)-CORT (the tracer) and 100 µl of CORT antiserum. Following incubation, 200 µl of dextran-coated charcoal was used to absorb and precipitate free steroids. This procedure was conducted at 4°C in order to prevent the stripping of bound steroids. The radioactivity in each sample was then quantified by liquid scintillation counting in Dupont Formula 989. Total CORT (bound plus free) expressed in µg/100 ml was determined by this method. The absolute amount of hormone in the sample was calculated by comparison with a standard curve generated by incubating varying amounts of known hormone (standards) with the tracer and antiserum.

Antiserum was obtained from Immunocorp, Montreal, PQ; (1,2,6,7,-^{3}H)-CORT was obtained from DuPont, New England Nuclear, Mississauga, ON; and unlabelled CORT for standards was obtained from Sigma, St. Louis, MO. Dextran-coated charcoal, prepared in the laboratory, was used to absorb and precipitate free steroids after incubation.

ACTH. ACTH was measured by RIA using a kit obtained from Incstar Inc, Stillwater, MN, USA. Samples were thawed and 50 µl of each plasma sample was added in duplicate to individual 12 x 75 mm glass tubes on ice. Only plastic equipment was used to transfer the plasma because ACTH binds readily to glass. The samples and standards were then incubated overnight at -4°C with 100 µl rabbit anti-ACTH antiserum and 100 µl synthetic ACTH labelled with I^{125} (tracer). Following incubation, 250 µl of normal rabbit serum pre-precipitated with goat anti-rabbit serum and polyethelene glycol was added to the tubes in order to separate the bound from the free hormone. The tubes were vortexed, incubated for 15-25 min at room temperature and centrifuged for 20 min at 3500 rpm at room temperature. The supernatant was immediately decanted from the sample tubes and the pellets were counted in a gamma counter. The absolute amount of hormone in the sample was calculated by comparison with a standard curve generated by incubating varying amounts of hormone with the tracer and binding proteins.

The antiserum has 100% cross-reactivity with porcine $ACTH_{1-39}$ and human $ACTH_{1-24}$, and less than 0.01% cross-reactivity with α-melanin stimulating hormone, β-endorphin, β-lipotropin, leucine enkaphalin, methionine enkephalin, bombesin, calcitonin, parathyroid hormone, FSH, vasopressin, oxytocin and substance-P. The lower limit of

sensitivity of this assay is 15 pg/ml. Total ACTH expressed in pg/ml was determined by this method.

Determination of Spleen and Thymus Weights and Cell Counts.

Spleens and thymuses were removed, weighed, and placed in 10 ml of media consisting of 0.05 M phosphate buffered saline (PBS) (1.31 g sodium phosphate monobasic and 5.75 g sodium phosphate dibasic in 1 L sterile saline at pH 7.4) and 10% fetal bovine serum to maintain cell viability. Single cell suspensions of lymphocytes were obtained by gently pressing the tissues through fine wire mesh screens using the flat end of a syringe plunger. Cells and media were collected in a petri dish and then transferred to a 15 ml conical centrifuge tube. The petri dish was rinsed with 2 ml of media and this was added to the centrifuge tube. Cells were centrifuged at 2400 rpm for 5 min and supernatant was discarded. Red blood cells were lysed by adding 2 ml of distilled water to the cell pellet and vortexing. Two min after vortexing, 10 ml of PBS was added to the sample which was then centrifuged at 2400 rpm for 5 min. After centrifuging, the supernatant was discarded. The cell pellet was resuspended in 1 ml PBS and 10% fetal calf serum and appropriate dilutions for enumeration were made, ranging from 1:100 to 1:500. Nucleated cell counts were obtained using a Coulter Counter (Model T660, Coulter Electronics, Hialeah, FL).

Statistical Analyses.

All data were analyzed by analysis of variance (ANOVA). Data on basal, pre-chronic stress and undisturbed, post-chronic stress CORT levels obtained via cardiac puncture from animals in the 6 d and 18 d chronic stress conditions were analyzed by a repeated measures

ANOVA. In addition, basal CORT levels obtained via cardiac puncture from animals in the 0 d, 6 d and 18 d chronic stress conditions were analyzed by a sex x prenatal treatment x chronic stress condition ANOVA. Significant main effects or interactions were further analyzed by Tukey post-hoc paired comparison tests.

Due to expected sex differences (Kitay, 1961), data on CORT and ACTH levels obtained at termination were analyzed separately for males and females using three independent variables: prenatal treatment, chronic stress condition and acute stress condition. When appropriate, ANOVAs were followed by Tukey post-hoc paired comparison tests.

Body weights at termination were analyzed as a percent of initial body weights to measure body weight gain. Organ weights were calculated as a proportion of total body weights at time of termination. Spleen and thymus cell counts were analyzed as a proportion of organ weights. The data were analyzed by sex x prenatal treatment x chronic stress condition ANOVAs. Data from males and females were then analyzed separately as above.

RESULTS

Developmental Data.

Ethanol intake by pregnant females was constantly high throughout gestation averaging 8.07 ± 0.28, 10.59 ± 0.27, 10.70 ± 0.14 g/kg bw for gestation wk 1, 2 and 3, respectively. Ethanol intake was significantly increased in wks 2 and 3 of gestation compared with intake in wk 1 [$F(2, 42) = 39.86$, $p < 0.001$; post hoc tests, p's < 0.001].

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group [$F(2, 44) = 23.14$, $p < 0.001$] and days [$F(3, 132) = 815.52$, $p < 0.001$], as well as a group x days interaction [$F(6, 132) = 31.21$, $p < 0.001$]. Post-hoc tests indicated that E and PF females weighed significantly less than C females on d 7, 14 and 21 of gestation (p's < 0.001). Analysis of maternal weights during lactation revealed a significant main effect of days [$F(3, 129) = 256.77$, $p < 0.001$] and a group x days interaction [$F(6, 129) = 7.40$, $p < 0.001$]. E and PF females weighed significantly less than C females on lactation d 1 (p's < 0.005). However, there were no significant differences among groups on lactation d 8, 15, and 22 (Table 1.1).

The length of gestation did not differ among E, PF or C females (23.1, 22.8 and 23.1 days, respectively) (Table 1.1). In addition, there were no significant differences among groups for litter size, numbers of male and female pups or number of stillborn pups. Repeated measure ANOVAs on male and female pup body weights indicated significant main effects of group [$F(2, 43) = 12.91$; $F(2, 43) = 12.53$, p's < 0.001, respectively] and days [$F(3, 129) = 4985.75$; $F(3, 129) = 5713.32$, p's < 0.001, respectively] as well as group x days interactions [$F(6, 129) = 10.63$ and $F(6, 129) = 19.85$, p's < 0.001, respectively]. Post hoc tests indicated that for both males and females, E and PF pups weighed significantly less than C pups on lactation d 1, 8, 15, and 22 (p's < 0.01) (Table 1.2).

Plasma CORT Levels.

Basal CORT. For animals in the 6 d and 18 d chronic stress conditions, basal CORT levels were determined in samples obtained by cardiac puncture one day before beginning the chronic stress regimens and one day after finishing the chronic stress regimens (i.e. 1 d prior

to termination). A repeated measures ANOVA was used to ascertain if basal CORT levels prior to the initiation of the chronic stress regimens were significantly different from CORT levels measured on the day following completion of the 6 d or 18 d chronic stress regimens. No significant differences were revealed between basal CORT levels prior to the 6 d and 18 d chronic stress conditions and CORT levels for undisturbed animals subsequent to the 6 d or 18 d chronic stress conditions (Fig 1.1). There were also no differences among E, PF and C animals.

For animals in the 0 d chronic stress condition, basal CORT levels were measured 1 d prior to termination. An overall ANOVA (i.e. sex x prenatal treatment x chronic stress condition) on basal CORT levels comparing animals in the 0 d, 6 d and 18 d chronic stress conditions revealed a significant main effect of sex [$F(1, 289) = 84.67$, $p < 0.001$]. As expected (Kitay, 1961), post-hoc analysis of the data collapsed across prenatal treatments and chronic stress conditions indicated that females had significantly higher basal CORT levels than males. Due to these sex differences in hormone levels, data on CORT and ACTH levels for males and females were analyzed separately.

Prenatal treatment (E, PF, C) x chronic stress condition (0 d, 6 d, 18 d) ANOVAs on basal CORT levels for both males and females revealed significant main effects of chronic stress on CORT levels for males [$F(2, 145) = 10.53$, $p < 0.001$] and for females [$F(2, 144) = 4.40$, $p < 0.05$]. Post hoc tests indicated that males and females in the 6 d and 18 d chronic stress conditions had significantly decreased basal CORT levels 1 d prior to beginning the chronic stress regimens compared to basal CORT levels for males and females in the 0 d chronic stress condition 1 d prior to termination (p's < 0.05) (Fig 1.2). These significant differences in pre-stress basal CORT levels among animals in the 0 d, 6 d and 18 d conditions

could not be explained by any identifiable factors in either the handling of the animals or in the experimental conditions. Despite the fact that both males and females from E, PF and C groups were randomly assigned to experimental conditions, there appeared to be a small but statistically significant bias in the basal hormone levels of animals assigned to the 0 d condition compared to animals assigned to the 6 d and 18 d conditions. Consequently, for analysis of CORT and ACTH levels obtained on the termination day, hormone levels of males and females in the 6 d and 18 d chronic stress conditions were analyzed omitting direct comparisons to animals in the 0 d chronic stress condition.

Plasma CORT and ACTH Levels.

CORT and ACTH levels from E, PF and C animals in the 6 d and 18 d chronic stress conditions were determined in samples obtained on the termination day under undisturbed (resting) conditions (i.e. no acute stress condition) or following an i.p. saline injection (i.e. acute stress condition).

CORT and ACTH Levels for Animals in the No Acute Stress Condition. For males and females in the no acute stress condition (i.e. blood samples obtained from undisturbed animals), CORT and ACTH levels were analyzed separately by prenatal treatment (E, PF, C) x chronic stress condition (6 d, 18 d) ANOVAs. There were no significant effects of prenatal treatment or previous exposure to chronic stress on undisturbed CORT levels for either males or females (Fig 1.3). In contrast, there was a significant effect of chronic stress condition on undisturbed ACTH levels for males [$F(1, 44) = 6.52$, $p < 0.05$]. Post hoc tests revealed that males in the 18 d chronic stress condition had higher undisturbed plasma ACTH levels than males in the 6 d chronic stress condition ($p < 0.05$) (Fig 1.4). There was no significant effect

of chronic stress condition on ACTH levels for females in the no acute stress condition. Furthermore, there were no significant differences in ACTH levels among prenatal treatment groups in either males or females in the 6 d and 18 d chronic stress conditions.

CORT and ACTH Responses to Acute Stress. CORT and ACTH levels were analyzed in all animals to determine if E, PF and C animals in the 6 d and 18 d chronic stress conditions showed differential pituitary-adrenal responses to an acute stressor.

Prenatal treatment x acute stress condition ANOVAs on male and female plasma CORT levels revealed significant main effects of acute stress in both the 6 d and 18 d chronic stress conditions [$F(1, 47) = 20.19$; $F(1, 45) = 13.19$, p's < 0.005, respectively]. For males and females within both the 6 d and the 18 d chronic stress conditions, CORT levels were significantly increased following the acute stressor compared to CORT levels for animals in the no acute stress condition (p's < 0.005) (Fig 1.5-1.6).

Analysis of plasma ACTH levels for males revealed significant main effects of acute stress for animals in the 6 d chronic stress condition [$F(1, 45) = 19.23$, $p < 0.001$]. Post hoc tests indicated that ACTH levels were significantly increased following the acute stress condition compared to levels in the no acute stress condition ($p < 0.001$). In contrast, males in the 18 d condition showed no significant ACTH response to the acute stress ($p = 0.656$) (Fig 1.7-1.8).

For females, prenatal treatment x acute stress condition ANOVAs on ACTH levels for animals in both the 6 d and 18 d chronic stress conditions revealed significant main effects of acute stress [$F(1, 47) = 12.13$; and $F(1, 47) = 5.98$, p's < 0.02]. For females in both the 6 d and 18 d chronic stress conditions, post hoc tests indicated that females exposed to acute

stress had significantly higher ACTH levels than females in the no acute stress condition (p's < 0.05) (Fig 1.7-1.8).

There were no significant differences in ACTH levels among E, PF and C males or females in either the 6 d or 18 d chronic stress conditions.

Further analyses were then done to compare CORT and ACTH levels among groups following acute stress. Prenatal treatment x chronic stress condition ANOVAs revealed a marginal effect of chronic stress condition on CORT levels for males [$F(1, 41) = 3.09$, $p = 0.086$]. Post hoc tests revealed that males in the 6 d chronic stress condition showed a trend toward higher CORT levels than males in the 18 d chronic stress condition ($p = 0.086$). For females, however, there were no significant differences between CORT levels for animals in the 6 d and 18 d chronic stress conditions (Fig 1.3).

As with CORT levels, there was also a marginal effect of chronic stress condition on ACTH levels for males [$F(1, 43) = 3.46$, $p = 0.070$]. Post hoc tests indicated that males in the 6 d chronic stress condition had a trend toward higher ACTH levels than males in the 18 d chronic stress condition ($p = 0.070$). For females in the acute stress condition, there were no significant differences in ACTH between animals in the 6 d and 18 d chronic stress conditions (Fig 1.4).

There were no significant differences in CORT or ACTH levels among E, PF and C males and females in either the 6 d or 18 d chronic stress condition.

Body Weights.

For initial body weights, separate prenatal treatment (E, PF, C) x chronic stress condition (0 d, 6 d, 18 d) ANOVAs revealed significant main effects of chronic stress for

males [$F(2, 151) = 25.80$, $p < 0.001$] and for females [$F(2, 160) = 21.29$, $p < 0.001$]. Post hoc tests indicated that, prior to exposure to the chronic intermittent stress regimens, males in 6 d chronic stress condition had significantly lower body weights than males in the 0 d chronic stress condition ($p < 0.005$). In addition, males and females in the 18 d chronic stress condition had significantly lower initial body weights than males and females in both the 0 d and 6 d chronic stress conditions (p's < 0.001).

For body weights at termination, separate prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of chronic stress condition for males [$F(2, 151) = 32.96$, $p < 0.001$] and for females [$F(2, 160) = 21.01$, $p < 0.001$]. Post hoc tests indicated that males in the 6 d chronic stress condition had significantly higher body weights at termination than males in the 18 d chronic stress condition ($p < 0.05$). Furthermore, males and females in the 6 d and 18 d chronic stress conditions had significantly lower body weights at termination than males and females in the 0 d chronic stress condition (p's < 0.001).

Due to differences in body weights among chronic stress conditions prior to exposure to the chronic stress regimens, body weights at termination were analyzed as a percent of initial body weight to obtain a measure of body weight gain. Separate prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of chronic stress condition for males [$F(2, 151) = 30.25$, $p < 0.001$] and females [$F(2, 160) = 27.34$, $p < 0.001$] on body weight gain. Post hoc tests indicated that males and females in the 6 d chronic stress condition gained less body weight compared to males and females in the 0 d and 18 d chronic stress conditions, respectively (p's < 0.001). There were no significant differences in body weight gain among prenatal treatment groups for either males or females in the 0 d, 6 d and 18 d chronic stress conditions (Fig 1.9).

Organ Weights.

Absolute Adrenal Weights. Separate prenatal treatment x chronic stress condition ANOVAs on absolute adrenal weights revealed significant main effects of group and marginal effects of chronic stress condition for males [$F(2\ 151) = 8.89$, $p < 0.001$; $F(2, 151) = 2.49$, $p = 0.087$] and females [$F(2, 160) = 3.73$, $p < 0.05$; $F(2, 160) = 2.55$, $p = 0.081$]. Post hoc tests indicated that E males had lower absolute adrenal weights than PF and C males while PF females had lower absolute adrenal weights than C females (p's < 0.05). Furthermore, there were trends for males and females in the 6 d chronic stress condition to have higher absolute adrenal weights than males and females in the 0 d chronic stress condition ($p = 0.099$; $p = 0.066$, respectively)

Absolute adrenal weights were calculated as a proportion of total body weight at time of termination in order to account for sex and age effects on body weight.

Adrenal Weight: Body Weight Ratios. Prenatal treatment x chronic stress condition ANOVAs on adrenal weight: body weight ratios (i.e. relative adrenal weights) revealed significant main effects of chronic stress condition for both males [$F(2, 151) = 23.24$, $p < 0.001$] and females [$F(2, 160) = 14.13$, $p < 0.001$]. Post hoc tests indicated that both males and females in the 6 d and 18 d chronic stress conditions had significantly larger relative adrenal weights than males and females in the 0 d chronic stress condition, respectively (p's < 0.001). There were no significant differences in relative adrenal weights among prenatal treatment groups for males or females in the 0 d, 6 d and 18 d chronic stress conditions (Fig 1.10).

Absolute Gonadal Weights. Separate prenatal treatment x chronic stress condition ANOVAs on absolute gonadal weights revealed a marginal effect of prenatal treatment for

males [$F(2, 149) = 2.91$, $p = 0.057$]. Further analysis indicated that there was a trend for PF males to have lower gonadal weights than C males ($p < 0.05$). There were no significant effects of prenatal treatment on gonadal weights for females nor were there significant effects of chronic stress condition on absolute gonadal weights for males or females.

Absolute gonadal weights were calculated as a proportion of total body weight at time of termination in order to account for age effects on body weight.

Gonadal Weight: Body Weight Ratios. For males, a prenatal treatment x chronic stress condition ANOVA revealed a significant main effect of chronic stress condition on gonadal weight: body weight ratios (i.e. relative gonadal weights) [$F(2, 149) = 28.76$, $p < 0.001$]. Post hoc tests indicated that males in the 6 d and 18 d chronic stress conditions had significantly higher relative gonadal weights compared to males in the 0 d chronic stress condition (p's < 0.001). In addition, males in the 18 d chronic stress condition had significantly larger relative gonadal weights than males in the 6 d chronic stress condition ($p < 0.05$). There were no significant differences in relative gonadal weights among E, PF and C males (Fig 1.11).

For females, there were no significant effects of prenatal treatment or chronic stress condition on relative gonadal weights.

Cell Count: Organ Weight Ratios.

Lymphoid cell counts were analyzed as a proportion of lymphoid organ weights.

Thymus Cell Counts. A sex x prenatal treatment x chronic stress condition ANOVA on thymus cell counts revealed significant main effects of sex [$F(1, 141) = 16.85$, $p < 0.001$] and chronic stress condition [$F(2, 141) = 26.42$, $p < 0.001$] as well as a marginal prenatal

treatment x chronic stress condition interaction [$F(4, 141) = 2.17$, $p = 0.075$]. Overall, collapsed across prenatal treatment groups and chronic stress conditions, females had higher thymus cell counts than males ($p = 0.075$).

When data from males and females were analyzed separately, prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of chronic stress condition on thymus cell counts for both males [$F(2, 67) = 11.66$, $p < 0.001$] and females [$F(2, 74) = 15.32$, $p < 0.001$]. Post hoc analysis indicated that males and females in the 6 d and 18 d chronic stress conditions had lower thymus cell counts than males and females in the 0 d chronic stress condition, respectively (p's < 0.001). There were no significant differences among prenatal treatment groups in either males or females (Fig 1.12).

Spleen Cell Counts. A sex x prenatal treatment x chronic stress condition ANOVA on spleen cell counts revealed significant main effects of sex [$F(1, 149) = 8.39$, $p < 0.005$] and chronic stress condition [$F(2, 149) = 3.20$, $p < 0.05$]. Overall, females had higher spleen cell counts than males.

When data from males and females were analyzed separately, there were no significant effects of chronic stress condition on spleen cell counts for males. For females, the ANOVA revealed a significant main effect of chronic stress condition [$F(2, 76) = 3.64$, $p < 0.05$]. Post hoc tests indicated that females in the 6 d chronic stress condition had significantly lower spleen cell counts than females in the 0 d chronic stress condition ($p < 0.05$). There was no significant effects of prenatal treatment on spleen cell counts for either males or females (Fig 1.13).

DISCUSSION

This is one of the first studies to investigate the interactive effects of prenatal ethanol exposure and exposure to chronic stress in adulthood on parameters of both endocrine and immune function. Specifically, this study examined pituitary-adrenal activity, body and organ weights and lymphoid cell counts for E, PF and C animals exposed to 0 d, 6 d or 18 d of a chronic intermittent stress regimen consisting of a combination of psychological and physiological stressors. The data show that pituitary-adrenal, gonadal, and immune measures in E, PF and C animals were not differentially affected by exposure to either chronic or acute stress. However, chronic stress did affect both the HPA and HPG axes as well as the immune system in all animals and the duration of exposure differentially influenced the impact of chronic stress on these systems.

The form of liquid ethanol administration used in this study has proven to be an effective method of administering ethanol to pregnant animals, consistently resulting in elevated blood ethanol levels (Lieber & DeCarli, 1982). In studies using the liquid diet method in mice, a pattern of malformations resembling that of human FAS has been produced (Chernoff, 1977; Randall, Taylor & Walker, 1977). In studies using the liquid diet method in rats, differences in body weight have been reported with E and PF offspring generally weighing less than C offspring (Weinberg, 1992b; Gallo & Weinberg, 1986). In accordance with these previous findings, the results from the current study show that E and PF pups weighed significantly less than C pups from birth through weaning, indicating that prenatal treatment produced changes in postnatal measures. The finding that both E and PF offspring had decreased weights compared to C offspring suggests that the effect of ethanol on body weight was at least partially nutritionally-mediated.

In the current study, E, PF and C offspring in the 6 d and 18 d chronic stress conditions did not exhibit any change in undisturbed CORT levels measured prior to and subsequent to exposure to the chronic stress regimens. There were also no significant differences in undisturbed CORT or ACTH levels for E, PF, and C animals in the 6 d and 18 d chronic stress conditions on the day of termination. Previous investigators have reported that, after weaning, E animals display hormonal hyperresponsive manifested as enhanced pituitary-adrenal activation to stressors as well as delayed or deficient recovery to basal levels when tested in adulthood (Taylor, Branch, Kokka & Poland, 1983; Weinberg, 1988; Weinberg, 1992b). However, this difference has been apparent only after stress. Most reports indicate that E offspring do not appear to differ from PF or C animals when tested in adulthood under basal conditions (Taylor, Branch, Van Zuylen and Redei, 1988; Nelson et al, 1986; Weinberg, 1991). Furthermore, previous studies typically used stressors that were of acute or relatively short duration. In the present study, pituitary-adrenal responses following exposure to a chronic stress regimen were examined.

The current study was designed to examine whether exposure to chronic stress produced differential effects on undisturbed or 'basal' pituitary-adrenal activity in E, PF and C offspring. The lack of significant differences in CORT and ACTH levels among prenatal treatment groups after 6 d and 18 d of chronic stress suggests that prenatal ethanol exposure does not influence basal activity in animals even after prolonged exposure to stress. It is also possible that the chronic stressors used in the present study may not have been well-suited to identifying differences among the prenatal treatment groups since it has been reported that some types of stressors do not distinguish among E, PF and C animals (Taylor, Branch, Liu & Kokka, 1982). Another possibility involves the issue of predictable and unpredictable

schedules of stress exposure. Weinberg (1992a) found that basal CORT levels were higher following prior exposure to predictable rather than unpredictable stressors, suggesting that the animals exhibited HPA activation due to the anticipation or expectancy of the predictable stressor. Therefore, the use of an unpredictable schedule of stressor presentation in the current study may have been less likely to result in alterations of basal CORT levels than a predictable schedule.

Although previous studies have found that prenatal ethanol exposure induces HPA hyperresponsiveness to acute stressors, research on responses to chronic stress has been limited. In one study examining the effects of repeated exposure to restraint, Weinberg, Taylor and Gianoulakis (in press) demonstrated that E males and females showed significantly increased plasma levels of ACTH compared to their respective PF and C controls following repeated exposures to the stressor. In addition, marginal increases in the CORT response of E males compared to controls were observed. These data indicate that animals prenatally exposed to ethanol may exhibit hormonal hyperresponsiveness following repeated exposures to a stressor. However, Weinberg, Taylor and Gianoulakis concluded that the patterns of response differed depending on the sex of the animal, the number and duration of exposures to the stressor and whether CORT or ACTH were measured.

In the present study, there were no differences in body weight gain and relative adrenal weights among E, PF and C animals in the 0 d, 6 d and 18 d chronic stress conditions. Body weight gain was assessed in order to account for initial differences in body weight among animals prior to exposure to the chronic stress conditions. Furthermore, although there were differences among prenatal treatment groups in absolute adrenal weights at termination, the

ratio of adrenal weight to body weight at termination was assessed to provide a more accurate measure of changes in adrenal weight relative to changes in body weight.

Since studies on the effects of chronic cold, isolation or restraint on rats have generally reported activation of the pituitary-adrenal system, reductions in body weight gain and increases in adrenal weight (Daniels-Severs, Goodwin, Keil & Vernikos-Danellis, 1973; Imms, 1967; Hashimoto, 1988), the finding that body weight gain and relative adrenal weights as well as pituitary-adrenal activation were not different in E, PF and C animals in the 6 d and 18 d chronic stress conditions suggests that exposure to chronic stress equally affected pituitary-adrenal activity for animals in all three prenatal treatment groups.

Another objective of this study was to determine if prior exposure to chronic stress differentially affected the pituitary-adrenal responses of E, PF and C animals to a novel, acute stressor. The data show that E, PF and C animals in the 6 d and 18 d chronic stress conditions did not display differences in pituitary-adrenal activity in response to the acute stress condition. The present finding was unexpected since previous investigators have reported hormonal hyperresponsiveness in E animals following acute stress (Taylor, Branch, Kokka & Poland, 1983; Weinberg, 1988; Weinberg, 1992b) and exposure to chronic stress has been reported to make the pituitary-adrenal system hyperresponsive to a novel, acute stressor (Vernikos, Dallman, Bonner, Katzen & Shinsako, 1982). The current finding suggests that the hormonal hyperresponsiveness to acute stressors previously reported in E animals compared to controls may be attenuated by prior exposure to chronic stress. In addition, the hormonal hyperresponsiveness to a novel, acute stressor demonstrated following exposure to chronic stress may not be differentially apparent in E, PF and C animals.

Procedural variations between this study and previous studies may account for some of the differences in findings. Taylor, Branch, Van Zuylen and Redei (1988) reported that ACTH levels only differed among E, PF and C animals 10 min after exposure to an acute stressor and appeared similar at 5 and 15 min after application of the stressor. Therefore, the 20 min sampling time used in the present study may not have been the optimal point to identify differences in CORT and ACTH levels among E, PF and C animals following the acute stress. Furthermore, Nelson, Taylor, Lewis, Poland, Redei and Branch (1986) reported hyperresponsiveness to stress in E offspring only at the peak of the circadian rhythm. In the current study, samples were obtained at the trough of the circadian rhythm since the magnitudes of the CORT and ACTH responses to stress are greatest when stress is presented during the trough of the diurnal rhythm (Nicholson, Lin, Mahmoud, Campbell, Gillham & Jones, 1985; Bradbury, Cascio, Scribner & Dallman, 1991). Consequently, the observation that the acute stress condition did not differentially affect pituitary-adrenal activity among E, PF and C suggests that differences among prenatal treatment groups might only be revealed at specific points in the circadian rhythm.

Although there were no effects of prenatal ethanol exposure on pituitary-adrenal activity in this study, chronic stress had a number of significant effects across all groups. The current study was designed to examine several specific questions concerning pituitary-adrenal activity in response to chronic stress. The first aim of the study was to determine whether basal pituitary-adrenal activity was altered by exposure to chronic stress. For animals in the 6 d and 18 d chronic stress regimens, basal CORT levels measured prior to beginning the chronic stress regimens were compared with undisturbed or 'basal' CORT levels subsequent to 6 d or 18 d of chronic stress. In agreement with previous investigations (Armario,

Restrepo, Castellanos & Balasch, 1985; Hashimoto, Suemaru, Takao, Sugawara, Makino & Ota, 1988), there were no significant changes in CORT levels in male or female animals after exposure to chronic stress in the current study.

Although the response of the pituitary-adrenal axis to novel or acute stress typically produces increases in glucocorticoid levels within 10 to 15 min after presentation of an aversive stimulus (Lovely, Pagano, & Paolino, 1972), exposure to more chronic or prolonged stress may result in alterations of pituitary-adrenal activity over time. In fact, early work by Hans Selye who defined the general adaptation syndrome to stress demonstrated that an animal's stress response decrements over time despite continued application of the stressor (Selye, 1937 cited in Pitman, Ottenweller & Natelson, 1988). Many others have also shown that chronic exposure to individual stressors such as restraint, cold, water deprivation and single housing may eventually lead to a reduction in CORT release compared to initial responses or may lead to an almost complete absence of an adrenal response to the stressor (Riegle, 1973; Hashimoto, Suemaru, Takao, Sugawara, Makino & Ota, 1988). This may occur more rapidly for some stressors such as single housing and restraint (minutes) and slower for others such as water restriction (weeks) (Sakellaris & Vernikos-Danellis, 1975).

These previous studies and the results of the present study provide evidence for a phenomenon commonly termed adaptation. Adaptation has been used to refer to that stage during continuous or chronic exposure to an aversive stimulus that is characterized by a return toward pre-stress levels of circulating corticosteroids (Dallman, 1993). Significantly, this is the first study to demonstrate that basal pituitary-adrenal levels are not altered after different lengths (6 d and 18 d) of exposure to a chronic intermittent stress regimen consisting of a set of different stressors. Whereas previous studies have focused on the effects of continual

exposure to one particular stressor (Hashimoto, Suemaru, Takao, Sugawara, Makino & Ota, 1988; Sakellaris & Vernikos-Danellis, 1975), the diversity of stressors used in the present study and the manner in which they were presented (e.g. superimposing one stressor onto another) indicate that adaptation may occur under a variety of conditions.

The manner in which the stressors were presented in the present study was chosen in order to reduce the possibility of habituation and maintain maximally elevated CORT levels throughout application of the stressor. In addition, unpredictability of the stressor presentation and lack of control are elements which have been shown to negatively impact on an animal's ability to cope with aversive stimuli (Weinberg & Levine, 1980). In fact, observations of rats exposed to chronic stress have indicated that the psychological aspects of the stressful situation are important in determining the magnitude of the physiological response (Bliss & Zwanziger, 1966). Thus, the findings in the present study add to the growing literature on pituitary-adrenal adaptation to physiological stressors by indicating that a combination of psychological and physiological stressors presented at random times may result in pituitary-adrenal adaptation.

As indicated previously, studies on the effect of chronic stress on rats have generally reported activation of the pituitary-adrenal system, reductions in body weight gain and increases in adrenal weight (Daniels-Severs, Goodwin, Keil, Vernikos-Danellis, 1973; Imms, 1967; Hashimoto et al, 1988). Furthermore, clinical studies have shown that in people who have died after low grade chronic stresses, adrenal weight was increased when measured immediately post-mortem (Landing, 1955 cited in Dallman et al, 1992). Thus, reductions in body weight gain and increases in adrenal weight may represent objective evidence of the excitatory state characteristic of prolonged stress (Weiss, 1970).

The results of this study confirm the general pattern of body weight gain and organ weight changes reported previously. Both males and females in the 6 d chronic stress condition showed lower body weight gain compared to males and females in the 0 d chronic stress condition, although body weight gain for animals in the 18 d chronic stress condition was not significantly different from animals in the 0 d chronic stress condition. In addition, both males and females in the 6 d and 18 d chronic stress conditions had increased relative adrenal weights compared to animals in the 0 d chronic stress condition, lending support to the general conclusion that the chronic stress regimens resulted in HPA activation. Although Selye reported that adrenal weight initially increased and then subsequently returned to baseline levels despite continued exposure to the aversive stimulus (Selye, 1937 as cited in Pitman, Ottenweller & Natelson, 1988), variations in the duration and intensity of the aversive stimuli may account for differences between studies. Measures of body weight gain did not differ significantly between animals in the 0 d and 18 d chronic stress conditions and continued exposure to the chronic stress regimen used in this study may have also resulted in a return of relative adrenal weights to pre-stress levels.

Although adaptation is characterized by a return toward pre-stress levels of pituitary-adrenal hormones during chronic stress, exposure to chronic stress has reportedly resulted in a triphasic pattern of response during continuous exposure to an aversive stimulus. Bohus (1969 cited in Sakellaris & Vernikos-Danellis, 1975) found that ACTH secretion in response to immobilization initially increased rapidly, decreased after four hours and increased again after twelve hours of exposure. Thus, the decline of HPA activity toward pre-stress levels following initial elevations may either be maintained or followed by a subsequent rise in circulating corticosterone or ACTH. The results of the present study are consistent with a

triphasic pattern of pituitary-adrenal response; males in the 18 d chronic stress condition had significantly elevated undisturbed ACTH levels compared to males in the 6 d chronic stress condition, suggesting that the length of exposure to chronic stress (6 d or 18 d) may differentially affect pituitary-adrenal activity in an undisturbed state. This finding lends support to the thesis of Dallman (Dallman, Jones, Vernikos-Danellis & Ganong, 1972) that repeated chronic stress may cause increases in ACTH-secreting mechanisms in order to compensate for or override negative feedback from glucocorticoids.

The observed increase in undisturbed ACTH levels for males in the 18 d chronic stress condition may have impacted on the ability of these males to mount a significant pituitary response to an acute stress, perhaps due to either a less pronounced release of CRF (Culman, Kopin & Saavedra, 1991) or a decrease in the readily releasable pituitary ACTH pool (Rivier & Vale, 1987). For males in the 6 d chronic stress condition as well as females in the 6 d and 18 d conditions, CORT and ACTH levels were significantly elevated in the acute stress condition compared to the no acute stress condition. However, for males in the 18 d chronic stress condition, ACTH levels between the no acute stress and acute stress conditions were not significantly different even though CORT levels were significantly increased in the acute stress condition compared to the no acute stress condition. These results indicate that exposure to different lengths of chronic stress does not alter the pituitary-adrenal response to an acute, novel stressor in females. In males, however, more prolonged exposure may alter undisturbed pituitary function and affect the response of the pituitary to an acute stressor.

The general ability of animals in the chronic stress regimens to respond to an acute stressor with increases in CORT and ACTH shows that the process of pituitary-adrenal adaptation which has been partially attributed to inhibition of the HPA axis by negative

feedback from circulating glucocorticoids (Rivier & Vale, 1987) still allows continued responsiveness of the system to stress. Altered secretion of vasopressin (AVP) may provide one possible mechanism for animals subjected to chronic stress to exhibit a continued ability to respond to acute stressors. Under normal circumstances, AVP acts in combination with CRF to release ACTH and, unlike CRF, AVP seems to be largely insensitive to negative feedback from CORT. Therefore, increased AVP-CRF ratios in neurons during chronic stress may be important in maintaining adequate ACTH secretion in response to a novel, acute stressor when negative feedback from elevated circulating CORT might normally result in reductions in pituitary activity. In support of this concept, research has indicated that the anterior pituitary in rats exposed to chronic immobilization becomes hypersensitive to AVP (Hashimoto et al, 1988).

Since several investigators (Akana & Dallman, 1992; Sakellaris & Vernikos-Danellis, 1975) have observed that exposure to chronic stress may increase an animal's pituitary-adrenal response to a novel, acute stressor, this study examined whether pituitary-adrenal activity following exposure to an acute stress differed depending on the length of exposure to a chronic stress regimen. The effects of 6 d and 18 d of chronic stress on pituitary-adrenal activity could not be compared to the effects of 0 d of chronic stress due to the initial elevations in CORT levels for animals in the 0 d chronic stress condition. However, the comparisons of animals in the 6 d and 18 d chronic stress regimens revealed trends for males in the 6 d chronic stress condition to have increased CORT and ACTH levels following exposure to an acute stress compared to males in the 18 d chronic stress condition, indicating that the length of exposure to chronic stress may impact on the level of pituitary-adrenal response to an acute stressor.

In summary, exposure to 6 d and 18 d of chronic stress did not significantly alter basal pituitary-adrenal activity. Although it could not be determined if animals exposed to 6 d or 18 d of chronic stress were hyperresponsive to an acute stress compared to animals in the 0 d chronic stress condition, animals subjected to different lengths of chronic stress did display differences in undisturbed hormone levels and hormone levels in response to acute stress conditions. In contrast, E, PF and C animals in 6 d and 18 d chronic stress conditions did not display differences in pituitary-adrenal activity nor did E offspring in the 6 d and 18 d chronic stress conditions exhibit hormonal hyperresponsiveness to acute stress compared to PF and C offspring.

Prenatal ethanol exposure has been reported to affect the HPG axis detrimentally at multiple levels in male offspring. Reductions in prenatal and postnatal testosterone surges (McGivern, Roselli & Handa, 1988; McGivern, Handa & Redei, 1993) as well as feminization in the size of the sexually dimorphic nucleus of the preoptic area of the hypothalamus (Barron, Tieman & Riley, 1988) have been observed in E males compared to PF and C males. However, the effects of prenatal ethanol exposure on testis weight have varied, with reports of increased, decreased or unchanged testis weight in adult E males compared to PF and C males (McGivern, Raum, Handa & Sokol, 1992, Udani, Parker, Gavaler & Van Thiel, 1985; McGivern, Holcomb & Poland, 1987). The present study did not find an effect of prenatal ethanol exposure on relative testicular weights, supporting recent conclusions by McGivern, Raum, Handa and Sokol (1992) that ethanol exposure does not induce long-term decreases in testicular mass in Sprague-Dawley males while it does in Wistar animals.

Although the effects of prenatal ethanol exposure on HPG function in females has been less studied than that in males, studies have reported delays in vaginal opening in female

rats prenatally exposed to ethanol (Esquifino, Sanchis & Guerri, 1986). In addition, decreased plasma LH (Esquifino, Sanchis & Guerri, 1986), and reduced hypothalamic LHRH levels (Morris, Harms, Peterson & MacArthur, 1989) have been demonstrated in E females compared to PF and C females. However, increased ovarian sensitivity to exogenous gonadotrophins (Rudeen & Hagaman, 1988) and increased ovarian weights (McGivern, Raum, Handa & Sokol, 1992) have also been reported in adult E females compared to PF and C females. Consistent with data for the males, the present study did not find an effect of prenatal ethanol exposure on relative gonadal (ovary) weights in females. Whether ethanol might affect reproductive function despite normal gonadal weights remains to be investigated.

Across all groups, there were significant effects of chronic stress on relative gonadal weights, and chronic stress differentially affected the HPG axes of males and females. Consistent with the effects on pituitary-adrenal activity in this study, chronic stress significantly affected relative gonadal weights only in males. Males in the 18 d chronic stress condition had significantly elevated relative gonadal weights compared to males in the 6 d chronic stress condition. In addition, males in both the 6 d and 18 d chronic stress conditions had increased relative gonadal weights compared to males in the 0 d chronic stress condition. In contrast, relative gonadal weights in females were not affected by the chronic stress conditions. Although absolute testicular weights were not altered by exposure to chronic stress, the increased relative gonadal weights in males in the 6 d and 18 d chronic stress conditions suggest that the testis may have been spared during exposure to chronic stress.

From these findings, it appears that chronic stress had a larger impact on physiological systems in males than in females. This corresponds to previous work showing marked effects of glucocorticoids on testosterone function in males. In fact, testosterone production has been

shown to vary depending on the type and length of the stress exposure. Acute stress has been reported to produce both increased (Gatenbeck, Eneroth, Johansson & Stromberg, 1987) and decreased testosterone levels (Armario, Restrepo, Hidalgo & Lopez-Calderon, 1987). In addition, several studies have reported reduced plasma tesosterone concentrations after prolonged exposure to stress (Bidzinska et al, 1993; Demura, Suzuki, Nakamura, Komatsu, Odagiri & Demura, 1989) whereas others have observed increased testosterone levels (Pollard, Bassett & Joss, 1980). However, changes in testosterone levels have not necessarily been associated with changes in testis weights (Pollard et al, 1980; Demura, et al 1989). Therefore, the elevations in relative gonadal weights reported in this study underscore the importance of examining several measures within a system when assessing the impact of chronic stress.

Few studies have been conducted on the effects of chronic stress on the HPG axis in females. The present study did not find any significant differences in relative ovarian weights among females in the 0 d, 6 d and 18 d chronic stress conditions. It is possible that estrogen may directly protect against the effects of chronic stress on various physiological systems or may indirectly influence these systems through its effect on HPA activity (Lesniewska, Miskowiak, Nowak & Malendowicz, 1990).

There were no significant differences among E, PF and C offspring in either thymus cell counts or spleen cell counts. Previous research has shown that prenatal ethanol exposure reduces thymus weight (Ewald & Walden, 1988), and alters the development of T lymphocyte populations, resulting in a decrease in thymus cell number in fetal mice (Ewald, 1989; Ewald & Walden, 1988). Species differences may therefore account for the current lack of differences among prenatal treatment groups in thymus cell counts, suggesting that thymus

cells in rats may not be as affected by prenatal ethanol exposure as thymus cells in mice. In addition, Redei, Clark and McGivern (1989) have reported increased thymus weights in E animals at 21 d of age compared to controls, indicating that age may also influence alterations in the thymus of E offspring.

Previous studies have reported functional impairments in splenocyte responsiveness to mitogen in E animals (Redei, Clark & McGivern, 1989; Norman, Chang, Castle, Van Zuylen & Taylor, 1989; Monjan & Mandell, 1980) compared to PF and C animals. However, changes in spleen cell counts in E, PF and C animals were not examined in these previous studies. Therefore, the present findings suggest that prenatal ethanol exposure may not alter the numbers of splenocytes even though previous studies indicate that it may affect their functional ability.

Although thymus cell counts and spleen cell counts were not affected by the prenatal treatment groups, they were significantly altered by exposure to chronic stress. Thymus cell counts were reduced in both males and females in the 6 d and 18 d chronic stress conditions compared to males and females in the 0 d chronic stress condition. Spleen cell counts were also decreased in females in the 6 d chronic stress condition compared to females in the 0 d chronic stress condition. However, spleen cell counts for females in the 18 d chronic stress condition were not significantly different from spleen cell counts for females in the 0 d chronic stress condition and spleen cell counts for males were unaffected by chronic stress.

These findings suggest that thymocytes may be more susceptible to the effects of chronic stress, whereas splenocytes may be only transiently affected. Exercise stress has been shown to decrease the percentages of pan T cells and $CD8^+$ T cells in the thymus while not altering the percentages of cells in the spleen or lymph nodes (Hoffman-Goetz, Thorne,

Simpson & Arumugam, 1989). Furthermore, the proportion of immature T-cells reportedly increases in the thymus, blood and spleen of stressed mice (Teshima, Sogawa, Kihara and Nakagawa, 1991) and immature thymocytes are localized in the thymic cortex, an area that has been demonstrated to be sensitive to steroids (Ishidate & Metcalf, 1963 as cited in Hori, 1993).

It is possible that the marked effect of chronic stress on thymus cell counts may be attributable to thymocyte vulnerability to the effects of glucocorticoids. In general, glucocorticoids appear to be primarily suppressive on immune function, particularly inhibiting cytokine production which may in turn reduce proliferation of immune cells. IL-1 and IL-2 production which enhance the proliferation of helper T cells are decreased by glucocorticoid administration (Knudsen, Dinarello & Strom, 1987; Grabstein, Dower, Gillis, Urdal & Larsen, 1986). In addition, CRF and ACTH have been shown to modulate cytokine activity on immune cells (Johnson, Torres, Smith, Dion & Blalock, 1984). Thus, hormones of the HPA axis can influence immune function and alterations in cytokine activity may impact more heavily on thymocytes than splenocytes since the thymus contains more immature immune cells which may require cytokines to develop.

The finding that spleen cell counts for females in the 0 d and 6 d chronic stress conditions were significantly different but spleen cell counts for females in the 0 d and 18 d chronic stress conditions were not suggests that splenocytes may be more resistant to the effects of prolonged chronic stress on the immune system. The current finding corresponds to previous reports that although stress in animals may be initially associated with immunosuppression, chronic stress may eventually result in a recovery of immune function. Monjan and Collector (1977) reported that sound stress initially suppressed splenocyte

proliferation to LPS and Con A in male rats, but that more extended exposure to the stress resulted in a recovery of responsiveness to these same mitogens. In addition, the suppressed reactivity of splenic lymphocytes to Con A was shown to diminish with repeated sessions of frequent electric footshock (Lysle, Cunnick & Rabin, 1990; Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988). Thus, the present study expands these previous reports to include adaptation of spleen cell counts after continued exposure to chronic stress.

The multiple effects of chronic stress on pituitary-adrenal activity, body weight gain and relative organ weights as well as lymphoid cell counts observed in the present study may reflect the mosaic of interactions among the HPA axis, the HPG axis and the immune system that can be altered by chronic stress. Thus chronic stress may alter functioning within these systems either directly or indirectly, by influencing the communication among systems. Furthermore, in assessing the effects of chronic and acute stress on the HPA, HPG and immune systems in E, PF and C animals, the findings of the present study suggest that the effects of prenatal ethanol exposure on the HPA axis in animals challenged with stress may not be as robust a phenomenon as previously thought and may be dependent upon methodological variations among experiments.

EXPERIMENT 2

Prenatal ethanol exposure is known to cause of variety of abnormalities of immune function in humans and animals. Children diagnosed with FAS show an increased incidence of life-threatening and minor infections (Johnson, Knight, Marmer & Steele, 1981). In addition, animal models of FAS have demonstrated reduced numbers of thymocytes (Ewald, 1989), altered T-cell responsiveness to mitogens (Weinberg & Jerrells, 1991; Wong, Chiappelli, Chang, Norman, Cooper, Branch & Taylor, 1992; Monjan & Mandell, 1980), and decreased responsiveness of T-lymphoblasts to IL-2 (Norman, Change, Castle, Van Zuylen & Taylor, 1989). Interestingly, immune aberrations associated with prenatal ethanol exposure may be sexually dimorphic, and male offspring appear more susceptible than female offspring to the adverse effects of prenatal ethanol exposure on immune function (Weinberg & Jerrells, 1991).

Exposure to chronic stress has been associated with immunosuppression which may eventually result in a return of immune function to pre-stress levels. Specifically, lymphocyte proliferative responses to LPS and Con A following exposure to sound stress (Monjan & Collector, 1977) and responses to Con A following exposure to electric footshocks (Lysle, Cunnick & Rabin, 1990; Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988) have been shown initially to decrease and then return to pre-stress levels. In contrast, Batuman, Sajewski, Ottenweller, Pitman and Natelson (1990) found decreased numbers of splenic $CD8^+$ T cells as well as reduced lymphocyte proliferative responses to PHA and Con A following exposure to a combined chronic stress regimen of restraint and mild electric footshock. These findings suggest that adaptation of immune function during chronic stress may be dependent upon the type and duration of the stressor.

The present study was designed to examine the interactive effects of prenatal ethanol exposure and exposure in adulthood to a chronic intermittent stress regimen (21 d) on lymphocyte proliferative responses to mitogens. Furthermore, this study examined the differential impact of prenatal ethanol exposure and exposure in adulthood to chronic stress on males and females since many of the previous studies tested only males. In order to characterize more completely the effects of prenatal ethanol exposure and chronic stress on immune function, three different mitogens are included in the study: (1) Con A which is able to selectively stimulate T cells; (2) PWM which is both a T and B cell mitogen and is able to stimulate B cells only in the presence of T cells; and (3) LPS which is a direct stimulator of B cell proliferation and differentiation independent of T cells (Paul, 1993).

METHODS

Male and female offspring were randomly selected from each of the three prenatal treatment groups (i.e. E, PF, C) at approximately 103 d of age for assignment to either 0 d or 21 d of chronic intermittent stress. Ten males and ten females from each of the prenatal treatment groups were each assigned to either the 0 d or 21 d chronic stress condition. As in the first study, no more than one male and one female from each litter were assigned to a prenatal treatment x chronic stress cell to control for litter effects since animals within a litter are not independent subjects. In addition, although some attrition did occur, each cell always contained a minimum of nine animals.

On the first day of the stress regimen (i.e. d 1), animals in the 21 d chronic stress condition were weighed and singly housed in 25 x 20 x 15 cm translucent plastic cages. On the same day, animals in the 0 d chronic stress condition were weighed and housed with other

animals to be terminated at the same time. Animals in the 21 d chronic stress condition were subjected to the chronic stress regimen described in the General Methods. Aside from routine cage cleaning, animals in the 0 d chronic stress were left undisturbed until termination. Consistent with the first study, animals in the 0 d chronic stress condition were isolated from animals in the 21 d chronic stress conditions at all times during the study to avoid disturbance from vocalizations and odor cues (Pitman, Ottenweller & Natelson, 1988).

On the morning of termination (i.e. d 22), animals in the 0 d and 21 d chronic stress condition were weighed and placed in separate rooms adjacent to the termination room. The order of termination was counterbalanced for prenatal treatment group and chronic stress condition with only one animal brought into the termination room at a time. Animals were terminated by decapitation and spleens were collected using aseptic techniques. The spleens were processed using the methods described below. Single cell suspensions of splenic lymphocytes were collected and lymphocyte proliferation in response to mitogens was assessed.

Isolation of lymphocytes from the spleen and lymphocyte proliferation to mitogens.

At the time of sacrifice, spleens were quickly removed using aseptic techniques and placed in room temperature RPMI 1640 medium supplemented with 10 mM Hepes, 10% fetal bovine serum, 1% glutamine, 5×10^{-5} M 2-mercaptoethanol and antibiotics (complete RPMI) to facilitate cell growth. In a sterile petri dish, single cell suspensions were obtained by gently pressing the spleen through a fine wire mesh screen using the end of a syringe plunger. Cells and media were transferred to a sterile 15 ml conical centrifuge tube; the petri dish was rinsed with 2 ml complete RPMI and this was added to the same tube. Debris and cell clumps were

allowed to settle for 3-5 min. The supernatant containing individual cells was transferred to a new sterile 15 ml centrifuge tube. Cells were centrifuged at 2400 rpm for 5 min and supernatant was discarded. The cell pellet was resuspended in 5 ml Tris-NH_4CL lysis buffer (to lyse red blood cells) and left to stand for 5 min. The sample was centrifuged at 2400 rpm for 5 min and the supernatant was discarded. The pellet was then washed twice (resuspended in 5 ml Hank's Balanced Salt Solutions [HBSS] and centrifuged) and resuspended in 1 ml complete RPMI. Nucleated cell counts were obtained using a Coulter Counter (Model T660, Coulter Electronics, Hialeah, FL) and the cell concentration was adjusted to 1×10^6 cells/ml using complete RPMI. Cell viability was determined using the trypan blue dye exclusion method and light microscopy. This method of lymphocyte isolation has been shown to result in greater than 85% viability.

The mitogens Concanavalin A (Con A) and pokeweed mitogen (PWM) at 3 different concentrations (0.5, 5.0, 50 μg/ml) were delivered in triplicate (50μl per well) to 96-well flat-bottom microtitre plates (Falcon 3075, Microtest, Ill); 200 μl of the cell suspension (2×10^5 cells/well) was then added to give final mitogen concentrations of 0.1, 1.0 and 10 μg/ml. The mitogen lipopolysaccharide (LPS) was tested at 4 concentrations (0.5, 5.0, 50, 500 μg/ml) yielding final mitogen concentrations of 0.1, 1, 10 and 100 μg/ml. Plates were incubated for 72 hr at 37°C in 5% CO_2. Six hours prior to harvesting, plates were pulsed with ^3H-thymidine (Amersham, Oakville ON) at a concentration of 1μCi per well. At the end of the incubation period, cells from each well were harvested onto glass fibre filters using an automated harvesting technique (Matrix 96, Packard, Meridien, CT) and incorporated radioactivity was determined using the Matrix 96 Direct Beta Counter (Packard, Meridien,

CT). Results were expressed as the mean counts per minute (cpm) of triplicate samples without adjusting for background ^{3}H-thymidine incorporation by unstimulated lymphocytes (e.g. Wong, Chiappelli, Chang, Norman, Cooper, Branch & Taylor, 1991).

Statistical Analyses.

All mitogen proliferation data were analyzed by sex x prenatal treatment x chronic stress condition ANOVAs. Data from males and females were then analyzed separately and, when appropriate, data from the 0 d and 21 d chronic stress conditions were analyzed separately. Significant main effects or interactions were further analyzed by Tukey post-hoc paired comparison tests.

Body weight at termination was analyzed as a percent of initial body weight to measure body weight gain. Adrenal weight was calculated as a proportion of total body weight at the time of termination. These ratios were analyzed by sex x prenatal treatment x chronic stress condition ANOVAs. Data from males and females were then analyzed separately as above.

RESULTS

Developmental Data.

Ethanol intake of pregnant females was constantly high throughout gestation averaging 9.54 ± 0.28, 12.74 ± 0.32, 11.80 ± 0.24 g/kg bw for gestation wks 1, 2 and 3, respectively. In addition, ethanol intake was significantly increased in wks 2 and 3 of gestation compared to intake in wk 1 [$F(2, 33) = 33.81$, $p < 0.001$; post hoc tests, p's < 0.001].

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group [$F(2, 42) = 6.24$, $p < 0.004$] and days [$F(3, 126) = 438.13$, $p < 0.001$], as well as a group x days interaction [$F(6, 126) = 9.34$, $p < 0.001$]. Post-hoc tests indicated that E and PF females weighed significantly less than C females on gestation d 7, 14 and 21 ($p < 0.05$). Analysis of maternal weights during lactation showed a significant main effect of days [$F(3, 111) = 303.42$, $p < 0.001$] and a group x days interaction [$F(6, 111) = 4.31$, $p < 0.001$]. However, post hoc tests did not reveal any significant differences among E, PF and C females on any day (Table 2.1).

There were significant differences in length of gestation among E, PF and C females [$F(2, 37) = 4.46$, $p < 0.018$]. Post-hoc tests revealed that E females had significantly longer gestation periods than C females ($p < 0.025$). In addition, there was a trend for PF females to have longer gestation periods than C females ($p = 0.06$) (Table 2.1). There were no significant differences among groups for litter size, numbers of male and female pups or number of stillborn pups.

A repeated measures ANOVA on body weights of male pups showed a significant main effect of days [$F(3, 111) = 1504.63$, $p < 0.001$]. All pups gained weight over the preweaning period but there were no differences among E, PF and C animals. A repeated measures ANOVA on body weights of female pups revealed significant main effects of group [$F(2, 36) = 4.38$, $p < 0.05$] and days [$F(3, 108) = 4572.19$, $p < 0.001$], as well as a marginal group x days interaction [$F(6, 108) = 2.00$, $p = 0.072$]. Post hoc tests indicated that E and PF female pups weighed less than C pups on lactation days 1 and 8 (p's < 0.01). In addition, E female pups weighed less than C pups on lactation day 22 ($p < 0.05$) (Table 2.2).

Body Weights.

There were no effects of prenatal treatment (E, PF, C) or chronic stress condition (0 d, 6 d, 18 d) on initial body weights for males or females. However, separate prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of chronic stress condition on body weight at termination for males [$F(1, 56) = 47.05$, $p < 0.001$] and females [$F(1, 57) = 31.74$, $p < 0.001$]. Post hoc tests indicated that males and females in the 21 d chronic stress conditions had lower body weights at termination than males and females in the 0 d chronic stress condition (p's < 0.001).

Body weights at termination were also analyzed as a percent of initial body weight to determine body weight gain. Separate prenatal treatment x chronic stress condition ANOVAs on body weight gain revealed significant main effects of chronic stress condition for males [$F(1, 56) = 248.80$, $p < 0.001$] and females [$F(1, 57) = 173.68$, $p < 0.001$]. Overall, males and females in the 21 d chronic stress condition gained less body weight than males and females in the 0 d chronic stress condition (p's < 0.001). There were no significant differences in body weight gain among E, PF and C males and females (Fig 2.1).

Adrenal Weights.

Absolute Adrenal Weights. There were no effects of prenatal treatment or chronic stress condition on absolute adrenal weights at termination.

Adrenal Weight: Body Weight Ratios. Absolute adrenal weights were also calculated as a proportion of total body weight at time of termination in order to account for sex and age effects on body weight. Separate prenatal treatment x chronic stress condition ANOVAs on adrenal weight: body weight ratios (i.e. relative adrenal weights) revealed significant main

effects of chronic stress condition for both males [$F(1, 56) = 15.71$, $p < 0.001$] and females [$F(1, 56) = 18.90$, $p < 0.001$]. Post hoc tests indicated that both males and females in the 21 d chronic stress condition had significantly larger relative adrenal weights than males and females in the 0 d chronic stress condition, respectively (p's < 0.001). There were no significant differences in relative adrenal weights among prenatal treatment groups for either males or females (Fig 2.2).

Lymphocyte Proliferative Responses.

Con A.

Data for lymphocyte proliferative responses to Con A for males and females is represented in Figures 2.3-2.5.

Sex x prenatal treatment x chronic stress ANOVAs on lymphocyte proliferative responses to all three concentrations of Con A (0.1, 1.0, 10.0 μg/ml) revealed significant main effects of sex at the highest concentration of Con A (10.0 μg/ml) [$F(1, 106) = 11.17$, $p < 0.005$] and of prenatal treatment at the lowest concentration of Con A (0.1 μg/ml) [$F(2, 106) = 6.58$, $p < 0.005$]. As expected (Grossman, 1989), females had elevated lymphocyte proliferative responses compared to males at the highest concentration of Con A when data were collapsed across prenatal treatment groups and chronic stress conditions ($p < 0.005$). Therefore, data from males and females were analyzed separately by prenatal treatment x chronic stress condition ANOVAs.

For males, there were no significant effects of prenatal treatment on lymphocyte proliferative responses at the two highest concentrations of Con A (1.0, 10.0 μg/ml). However, separate ANOVAs on males in the 0 d and 21 d conditions revealed a significant

effect of prenatal treatment on lymphocyte proliferative responses at the lowest concentration of Con A for males in the 21 d chronic stress condition [$F(2, 27) = 4.37$, $p < 0.05$]. Post hoc tests indicated that E males displayed significantly increased lymphocyte proliferative responses compared to C males ($p < 0.05$). In addition, E males showed a trend toward enhanced lymphocyte proliferative responses compared to PF males ($p = 0.073$). There was no significant effect of prenatal treatment on lymphocyte proliferative responses at the lowest concentration of Con A for males in the 0 d chronic stress condition.

When data from females in the 0 d and 21 d chronic stress conditions were analyzed separately, there was a significant main effect of prenatal treatment on lymphocyte proliferative responses at the lowest concentration of Con A for females in the 0 d chronic stress condition [$F(2, 26) = 6.71$, $p < 0.005$] and a marginal effect of prenatal treatment on lymphocyte proliferative responses at the intermediate concentration of Con A (1.0 μg/ml) for females in the 21 d chronic stress condition [$F(2, 26) = 3.27$, $p = 0.054$]. Post hoc tests revealed that, in the 0 d condition, PF females had significantly elevated lymphocyte proliferative responses compared to C females at the lowest concentration of Con A ($p < 0.005$). In addition, there was a trend for E females to have increased lymphocyte proliferative responses compared to C females ($p = 0.058$). Post hoc tests on lymphocyte proliferative responses at the intermediate concentration of Con A indicated that PF females in the 21 d chronic stress condition had higher responses than C females in the 21 d chronic stress condition ($p < 0.05$).

There were no significant effects of prenatal treatment on lymphocyte proliferative responses at the lowest concentration of Con A for females in the 21 d chronic stress condition nor on lymphocyte proliferative responses at the intermediate concentration of Con

A for females in the 0 d chronic stress condition. In addition, there were no effects of prenatal treatment on lymphocyte proliferative responses at the highest concentration of Con A for females in either the 0 d or 21 d chronic stress conditions.

There were no significant effects of chronic stress on lymphocyte proliferative responses for males and females at any of the three concentrations of Con A.

PWM.

Data for lymphocyte proliferative responses to PWM for males and females is represented in Figures 2.6-2.8.

Sex x prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of sex [$F(1, 106) = 22.59$, $p < 0.001$; $F(1, 106) = 8.32$, $p < 0.01$] and prenatal treatment [$F(2, 106) = 4.06$, $p < 0.05$; $F(2, 106) = (4.07)$, $p < 0.05$] on lymphocyte proliferative responses at the two lowest concentrations of PWM (0.1, 1.0 μg/ml). As expected, females had higher lymphocyte proliferative responses than males to PWM at these two concentrations when data were collapsed across prenatal treatment groups and chronic stress conditions (p's < 0.05).

When data from males and females were analyzed separately, there was a marginal effect of prenatal treatment on lymphocyte proliferative responses at the lowest concentration of PWM (0.1 μg/ml) for males across chronic stress conditions [$F(2, 54) = 2.82$, $p = 0.068$]. Further analysis revealed a trend for E males to show increased proliferative responses compared to C males at the lowest concentration of PWM ($p = 0.059$). For males, there were no significant effects of prenatal treatment on lymphocyte proliferative responses at the two highest concentrations of PWM (1.0, 10.0 μg/ml). Furthermore, there were no significant

effects of chronic stress on lymphocyte proliferative responses for males at any of the three concentrations of PWM (0.1, 1.0, 10.0 μg/ml).

For females, prenatal treatment x chronic stress condition ANOVAs on lymphocyte proliferative responses revealed a significant main effect of prenatal treatment at the intermediate concentration of PWM (1.0 μg/ml) [$F(2, 52) = 5.89$, $p < 0.01$]. When data from females in the 0 d and 21 d chronic stress conditions were analyzed separately, there was a significant effect of prenatal treatment for females in the 21 d chronic stress condition [$F(2, 26) = 3.36$, $p = 0.05$] on lymphocyte proliferative responses at the intermediate concentration of PWM. Post hoc tests indicated that E females had decreased lymphocyte proliferative responses compared to PF females ($p < 0.05$). There was no significant effect of prenatal treatment on lymphocyte proliferative responses at the intermediate concentration of PWM for females in the 0 d chronic stress condition. In addition, there were no significant effects of prenatal treatment on lymphocyte proliferative responses for females in either the 0 d or 21 d chronic stress conditions at the lowest (0.1 μg/ml) or the highest (10.0 μg/ml) concentrations of PWM.

An ANOVA also revealed a significant main effect of chronic stress for females at the lowest concentration of PWM [$F(1, 52) = 6.02$, $p < 0.05$]. Overall, females in the 21 d chronic stress condition had increased lymphocyte proliferative responses compared to females in the 0 d chronic stress condition at the lowest concentration of PWM, regardless of prenatal treatment ($p < 0.05$). There were no significant effects of chronic stress on lymphocyte proliferative responses for females at the two highest concentrations of PWM (1.0, 10.0 μg/ml).

LPS.

Data for lymphocyte proliferative responses to LPS for males and females is represented in Figures 2.9-2.11.

Sex x prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of sex on lymphocyte proliferative responses at the two highest concentrations of LPS (10.0, 100.0 μg/ml) [$F(1, 106) = 4.32$, $p < 0.05$; $F(1, 106) = 12.82$, $p < 0.002$] and a marginal effect of sex at the second lowest concentration of LPS (1.0 μg/ml) [$F(1, 106) = 2.90$, $p = 0.091$]. In addition, there were significant main effects of prenatal treatment on lymphocyte proliferative responses at all four concentrations of LPS (0.1 1.0 10.0, 100.0 μg/ml) [$F(2, 106) = 3.81$, $p < 0.05$; $F(2, 106) = 10.80$, $p < 0.001$; $F(2, 106) = 8.40$, $p < 0.001$; $F(2, 106) = 5.04$, $p < 0.01$] and a marginal sex x chronic stress interaction at the highest concentration of LPS (100.0 μg/ml)[$F(1, 106) = 3.41$, $p = 0.067$]. Data for males and females were then analyzed separately. For males, there were no significant effects of prenatal treatment or chronic stress on lymphocyte proliferative responses at any of the four concentrations of LPS (0.1, 1.0, 10.0, 100.0 μg/ml).

For females, however, prenatal treatment x chronic stress condition ANOVAs revealed significant main effects of prenatal treatment at the three highest concentrations of LPS (1.0, 10.0, 100.0 μg/ml) [$F(2, 52) = 7.93$, $p < 0.005$; $F(2, 52) = 8.68$, $p < 0.005$; and $F(2, 52) = 4.28$, $p < 0.02$] and a marginal effect of prenatal treatment at the lowest concentration of LPS (0.1 μg/ml) [$F(2, 52) = 2.50$, $p = 0.092$].

When data from females in the 0 d and 21 d chronic stress conditions were analyzed separately, there were significant effects of prenatal treatment on lymphocyte proliferative responses for females in the 0 d chronic stress conditions at the two lowest concentrations of

LPS (0.1, 1.0 μg/ml) [$F(2, 26) = 8.65$, $p < 0.002$; $F(2, 26) = 6.978$, $p < 0.005$], for females in the 0 d and 21 d chronic stress conditions at the second highest concentration of LPS (10.0 μg/ml) [$F(2, 26) = 4.37$; $F(2, 26) = 4.39$, p's < 0.05], and for females in the 21 d chronic stress condition at the highest concentration of LPS (100.0 μg/ml) [$F(2, 26) = 3.79$, $p < 0.05$]. Post hoc tests revealed that, for females in the 0 d chronic stress conditions, PF females had increased lymphocyte proliferative responses compared to E and C females at the two lowest concentrations of LPS (0.1, 1.0 μg/ml) (p's < 0.05). For females in the 0 d and 21 d chronic stress conditions, post hoc tests indicated that PF females had elevated lymphocyte proliferative responses compared to C females at the second highest concentration of LPS (10.0 μg/ml) (p's < 0.05). There were also trends for E and PF females in the 21 d chronic stress condition to have increased lymphocyte proliferative responses compared to C females in the 21 d chronic stress condition at the highest concentration of LPS (100.0 μg/ml) ($p = 0.070$; $p = 0.058$, respectively).

There was a significant main effect of chronic stress for females at the highest concentration of LPS [$F(1, 52) = 4.50$, $p < 0.05$]. Post hoc tests indicated that females in the 21 d chronic stress condition had increased lymphocyte proliferative responses compared to females in the 0 d chronic stress condition ($p < 0.05$).

DISCUSSION

The present study is the first to examine the possible interactive effects of prenatal ethanol exposure and exposure to stress in adulthood on lymphocyte proliferative responses to mitogens in adult rats. The data demonstrate that males and females displayed expected sexually dimorphic immune responses. Furthermore, prenatal ethanol exposure affected

lymphocyte proliferative responses to mitogens and the additional challenge of chronic stress in adulthood appeared to differentially influence E, PF and C offspring. In addition, some of the effects of prenatal ethanol exposure on lymphocyte proliferative responses appeared to be nutritionally-mediated, and pair-feeding, itself, is an experimental procedure that markedly influenced immune function, particularly in female offspring.

The developmental data indicate that the prenatal treatments resulted in differences in the pregnant dams and their offspring. E and PF dams had significantly decreased body weights compared to C dams during all but the initial stages of gestation. In addition, E females had significantly longer gestation periods than C females, corresponding to reports of longer gestation periods in studies were appreciable blood ethanol concentrations were obtained (Abel & Dintcheff, 1978). In addition, in agreement with previous studies using the liquid diet method of ethanol administration in rats (Weinberg, 1992b; Gallo & Weinberg, 1986), the present study found that E and PF female offspring weighed less than C female offspring on d 1 and d 8 of lactation and E females weighed less than C females at weaning (i.e. d 22). Although these findings demonstrate that the prenatal treatments produced changes that persisted into the postnatal period, similiarities between E and PF groups suggest that the effects of ethanol on body weight may have been at least partially nutritionally-mediated.

In agreement with previous research which has demonstrated that females display more active humoral and cell-mediated immune responses than males (Grossman, 1984; Grossman, 1985), the present study found that females exhibited increased lymphocyte proliferative responses compared to males at the highest concentration of Con A (10.0 μg/ml), the two lowest concentrations of PWM (0.1, 1.0 μg/ml) and the three highest concentrations of

LPS (1.0, 10.0, 100.0 μg/ml). Each of these mitogens was used to examine a particular type of immune cell response. Con A is a polyclonal activator of T cells. PWM stimulates the proliferation of B cells and T cells through the actions of helper T cell-derived cytokines. Lastly, LPS directly stimulates B cell proliferation and differentiation without the assistance of T cells or their cytokines (Abbas, Litchtman & Pober, 1991; Paul, 1993).

The current finding that females had increased lymphocyte proliferative responses to each of the mitogens compared to males, suggests that, following stimulation, females were capable of responding with elevated levels of T cell and B cell activity as well as increased levels of such T cell-B cell interactions as B cell presentation of antigen to T cells. The differences observed in the present study between males and females may be attributable to the actions of hormones of the endocrine system (Grossman, 1989). In fact, it has been hypothesized that testosterone may impact negatively on immune function whereas estrogen may enhance adult T cell and B cell function (Grossman, 1989).

There are several lines of evidence which provide support for this hypothesis. The response of adult human B-lymphocytes to PWM was reportedly inhibited by in vitro incubation with testosterone and stimulated by in vitro incubation with estrogen (Sthoeger, Chiorazzi & Lahita, 1988). Castration in male rats significantly increased the numbers of helper T cells and CD8+ T cells in thymic tissue (Grossman, 1989). In addition, steroid receptors for estrogens and androgens have been reported in cells of the thymus (Grossman, 1984; Grossman, 1985), and androgen treatment of chick embryos reportedly mediated the development of B lymphocytes (Hirota, Suzuki, Chazono & Bito, 1976). Consequently, it has been proposed that sex steroids may influence stem cells, pre-T and pre-B lymphocytes as

well as adult T cells and cells of the monocyte-macrophage system (Ahmed, Penhale & Talal, 1985).

As previously mentioned, an objective of the present study was to examine the possible interactive effects of prenatal treatment and exposure to chronic stress on lymphocyte proliferative responses to mitogens. The results show that, following exposure to 21 d of chronic intermittent stress, E males had significantly increased lymphocyte proliferative responses compared to C males at the lowest concentration of Con A (0.1 μg/ml). In addition, E males showed a trend toward enhanced responses compared to PF males at the lowest concentration of Con A. E males also showed a trend toward increased lymphocyte proliferative responses compared to C males at the lowest concentration of PWM (0.1 μg/ml) For females in the 21 d chronic stress condition, E animals had significantly decreased lymphocyte proliferative responses at the intermediate concentration of PWM (1.0 μg/ml) compared to PF females.

These findings are in partial agreement with previous research which has demonstrated that while E females appear to have relatively normal immune responses compared to PF and C females, E males showed long-term alterations in immune function (Weinberg & Jerrells, 1991), especially in T-cell responses. However, the results of the present study which demonstrate enhanced lymphocyte proliferative responses to Con A in E males compared to PF and C males contrasts with previous observations that prenatal ethanol exposure adversely affects immune function (Monjan & Mandell, 1980; Redei, Clark & McGivern, 1989). Weinberg and Jerrells (1991) found E males at 3 months of age showed significantly lower thymocyte counts as well as reduced splenic T-cell proliferative responses to 1.0 μg/ml and 5.0 μg/ml Con A compared to PF and C males. In addition, Redei, Clark and McGivern

(1989) showed that, in 21 day old animals, E males showed a decrease in lymphocyte proliferative responses to 3.0 μg/ml Con A compared to PF and C males. Prenatal ethanol exposure has also reportedly resulted in immune deficits in both males and females. Monjan and Mandell (1980) showed suppressed lymphocyte proliferative responses to 4 μg/ml Con A in 7 and 11 month old E offspring compared to PF and C offspring. However, these effects only occurred in animals whose mothers were intubated with high doses (6 g/kg) of ethanol during gestation. Furthermore, interpretation of their data is limited by a small sample size (i.e. their 7 months data were from two pooled spleens).

Consequently, even though reduced splenocyte proliferative responses to Con A in adult E animals has been reported, studies vary widely in terms of the age at which animals were assessed as well as the concentrations of Con A used. Norman, Chang, Castle, Van Zuylen and Taylor (1989) showed no decrease in Con A-induced proliferative responses of splenic T cells from 3 month old E males and females compared to controls; a result which they suggested was due to wide variability in response between individual rats. However, Norman et al (1989) did demonstrate that Con A-activated T lymphocytes from adult E animals were less responsive to stimulation with IL-2 than were splenocytes from controls. Although this deficit may have a profound effect on the immunocompetence of an organism since IL-2 plays a key role in stimulating and promoting a variety of immune functions, further investigations (Norman, Chang, Wong, Branch, Castle & Taylor, 1991) indicated that this effect of prenatal ethanol exposure varied with the age of the animal. Although diminished splenic T cell proliferative responses to IL-2 in E animals was maximal at 6 wks and 3 months of age, it could not be detected at 2 wks of age and was indistinguishable from

controls at 8 months of age (Norman et al, 1991), suggesting that impairments due to prenatal ethanol exposure may be age-dependent.

In addition, reductions in proliferative responses to Con A due to prenatal ethanol exposure differ depending on whether splenocytes or thymocytes are examined. Redei et al (1989) reported that in 21 d old male rats, the lymphocyte proliferative responses to Con A was suppressed eightfold in splenocytes and twofold in thymocytes of E animals compared to control animals. In addition, Ewald (1989) found in mice on gestation d 18 and d 19, E fetuses had reductions in thymic lymphocyte proliferative responses to Con A plus a source of IL-2 compared to PF and C fetuses. Other investigators (Wong, Chiappelli, Chang, Norman, Cooper, Branch, & Taylor, 1991; Chiappelli, Tio, Tritt, Pilati, & Taylor, 1992) reported age dependent effects of thymocyte proliferative responses. At 44 d of age, E males reportedly demonstrated enhanced thymocyte proliferative responses to 2.5 μg/ml Con A compared to controls although thymocyte proliferative responses in E males decreased to close to half that of control responses by 51 d of age. These differences normalized by 70 d of age with thymocyte proliferative responses in E males indistinguishable from responses in control animals (Wong, Chiappelli, Chang, Norman, Cooper, Branch, & Taylor, 1991; Chiappelli, Tio, Tritt, Pilati, & Taylor, 1992). Based on these observations, it appears that prenatal ethanol exposure exerts effects on lymphocyte proliferative responses, however, these effects may be age-dependent.

The present study is the first to report that E females display decreased lymphocyte proliferation responses to PWM, indicating that prenatal ethanol exposure may adversely influence interactions between T and B cells. In addition, this is the first study to report enhanced splenic lymphocyte proliferative responses to 0.1 μg/ml Con A in E males compared

to PF and C males as well as increased splenic lymphocyte proliferative responses to 1.0 μg/ml PWM in E compared to C males. The differences in results between this study and previous studies may be attributable to differences in both the age of testing and the concentrations of mitogens used. Low concentrations of Con A may reveal alterations in immune function in E animals not apparent at higher concentrations of mitogen.

In addition, the effects of prenatal ethanol exposure were predominantly observed in animals in the 21 d chronic stress condition, suggesting that chronic stress may reveal differences among E, PF and C animals that are not observed under basal or nonstressed conditions. Although there was no effect of prenatal treatment on either body weight gain or relative adrenal weights, there were effects of chronic stress on these measures. Body weight gain was significantly lower in both males and females in the 21 d chronic stress condition compared to males and females in the 0 d chronic stress condition, respectively. In addition, relative adrenal weights were increased for males and females in the 21 d chronic stress condition compared to males and females in the 0 d chronic stress condition. Studies on the effects of chronic stress on rats have generally reported activation of the pituitary-adrenal system, reductions in body weight gain and increases in adrenal weight (Daniels-Severs, Goodwin, Keil, Vernikos-Danellis, 1973; Imms, 1967; Hashimoto et al, 1988). Thus, the current results, when viewed in the context of previous research, suggest that animals in the 21 d chronic stress condition displayed evidence of an excitatory state characteristic of prolonged stress (Weiss, 1970).

Stress has been shown to alter immune function. Taylor and Ross (1989) examined the effects of neonatal restraint stress on the antibody response to type III Streptococcus pneumonia and found that both males and females showed deficits in immune response,

although the suppression was more profound and persisted for longer in males. Studies have also shown that psychosocial stressors (e.g. social housing condition) can modulate tumor growth rate (Sklar & Anisman, 1980; Weinberg & Emerman, 1989). In addition, other immune measures, such as T lymphocyte mitogenesis, antibody production and cytokine production, have been shown to be altered by stress (Batuman, Sajewski, Ottenweller & Pitman, 1990; Esterling & Rabin, 1987; Sonnenfeld, Cunnick, Armfield, Wood & Rabin, 1992).

Therefore, in the present study, the additional challenge to the immune system of chronic stress may have differentially affected E animals. In other words, specific effects on the immune system may not be observed under basal or nonstressed conditions, but may only become apparent when E animals are exposed to chronic stress. The findings of the present study extend previous data demonstrating that E offspring are often similar to control animals in behavioral and hormonal responses under basal conditions but respond differently when challenged with stressors (Taylor, Branch, Van Zuylen & Redei, 1988; Weinberg, 1988). In fact, Giberson and Weinberg (in press) demonstrated that E males exposed to chronic stress had reduced numbers of pan T cells in the thymus and whole blood compared to nonstressed E males.

Although the present study did not examine activation of the HPA axis, E animals may be more susceptible to the immune-altering effects of stress because of long-term alterations in endocrine function. In particular, E animals show hyperresponsiveness to stressors, exhibiting increased and/or more sustained activation of the HPA axis and pituitary β-endorphin system following stress compared to control animals (Angelogianni & Gianoulakis, 1989; Taylor, Branch, Van Zuylen & Redei, 1988). It has been demonstrated that cells of the

immune system possess receptors for glucocorticoids as well as peptide hormones (Blalock, 1989) and, therefore, differences among E, PF and C animals in activation of the HPA axis following stress may result in alterations of the immune system.

In addition to direct effects of ethanol on immune function, the present study demonstrated nutritionally-mediated effects of ethanol. For females, such nutritionally-mediated effects were apparent in both the 0 d and 21 d chronic stress conditions. In the 0 d chronic stress condition, E and PF females had increased lymphocyte proliferative responses compared to C females at the lowest concentration of Con A (0.1 μg/ml). Furthermore, for females in the 21 d chronic stress condition, there were trends for E and PF animals to have increased lymphocyte proliferative responses compared to C females at the highest concentration of LPS (100 μg/ml).

Giberson and Weinberg (in press) demonstrated that nutritionally-mediated effects of ethanol may be apparent for females under both stressed and nonstressed conditions. They showed that E and PF females had decreased numbers of lymph node helper T cells and increased numbers of thymic CD8+ T cells compared to C females. These results (Giberson & Weinberg, in press) and the findings of the present study reflect the effects of ethanol on absorption and/or utilization of nutrients as well as the nutritional effects of mildly reduced food intake due to ethanol consumption on the fetal immune system (Weinberg, 1984). Nutrition has been shown to influence all aspects of immunity, including humoral responses, phagocytosis, and cell-mediated immunity (Gross and Newberne, 1980). Although mild undernutrition may result in increased susceptibility to infectious disease (Keusch & Farthing, 1986), it may also, in some situations, stimulate the immune system, enhancing T-cell mediated immune responses (Good & Lorenz, 1992) and increasing humoral aspects of

immunity such as antibody responses to pneumococcal vaccine and smallpox (Mendenhall, 1989). However, such enhanced responses may not result in a greater degree of protection but, instead, may reflect disturbances in the regulation of stimulatory and suppressive elements in the immune system (Mendenhall, 1989).

The present study provides evidence that the effects of prenatal exposure to ethanol on nutrition may produce long-term changes in female offspring under both basal (0 d) and chronic stress (21 d) conditions. In addition to the findings of nutritionally mediated effects of ethanol, there were also effects of pair-feeding. Consistent with previous reports that PF animals show consistently greater splenic lymphocyte proliferative responses to LPS than both E and C animals (Weinberg & Jerrells, 1991; Giberson & Weinberg, in press), the current study found that PF females in the 0 d chronic stress condition had higher lymphocyte proliferative responses than E and C females in the 0 d chronic stress condition at the lowest concentration of LPS (0.1 μg/ml). In addition, for females in both the 0 d and 21 chronic stress conditions, PF females had higher lymphocyte proliferative responses than E and C females at the two intermediate concentrations of LPS (1.0, 10.0 μg/ml).

The results of the present study demonstrate that PF females exhibit long-term changes in lymphocyte proliferative responses to LPS irrespective of chronic stress conditions and emphasize the affect pair-feeding may have on humoral immunity in adulthood. Previous findings have demonstrated that although pair-feeding provides an essential nutritional control group, pair-feeding in itself is a type of experimental treatment (Gallo, 1984; Weinberg, 1984). For example, PF or restricted meal feeding can produce a shift in the circadian rhythm of biogenic amines, body temperature, activity and hormones (Gallo & Weinberg, 1981; Krieger, 1974) as well as alter body weights, organ weights and behavioral and physiological

responses of both the maternal female and the offspring (Weinberg, 1985; Weinberg & Gallo, 1982; Weinberg & Bezio, 1987). The findings of the present study extend these previous reports to include long-term changes on immune responses in adulthood and underscore the importance of including both pair-fed and *ad libitum*-fed control groups when examining the effects of prenatal ethanol exposure.

In addition to the effects of prenatal treatment, the present study found a main effect of chronic stress on lymphocyte proliferative responses in adult females across prenatal treatment groups. The results show that females in the 21 d chronic stress condition had higher lymphocyte proliferative responses than females in the 0 d chronic stress conditions at the lowest concentration of PWM (0.1 μg/ml) and the highest concentration of LPS (100.0 μg/ml), suggesting that chronic stress affects B-cell mitogen responses that are both T cell-dependent and T cell-independent. The present findings concur with previous research demonstrating stress-induced increases in splenic $CD8^+$ cell numbers and thymic CD8 antigen expression in males and females as well as an increase in splenic lymphoid cell counts in females (Giberson & Weinberg, in press).

Other researchers have also reported that although stress in animals may be initially associated with immunosuppression, chronic stress may eventually result in a return to pre-stress levels of immune function. Monjan and Collector (1977) reported that sound stress initially suppressed lymphocyte proliferative responses to LPS and Con A in male rats, but that more extended exposure to the stressor resulted in a return to pre-stress levels of response to these same mitogens. In addition, the suppressed reactivity of rat splenic lymphocytes to Con A was shown to diminish with repeated sessions of frequent footshocks (Lysle, Cunnick

& Rabin, 1990; Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988).

This is the first study to demonstrate enhanced lymphocyte proliferative responses to PWM as well as LPS in animals exposed to chronic intermittent stress. In agreement with previous studies showing a recovery of lymphocyte proliferative responses to Con A to pre-stress levels during chronic stress, the present study found no differences between splenic lymphocyte proliferative responses to Con A in animals in the 0 d and 21 d chronic stress conditions. The present study extends previous findings by showing that splenic lymphocyte proliferative responses to PWM and LPS may actually be enhanced following prolonged chronic stress. Furthermore, this effect was only observed in females. Therefore, the present findings extend previous research which only used male rats (Lysle, Lyte, Fowler & Rabin, 1987; Cunnick, Lysle, Armfield & Rabin, 1988), and indicates that chronic stress may differentially affect immune function in males and females.

In summary, prenatal exposure to ethanol leads to many alterations in immune competence in adulthood, some aspects of which may only become apparent following the additional challenge of stress. Furthermore, the effects of prenatal exposure to ethanol on nutrition as well as the pair-feeding procedure designed to control for the effects of ethanol consumption on food intake may both impact on immune function in adulthood, especially in female offspring. Lastly, not only may chronic stress differentially affect immune responses in E animals but it may also result in enhanced lymphocyte proliferative responses in females regardless of prenatal treatment.

GENERAL DISCUSSION

The studies presented in this dissertation were designed to examine the possible interactive effects of prenatal ethanol exposure and exposure to chronic intermittent stress in adulthood on the endocrine and immune systems. The developmental data indicate that the prenatal treatments produced effects on the pregnant dams and on their offspring. Although the pregnant dams in the E and PF conditions were not undernourished in terms of nutrient intake required for pregnancy, their body weights were less than those of dams in the C condition during all but the initial stages of gestation. Furthermore, in the second study, E females had significantly longer gestation periods than C females. The body weights of the offspring were also differentially affected by the prenatal treatments. In the first study, E and PF males and females weighed significantly less than C males and females throughout lactation. In the second study, there were no differences among E, PF and C males. However, E and PF females weighed significantly less than C females on d 1 and d 8 of lactation and E females weighed less than C females at weaning.

These findings are consistent with previous reports of developmental differences among E, PF and C offspring. The length of gestation has been reported to be increased in E dams in studies where appreciable blood ethanol concentrations were obtained (Abel & Dintcheff, 1978). In addition, maternal weight gain has been reportedly suppressed in both E and PF females compared to C females, regardless of protein content of the diet (Weinberg, 1987). Even highly nutritious diets cannot overcome the adverse effects of ethanol on fetal growth and development if ethanol is administered as a relatively large proportion (i.e. 36%) of an animal's total daily caloric intake (Weinberg, 1984). However, the effects of ethanol on body weights in the current studies may be partially nutritionally-mediated since E and PF

animals showed the same reductions in weight compared to C animals. Lastly, in agreement with previous studies (Abel & Dintcheff, 1978, Wiener, Shoemaker, Koda & Bloom, 1981), neither of the studies in this paper found differences among E, PF and C animals in the number of live births.

Previous studies have provided evidence that E animals exhibit HPA hyperresponsiveness to stressors manifested as enhanced pituitary-adrenal activation as well as delayed or deficient recovery to basal levels when tested in adulthood. Stressors such as footshock, ether, restraint, cold, and acute challenges with ethanol and morphine have all been reported to produce increases in pituitary-adrenal activity in E animals compared to controls (Nelson, Taylor, Lewis, Poland & Branch, 1986; Taylor, Branch, Liu & Kokka, 1982; Weinberg, 1992a; Weinberg & Gallo, 1982). However, this difference is only apparent following stress; E offspring do not appear to differ from PF and C animals when tested in adulthood under basal conditions (Weinberg, 1988; Weinberg, 1992a).

Since previous studies typically used either acute stressors or repeated exposure to the same stressor (Weinberg, 1988; Weinberg, Taylor & Gianoulakis, in press), the first study in this dissertation examined the effects of prenatal exposure on offspring responses to a chronic intermittent stress regimen of 6 d or 18 d duration. Animals were tested in adulthood to determine if basal or undisturbed levels of CORT and ACTH were altered from pre-stress levels and if E, PF and C animals were differentially affected by exposure to the chronic intermittent stress regimens.

Previous studies have shown that repeated exposure to one particular stressor may result in habituation or adaptation of the pituitary-adrenal response. In other words, the CORT and/or ACTH response to a stressor may decrease following multiple exposures

compared to that following the first exposure (Rivier & Vale, 1987; Hashimoto, 1988). In addition, animals may show a more rapid return of circulating CORT to resting levels during the period of exposure to the stressor (Rivier & Vale, 1987; Hashimoto, 1988; Culman, Kopin & Saavedra, 1991). Exposure to chronic stress has also been reported to result in pituitary-adrenal hyperresponsiveness or sensitization to novel, acute stressors (Sakellaris & Vernikos-Danellis, 1975; Armario, Hidalgo & Giralt, 1988). Therefore, the first study also examined whether exposure to chronic stress differentially affected the pituitary-adrenal responses of E, PF and C animals to an acute stressor.

The results of the first study indicated that prenatal ethanol exposure did not alter undisturbed pituitary-adrenal activity following exposure to chronic stress in adulthood. In addition, the data demonstrate that there were no significant differences among E, PF and C animals in pituitary-adrenal responses to the acute stressor following exposure to a chronic stress regimen. Thus, the current findings suggest that the hormonal hyperresponsiveness previously reported in E animals may not occur following exposure to chronic intermittent stress and, following exposure to chronic stress, E animals do not show differences in sensitization to a novel, acute stressor compared to controls.

Although there were no effects of prenatal treatment on pituitary-adrenal activity, body weight gain or relative organ weights, chronic stress had a number of effects across all groups. In both of the present studies, animals exposed to the chronic stress regimens showed increases in relative adrenal weights compared to their nonstressed controls. In addition, in the first study, there was a transient decline in body weight gain for males and females in the 6 d chronic stress regimen, although animals in the 18 d chronic stress condition were not significantly different in weight gain than animals in the 0 d chronic stress condition. In the

second study, males and females in the 21 d chronic stress condition showed less weight gain than animals in the 0 d chronic stress condition. Thus, these findings, in general, confirm previous reports that chronic stress in rats results in activation of the pituitary-adrenal system, reductions in body weight gain and increases in adrenal weight (Daniels-Severs, Goodwin, Keil, Vernikos-Danellis, 1973; Hashimoto, 1988), supporting the conclusion that the chronic stress regimen used in the present studies resulted in a state characteristic of prolonged stress.

The effects of exposure to chronic stress on undisturbed CORT levels in male and female animals in the first study support previous findings of adaptation of the pituitary-adrenal system. There were no significant changes in undisturbed CORT levels in either male or female animals after exposure to chronic stress. However, there were significant differences in CORT levels overall in males exposed to 6 d compared to 18 d of chronic stress. Males in the 18 d chronic stress condition had significantly elevated undisturbed ACTH levels compared to males in the 6 d chronic stress condition, indicating that, although pituitary-adrenal adaptation may have occurred, the length of exposure to chronic stress differentially affected resting pituitary-adrenal activity. The differences between undisturbed ACTH levels in the 6 d and 18 d chronic stress conditions may be attributable to increases in ACTH-secreting mechanisms which compensate for or override negative feedback from high levels of circulating glucocorticoids during or following stress (Dallman, 1992; Rivier & Vale, 1987).

The elevations in undisturbed ACTH levels for males in the 18 d chronic stress condition may have affected the response of these males to the acute stressor. Males in the 6 d and females in both the 6 d and 18 d chronic stress conditions had significantly increased CORT and ACTH levels in response to the acute stressor compared to undisturbed levels.

However, males in the 18 d chronic stress condition did not have significantly elevated ACTH levels in response to the acute stressor, even though their CORT levels were significantly increased. Consequently, it appears that adaptations of the pituitary-adrenal system during chronic stress may affect the ability of an animal to respond to an acute stressor. Furthermore, ACTH may be more sensitive than CORT in revealing this effect.

As mentioned previously, studies have shown hormonal hyperresponsiveness to a novel, acute stressor following chronic exposure to one particular stressor (Sakellaris & Vernikos-Danellis, 1975; Armario, Hidalgo & Giralt, 1988). Consequently, this study investigated whether exposure to a chronic intermittent stress regimen consisting of six different stressors would also result in hormonal hyperresponsiveness to a novel, acute stressor. Since comparisons were not made between chronically-stressed and nonstressed animals, it could not be determined whether animals in the 6 d and 18 d chronic stress conditions showed sensitization to the novel, acute stressor. However, there were trends for males in the 6 d chronic stress condition to have increased CORT and ACTH levels compared to males in the 18 d chronic stress condition following exposure to an acute stressor, indicating that the length of exposure to chronic stress may impact on the level of pituitary-adrenal response to an acute stressor.

The effects of chronic stress are often evaluated based upon pituitary-adrenal activity and an underlying assumption of many stress studies has been that any effects on the immune system are at least partially consequent to the stress-induced release of corticosteroids. While there is no question that steroids secreted following stress may act upon components of the immune response (Blalock, 1989), glucocorticoids may only partially mediate effects of stress on immune function. For example, stress in adrenalectomized rats may only induce the

suppression of some types of immune activity (Cunnick, Lysle, Kucinski & Rabin, 1990). Nevertheless, differences in hormonal responsiveness among E, PF and C animals may result in differential effects of stress on immune function, particularly in view of clinical and animal studies which have demonstrated immune deficits following prenatal ethanol exposure (Johnson, Knight, Marmer & Steele, 1981; Ewald & Walden, 1988; Norman, Chang, Castle, Van Zuylen & Taylor, 1989).

Both of the studies presented here assessed the interactive effects of prenatal ethanol exposure and exposure to chronic stress in adulthood on the immune system. The first study examined thymus and spleen cell counts. Although previous research has shown that prenatal ethanol exposure reduces thymus weight and alters the development of T lymphocyte populations in mice (Ewald, 1989), there were no significant differences in thymus cell counts or spleen cell counts among E, PF and C animals. The second study assessed lymphocyte proliferative responses to mitogen and the data demonstrate significant differences in lymphocyte proliferative responses among E, PF and C offspring. For animals in the 21 d chronic stress condition, E males had significantly increased lymphocyte proliferative responses compared to C males at the lowest concentration of Con A (0.1 μg/ml). There was also a trend for E males to have enhanced responses compared to PF males at the lowest concentration of Con A, and to have increased lymphocyte proliferative responses compared to C males at the lowest concentration of PWM (0.1 μg/ml). In contrast, E females in the 21 d chronic stress condition had significantly decreased responses compared to PF females at the intermediate concentration of PWM (1.0 μg/ml).

The enhanced lymphocyte proliferative responses observed in E males in the second study contrasts with previous findings of reduced lymphocyte proliferative responses to Con

A in animals prenatally exposed to ethanol (Monjan & Mandell, 1980; Redei, Clark & McGivern, 1989; Weinberg & Jerrells, 1991). The lack of correspondence between previous research and the current findings may be due to differences in the concentration of mitogen assessed, the ages of the animals examined, or the effects of chronic stress on E animals in this study which may have revealed differences among E, PF and C animals that are not apparent under basal conditions. The larger number of prenatal ethanol exposure effects found for males in the present study corresponds with previous reports that E males show long-term alterations in immune function while E females appear to have relatively normal immune responses compared to controls (Weinberg & Jerrells, 1991).

In addition, the second study demonstrated nutritionally-mediated effects of ethanol as well as effects of pair-feeding. For females in the 0 d chronic stress condition, E and PF animals had increased lymphocyte proliferative responses compared to C females at the lowest concentration of Con A (0.1 μg/ml). Furthermore, for females in the 21 d chronic stress condition, there were trends for E and PF animals to have increased lymphocyte proliferative responses compared to C females at the highest concentration of LPS (100 μg/ml). The similarities between E and PF animals suggest that the observed effects may, at least partially, be attributable to the effects of ethanol on nutrition since ethanol consumption reduces food intake and the PF group is matched to the E group for food intake.

The second study also found data consistent with previous findings that PF animals show consistently greater splenic lymphocyte proliferative responses to LPS than both E and C animals (Weinberg & Jerrells, 1991). The data demonstrate that PF females in the 0 d chronic stress condition had higher lymphocyte proliferative responses than E and C females in the 0 d chronic stress condition at the lowest concentration of LPS (0.1 μg/ml). In addition,

for females in both the 0 d and 21 chronic stress conditions, PF females had higher lymphocyte proliferative responses than E and C females at the two intermediate concentrations of LPS (1.0, 10.0 μg/ml). These results emphasize that although pair-feeding provides an essential nutritional control group in studies of the effects of prenatal ethanol exposure, pair-feeding in itself is a type of experimental treatment that may impact on immune function. Thus, it appears that pair-feeding or mild prenatal nutritional restriction may actually enhance immune responses, particularly humoral responses.

Chronic stress had effects on measures of the immune system in both studies. In the first study, in agreement with previous studies (Hoffman-Goetz, Thorne, Simpson & Arumugam, 1989; Teshima, Sogawa, Kihara & Nakagawa, 1991), chronic stress resulted in reductions in thymus cell counts for both males and females. Spleen cell counts were not affected by chronic stress conditions for males. In contrast, spleen cell counts were decreased in females after 6 d of chronic stress, whereas spleen cell counts for females in the 18 d and 0 d chronic stress conditions were not significantly different. In the second study, females exposed to chronic stress displayed enhanced splenic lymphocyte proliferative responses at the lowest concentration of PWM (0.1 μg/ml) and the highest concentration of LPS (100.0 μg/ml). There were no effects of chronic stress for males in the second study.

These findings suggest that thymocytes may be more susceptible to the effects of chronic stress than splenocytes. Glucocorticoids released during exposure to stressors may differentially affect the thymus and spleen, perhaps because the majority of thymocytes are immature (Paul, 1993) although this possibility was not directly assessed in the present study. Furthermore, chronic stress may differentially affect immune function depending upon the duration or type of stress. Immune function may be initially suppressed only to return to pre-

stress levels after continued exposure to stress (Monjan & Collector, 1977) or it may be enhanced following continued exposure to stress (Jessop & Bayer, 1989). These differences may possibly be due to the direct effects of stress on the immune system or the effects of endocrine hormones on the immune system.

The results of the first study suggest that splenocytes were only transiently affected by chronic stress and exhibited a recovery to pre-stress levels following prolonged exposure to chronic stress. Consequently, splenocyte activity may have returned to pre-stress levels following prolonged exposure to the chronic stress regimen (21 d) in the second experiment, possibly resulting in enhanced lymphocyte proliferative responses to mitogen at specific concentrations of mitogen. The findings of increased lymphocyte proliferative responses in females exposed to the 21 d chronic stress regimen compared to females in the 0 d chronic stress condition do correspond to previous reports of immunoenhancement following exposure to chronic stress (Jessop & Bayer, 1989).

As suggested previously, stress may either directly exert effects on immune function or may indirectly impact immune function through its effects on the endocrine system. Numerous studies have demonstrated that the endocrine system can control immune functions and, in turn, the immune system may affect endocrine function (Blalock, Harbour-McMenamin & Smith, 1985). Futhermore, the molecular basis for this bidirectional communication is becoming increasingly apparent. Classically, stress responses were thought to be mediated and regulated entirely by the HPA axis. However, recent studies have reported that immune cells may produce ACTH following exposure to viruses and this response may occur independently of the pituitary (Smith, Meyer & Blalock, 1982). In addition, production

of ACTH by cells of the immune system may be controlled by negative feedback from corticosteroids (Smith, Meyer & Blalock, 1982).

Therefore, an immunoadrenal axis may exist in which cells of the immune system serve a sensory function for stimuli such as viruses, bacteria or tumors. This information may then be conveyed to the adrenal glands by immune cell-derived ACTH and the release of adrenal glucocorticoids may inhibit further ACTH production. In turn, central nervous system recognition of cognitive stimuli may result in similar hormonal information from the endocrine system being conveyed to and recognized by hormone receptors on immune cells (Smith, Meyer & Blalock, 1982). Thus, the response of the immune system to stress may be dependent upon interactions between hormones produced by immune cells and those produced by the endocrine system. Consequently, differences in lymphocyte proliferative responses to mitogen among studies examining the effects of chronic stress may be attributable to slight variations in the duration and type of stress which may then either independently affect the immune or endocrine systems as well as altering the interactions between them.

Prenatal ethanol exposure and chronic stress appeared to differentially affect males and females in both of the studies presented in this dissertation. For males, prenatal ethanol exposure affected lymphocyte proliferative responses to mitogens whereas chronic stress affected the HPA and HPG axes. In contrast, females showed effects of chronic stress on spleen cell counts and lymphocyte proliferative responses to mitogens that were not observed in males. Furthermore, the nutritionally-mediated effects of ethanol as well as the effects of pair-feeding on lymphocyte proliferative responses to mitogens were most strongly apparent in females. Lastly, chronic stress differentially affected the HPG axes of males and females.

Males in the 6 d and 18 d chronic stress regimens displayed increased relative gonad weights compared to males in the 0 d chronic stress condition, whereas chronic stress did not affect relative gonadal weights in females. These differential responses of E, PF and C males and females to chronic stress emphasize the distinct influence chronic stress may have on each sex and highlight the importance of including both males and females in studies investigating the effects of prenatal treatment and chronic stress.

This sexual dimorphism may be attributable to interactions among the HPG axis, the HPA axis and the immune system. As with the bidirectional communication between the HPA and immune systems, the molecular basis for interactions between the HPG and immune systems is becoming increasingly apparent. Hormone receptors for estrogens and androgens are present in the thymus and various classes of lymphocytes (Grossman, 1989). Furthermore, it has been hypothesized that gonadal hormones may mediate the release of thymic hormones which, in turn, regulate lymphocyte function (Grossman & Roselle, 1983). In addition, direct effects of sex hormones on T lymphocyte function (Grossman, 1989) may produce widespread effects on other aspects of the immune response such as B cells since cytokines released by T lymphocytes can regulate B cell production of antibodies.

Hormonal regulation of one or more stages in lymphocyte maturation by products of the gonads, adrenals and/or pituitary may exert long-lasting effects on immune function. During the early stages of fetal life, endogenous hormones may promote differences in the development of immune tissue or cells which may result in a dimorphic immune response later in life (Grossman, 1985). Significantly, sex steroids may regulate the development of lymphocytes while glucocorticoids may control their final responses since adult T cells may

not possess sex steroid receptors although they may retain receptors for glucocorticoids (Grossman, 1989).

Although the evidence for interactions between the HPG and immune systems is growing, the exact effects of gonadal hormones on immune function *in vivo* are only beginning to be delineated. In the male, the lack of estrogen and the elevation of androgen may lead to less immunological responsiveness than in females. It is also possible that the self-limiting mechanisms designed to inhibit further responses after activation are less sensitive in females than in males (Grossman, 1989). Such limiting mechanisms could include negative feedbacks within the HPA and HPG axes as well as cytokine-mediated feedbacks to the hypothalamus and/or pituitary, the adrenal or directly to lymphocytes.

In conclusion, the results of the studies presented in this disseration indicate that chronic stress may produce changes in pituitary-adrenal activity, HPG activity, immune cell populations and immune cell function. In addition, these effects may occur differentially in males and females due to the influence of gonadal hormones. Furthermore, differences in the effects of prenatal ethanol exposure and exposure to chronic stress on immune cell populations and immune cell responses to mitogens observed in the present studies emphasize the importance of assessing multiple elements within a system. Significantly, the current findings indicate that repeated exposure to a combination of stressors may alter physiological systems in adult animals even under basal conditions. Most importantly, chronic stress may reveal effects of prenatal ethanol exposure that are not evident under basal conditions.

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TABLE 1.1. MATERNAL BODY WEIGHTS AND GESTATION LENGTHS
(Mean ± S.E.M.)

Maternal Females									
	Gestational Weights (g)				Gestation Length (days)	Lactational Weights (g)			
	D1	D7	D14	D21		D1	D8	D15	D22
E (16)	271.5 ± 2.6	268.1# ± 2.7	289.2# ± 4.1	346.7# ± 7.3	23.1 ± 0.1	291.5⊗ ± 3.7	335.2 ± 4.9	356.9 ± 4.5	339.5 ± 4.5
PF (16)	270.5 ± 3.3	266.0# ± 3.7	287.0# ± 4.8	353.1# ± 6.9	22.8 ± 0.1	298.4⊗ ± 5.5	330.7 ± 5.2	357.7 ± 5.8	341.0 ± 6.2
C (15)	270.6 ± 3.1	296.0 ± 3.0	323.8 ± 4.8	416.2 ± 5.5	23.1 ± 0.1	318.6 ± 3.1	341.4 ± 5.6	358.7 ± 5.2	340.2 ± 5.4

#E = PF < C, p's < 0.001

⊗E = PF < C, p's < 0.005

TABLE 1.2. OFFSPRING BODY WEIGHTS AND NUMBER OF LIVEBORN
(Mean ± S.E.M.)

	Pups									
	Offspring Weight (g) Males				Number of Liveborn Males	Offspring Weight (g) Females				Number of Liveborn Females
	D1	D8	D15	D22		D1	D8	D15	D22	
E	5.7# ± 0.1	14.7# ± 2.7	30.6# ± 0.6	46.8# ± 1.0	7.9 ± 0.5	5.3⊗ ± 0.1	14.0⊗ ± 0.4	29.4⊗ ± 0.6	45.0⊗ ± 0.7	7.3 ± 0.5
PF	5.9# ± 0.2	14.6# ± 0.6	30.9# ± 0.9	48.5# ± 1.2	7.9 ± 0.6	5.4⊗ ± 0.2	13.6⊗ ± 0.6	29.4⊗ ± 1.0	46.1⊗ ± 1.2	6.6 ± 0.6
C	6.7 ± 0.1	17.7 ± 0.5	34.3 ± 0.7	54.8 ± 1.1	7.6 ± 0.5	6.3 ± 0.2	16.8 ± 0.5	32.8 ± 0.7	52.2 ± 1.1	8.2 ± 0.4

#E = PF < C, p's < 0.001

⊗E = PF < C, p's < 0.01.

FIGURE LEGENDS: EXPERIMENT 1

Figure 1.1. Plasma CORT levels (mean ± S.E.M.) for resting animals one day before beginning the 6 d or 18 d chronic stress regimens (i.e. basal) and one day after finishing the 6 d or 18 d chronic stress regimens (i.e. undisturbed). No significant effects of chronic stress on CORT levels.

Figure 1.2. Basal plasma CORT levels (mean ± S.E.M.) for resting animals. For animals in the 0 d chronic stress condition, CORT levels were measured one day prior to termination. For animals in the 6 d and 18 d chronic stress conditions, CORT levels were measured one day before beginning the chronic stress regimens.

Males and Females. *Effects of chronic stress condition, 6 d = 18 d < 0 d, p's < 0.05.

Figure 1.3. Plasma CORT levels (mean ± S.E.M.) for animals left undisturbed and for animals in the acute stress condition. No significant effects of exposure to chronic stress on CORT levels in either the undisturbed or acute stress conditions.

Figure 1.4. Plasma ACTH levels (mean ± S.E.M.) for animals left undisturbed and for animals in the acute stress condition.

Males. *Effect of chronic stress condition; 18 d > 6 d, $p < 0.05$.

Figure 1.5. Plasma CORT levels (means ± S.E.M.) after 6 d of chronic stress for E, PF and C animals left undisturbed and for E, PF and C animals in the acute stress condition.

Males. *Effect of acute stress; Acute stress > Undisturbed, $p < 0.005$.

Females. #Effect of acute stress; Acute stress > Undisturbed, $p < 0.005$.

Figure 1.6. Plasma CORT levels (means ± S.E.M.) after 18 d of chronic stress for E, PF and C animals left undisturbed and for E, PF and C animals in the acute stress condition.

Males. *Effect of acute stress; Acute stress > Undisturbed, $p < 0.005$.

Females. #Effect of acute stress; Acute stress > Undisturbed, $p < 0.005$.

Figure 1.7. Plasma ACTH levels (means ± S.E.M.) after 6 d of chronic stress for E, PF and C animals left undisturbed and for E, PF and C animals in the acute stress condition.

Males. *Effect of acute stress; Acute stress > Undisturbed, $p < 0.001$.

Females. #Effect of acute stress; Acute stress > Undisturbed, $p < 0.05$.

Figure 1.8. Plasma ACTH levels (means ± S.E.M.) after 18 d of chronic stress for E, PF and C animals left undisturbed and for E, PF and C animals in the acute stress condition.

Females. #Effect of acute stress; Acute stress > Undisturbed, $p < 0.05$.

Figure 1.9. Body weight gain (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d, 6 d, and 18 d chronic stress conditions.

Males and Females. * ^ Effects of chronic stress condition; 6 d < 18 d = 0 d, p's < 0.001.

Figure 1.10. Relative adrenal weights (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d, 6 d, and 18 d chronic stress conditions.

Males and Females. #Effects of chronic stress condition; 6 d = 18 d > 0 d, p's < 0.001.

Figure 1.11. Relative gonad weights (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d, 6 d, and 18 d chronic stress conditions.

Males. # ^ Effects of chronic stress condition; 18 d > 6 d > 0 d, p's < 0.05.

Figure 1.12. Thymus cell counts (mean ± S.E.M) for E, PF and C males (A) and females (B) in the 0 d, 6 d, and 18 d chronic stress conditions.

Males and Females. #Effects of chronic stress condition; 18 d = 6 d > 0 d, p's < 0.001.

Figure 1.13. Spleen cell counts (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d, 6 d, and 18 d chronic stress conditions.

Females. #Effect of chronic stress condition; 6 d < 0 d, p < 0.05.

Figure 1.1

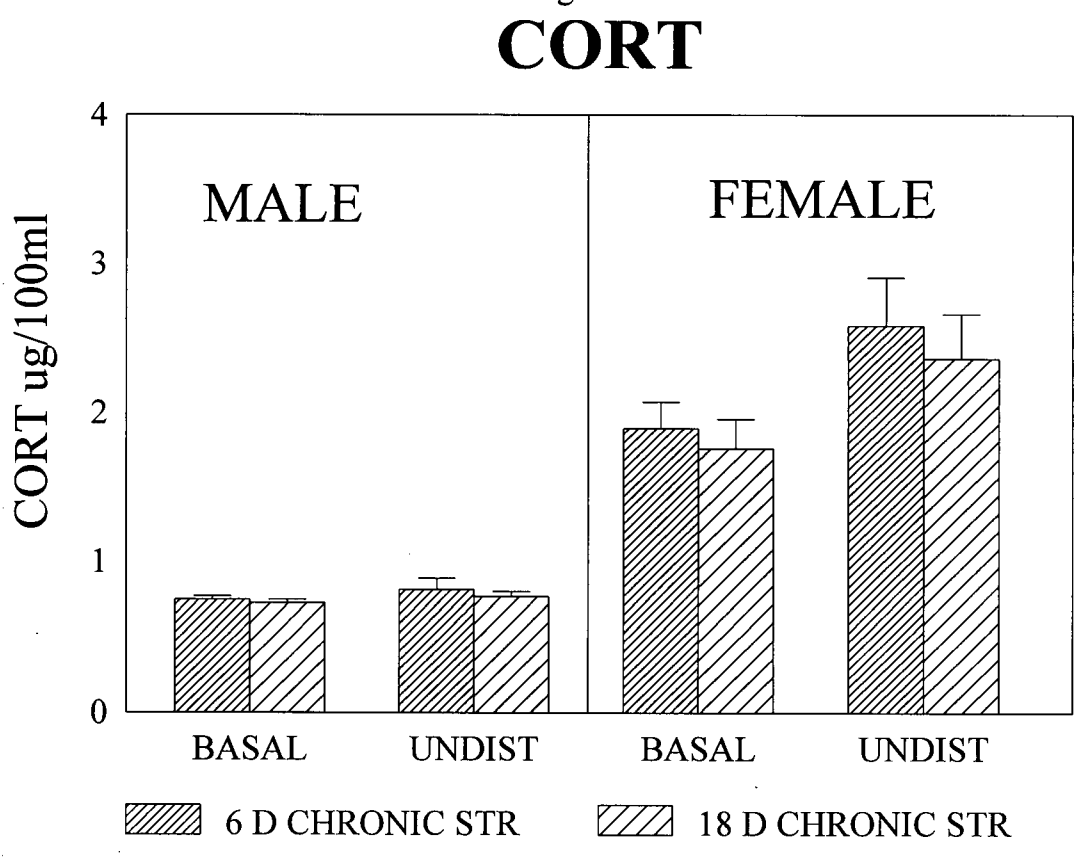

CORT
CORT ug/100ml
4
3
2
1
0
MALE
FEMALE
BASAL
UNDIST
BASAL
UNDIST
6 D CHRONIC STR
18 D CHRONIC STR

Figure 1.2

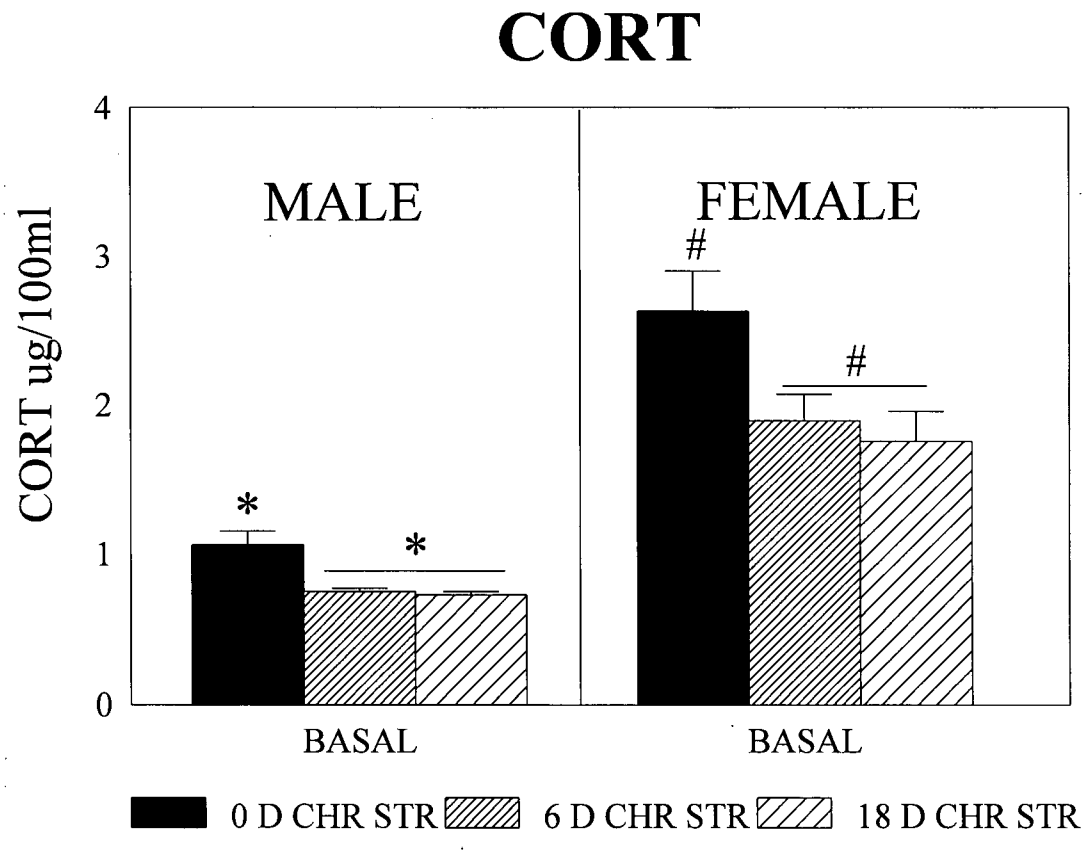

CORT
CORT ug/100ml
4
3
2
1
0
MALE
FEMALE
#
#
*
*
BASAL
BASAL
0 D CHR STR
6 D CHR STR
18 D CHR STR

Figure 1.3

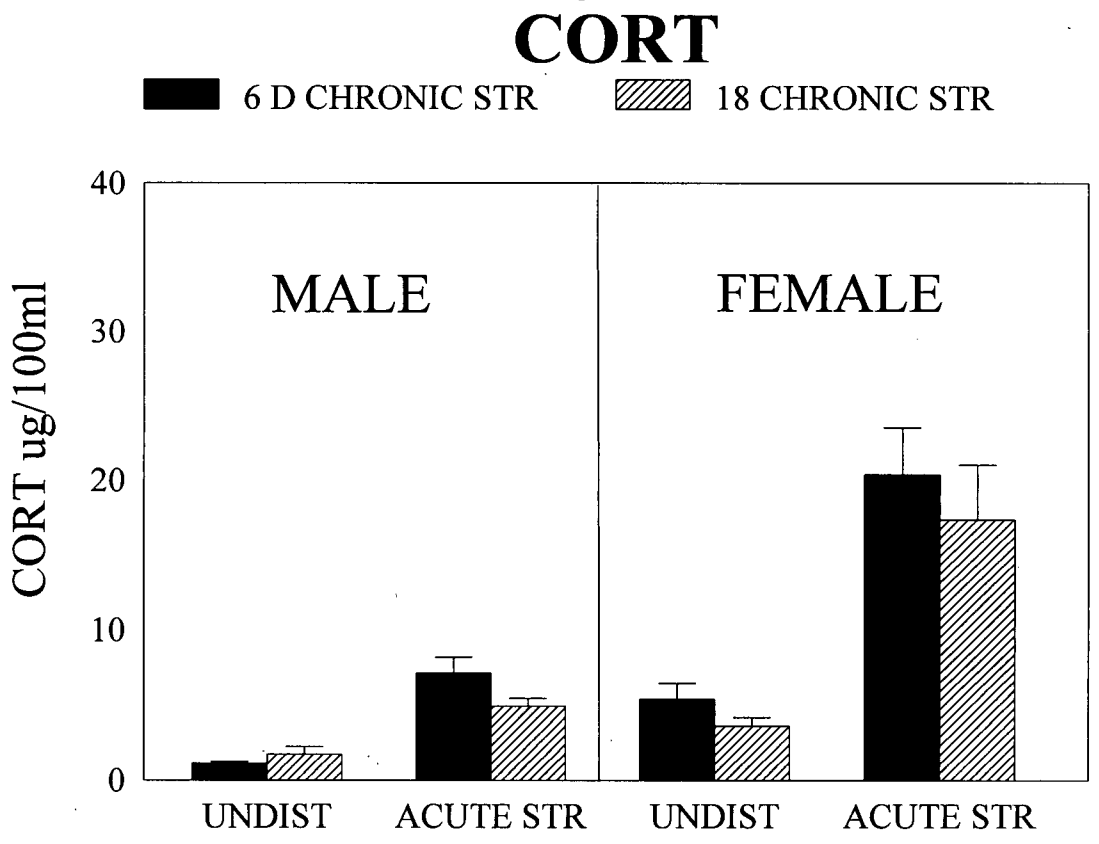

CORT
6 D CHRONIC STR
18 CHRONIC STR
CORT ug/100ml
40
30
20
10
0
MALE
FEMALE
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.4

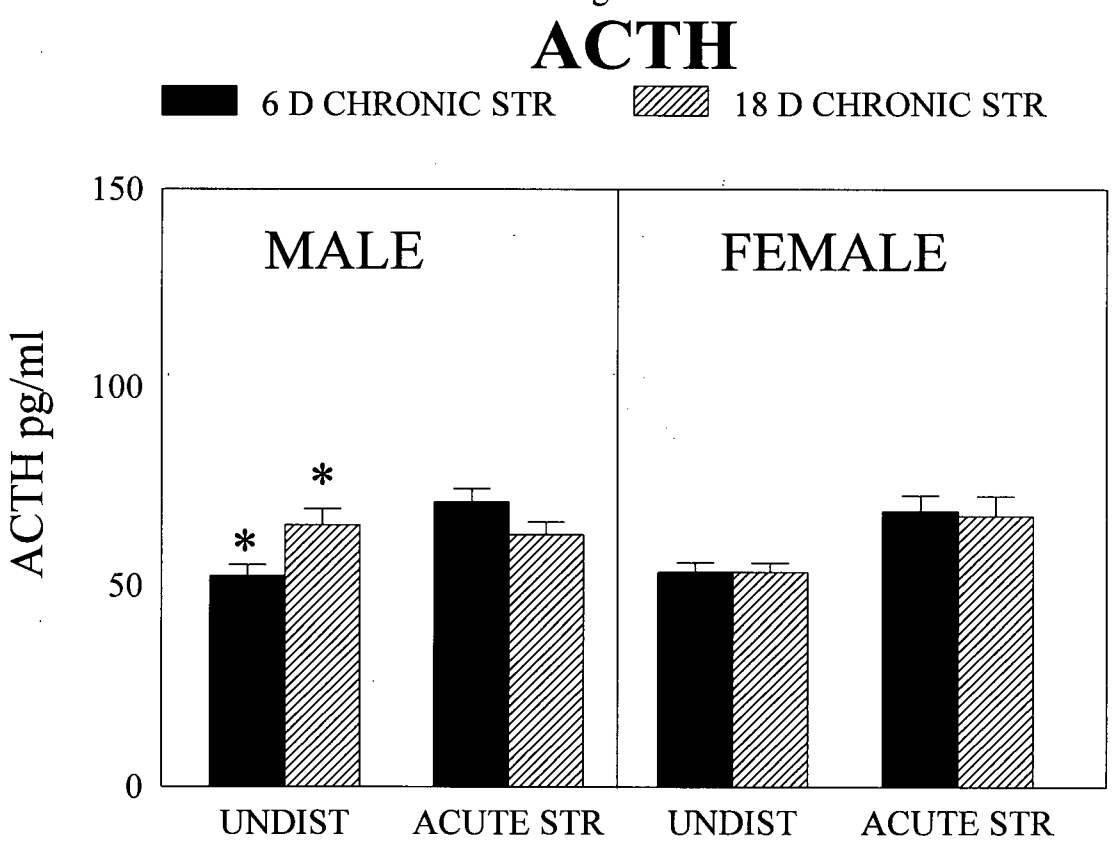

ACTH
6 D CHRONIC STR
18 D CHRONIC STR
150
100
50
0
ACTH pg/ml
MALE
FEMALE
*
*
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.5

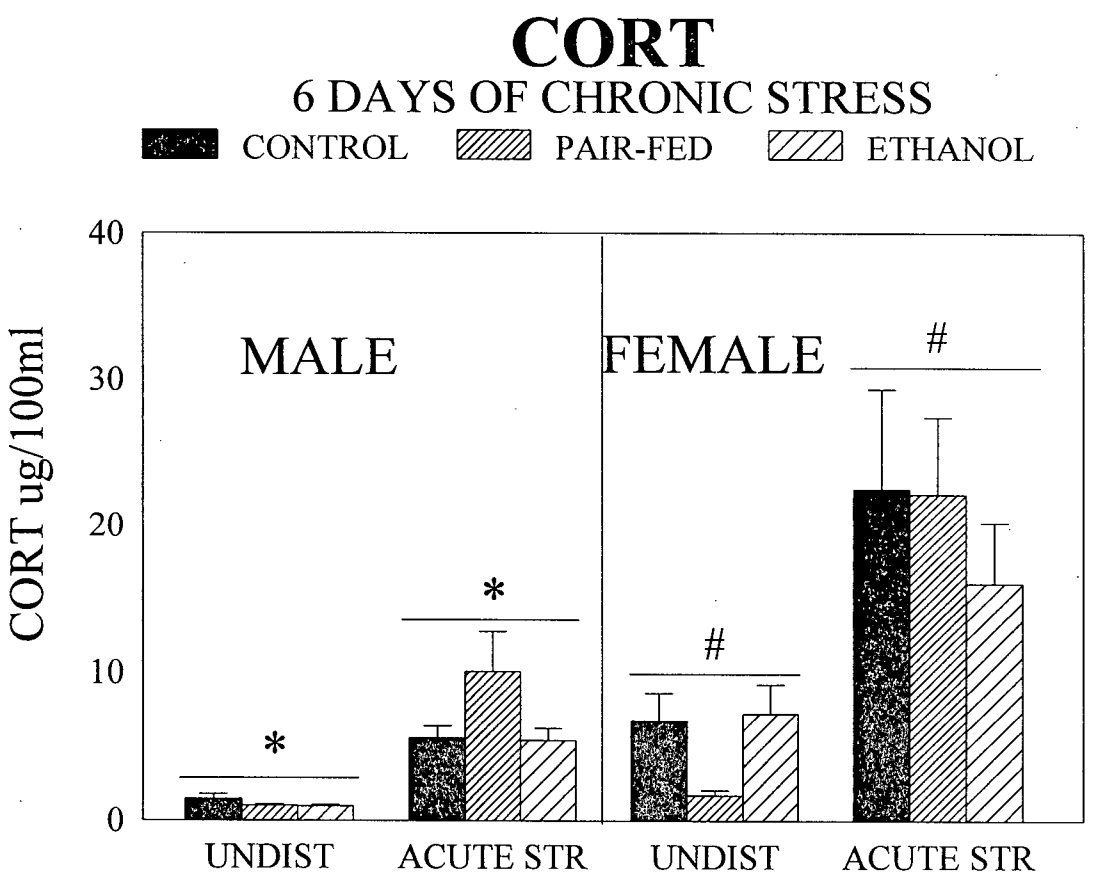

CORT
6 DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
CORT ug/100ml
40
30
20
10
0
MALE
FEMALE
*
*
#
#
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.6

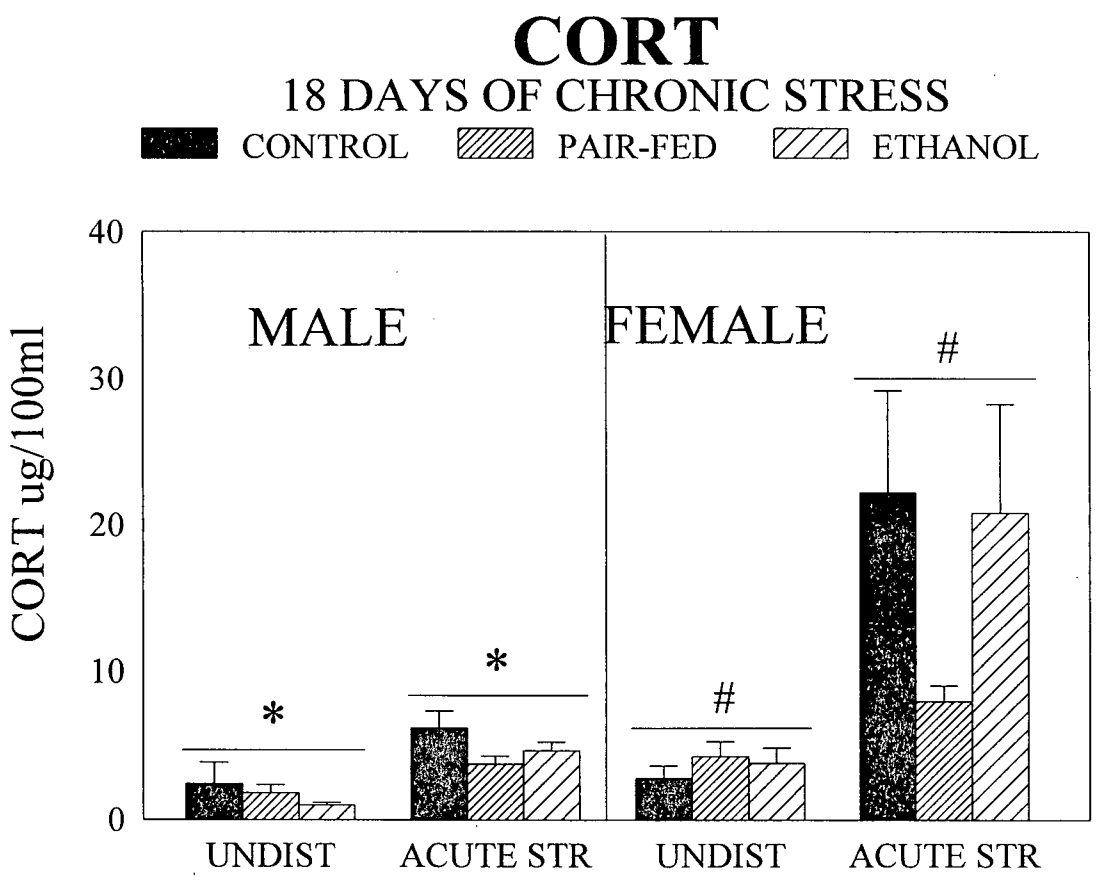

CORT
18 DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
CORT ug/100ml
40
30
20
10
0
MALE
FEMALE
*
*
#
#
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.7

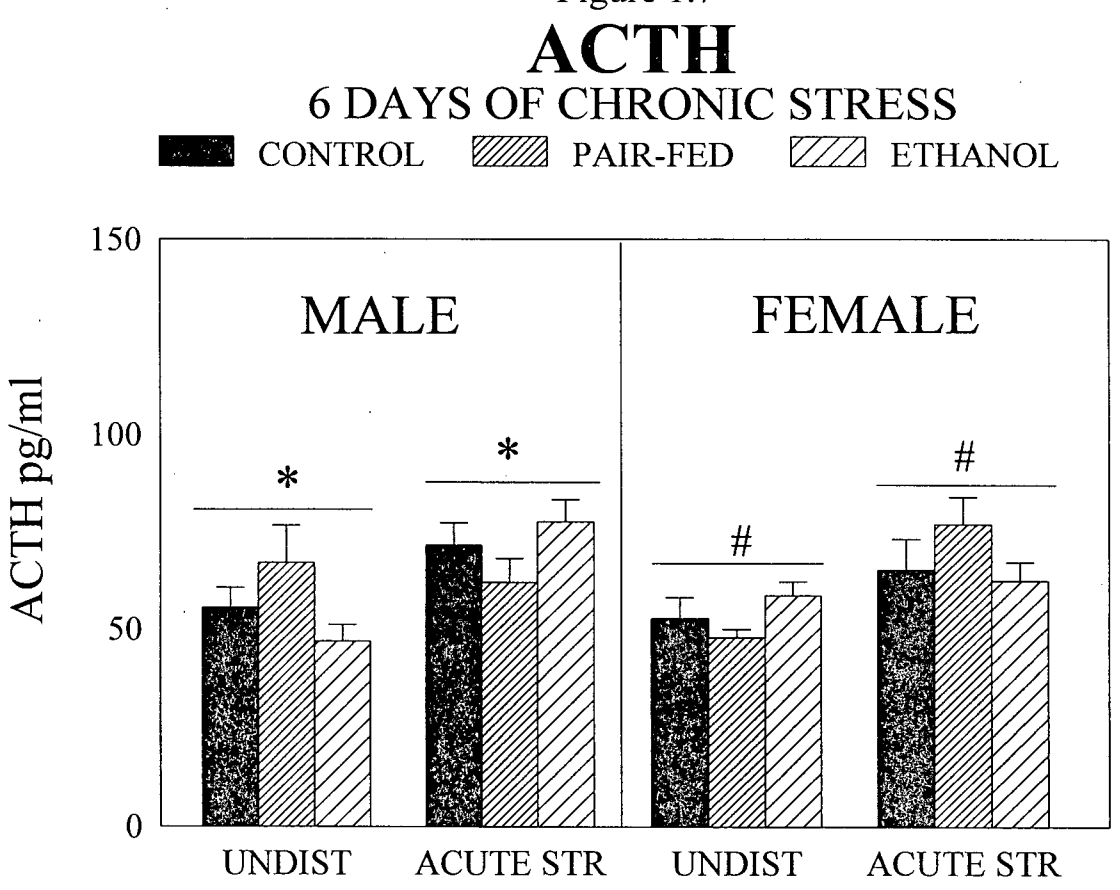

ACTH
6 DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
ACTH pg/ml
150
100
50
0
MALE
FEMALE
*
*
#
#
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.8

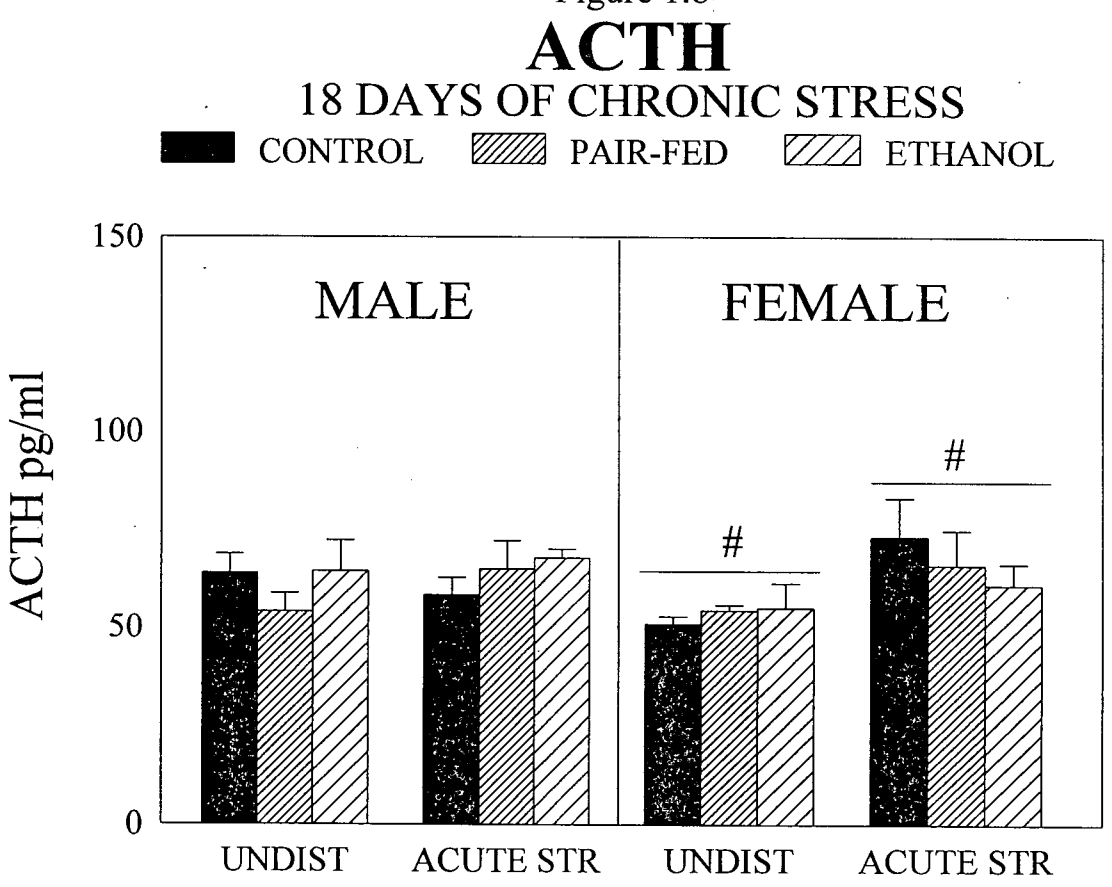
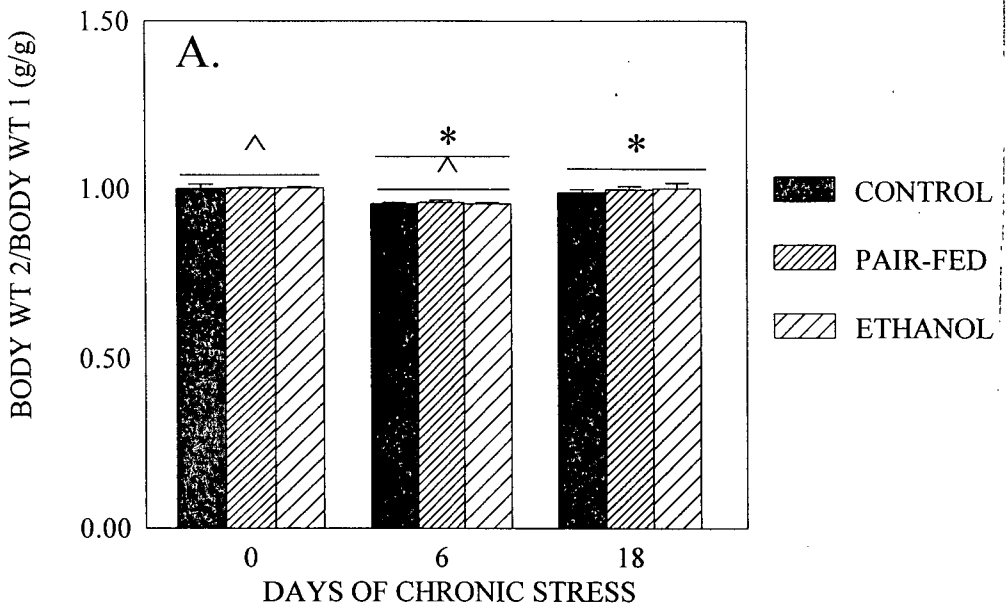

ACTH
18 DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
MALE
FEMALE
ACTH pg/ml
150
100
50
0
#
#
UNDIST
ACUTE STR
UNDIST
ACUTE STR

Figure 1.9

BODY WEIGHT GAIN

MALE



FEMALE

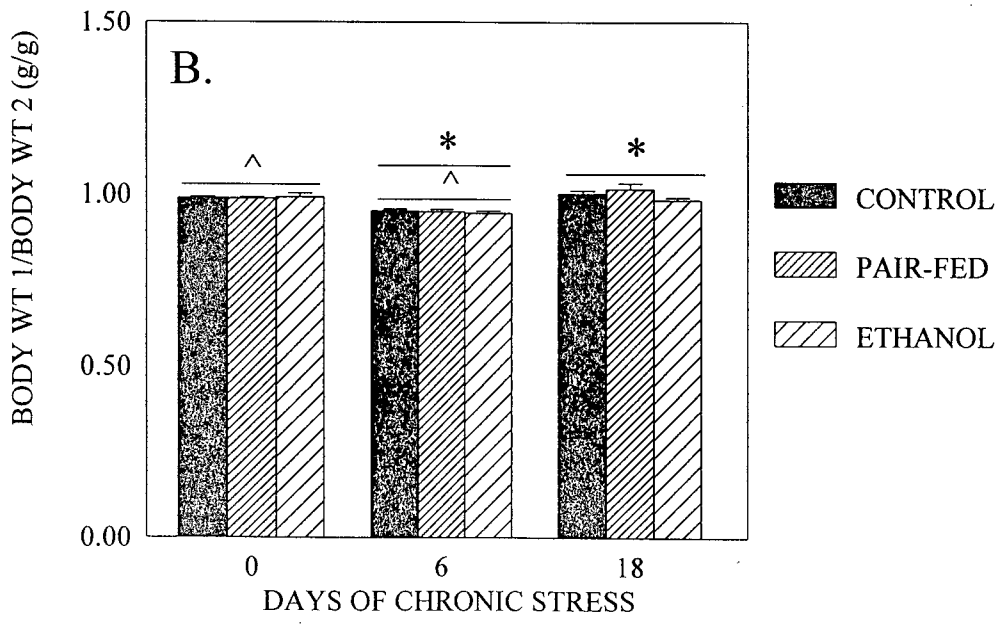
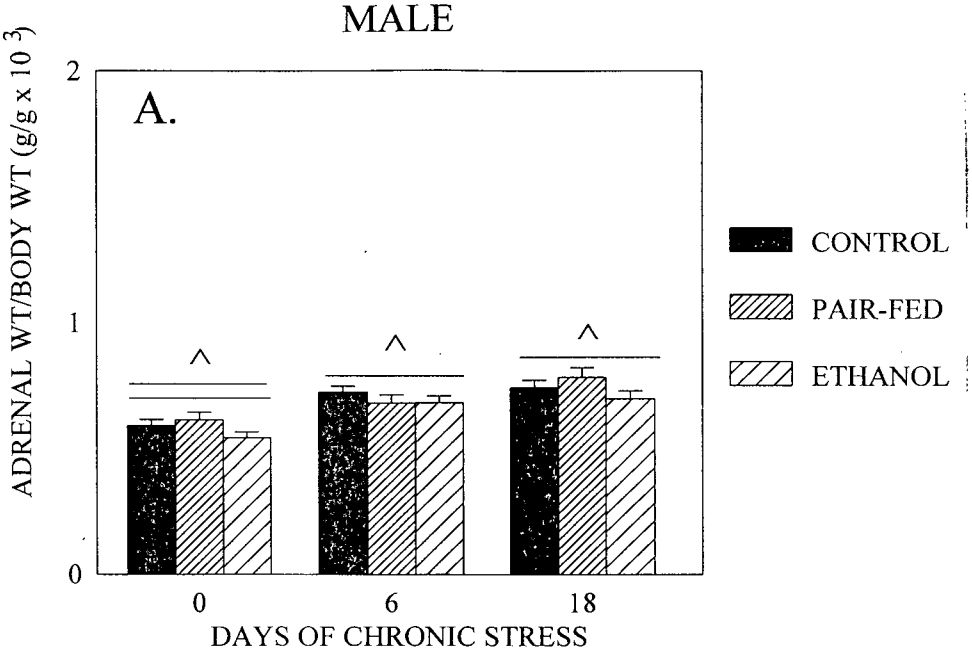


Figure 1.10

RELATIVE ADRENAL WEIGHTS

MALE



FEMALE

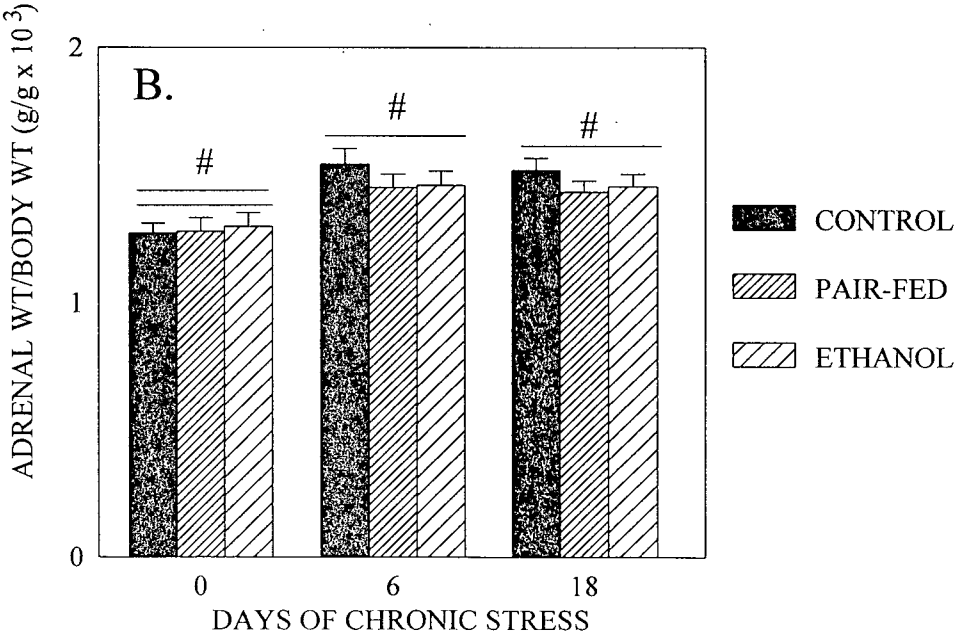
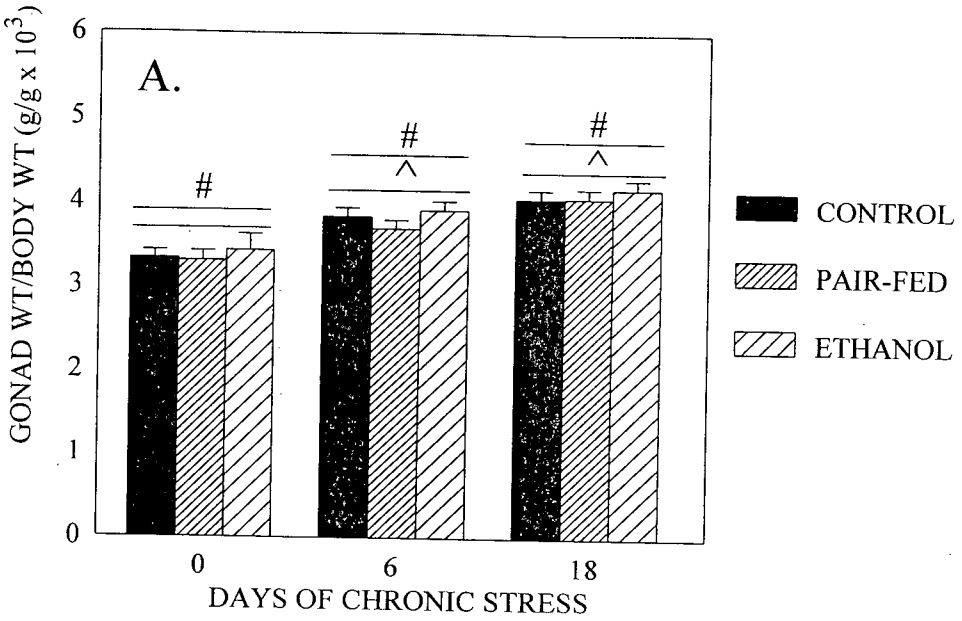


Figure 1.11

RELATIVE GONADAL WEIGHTS

MALE



FEMALE

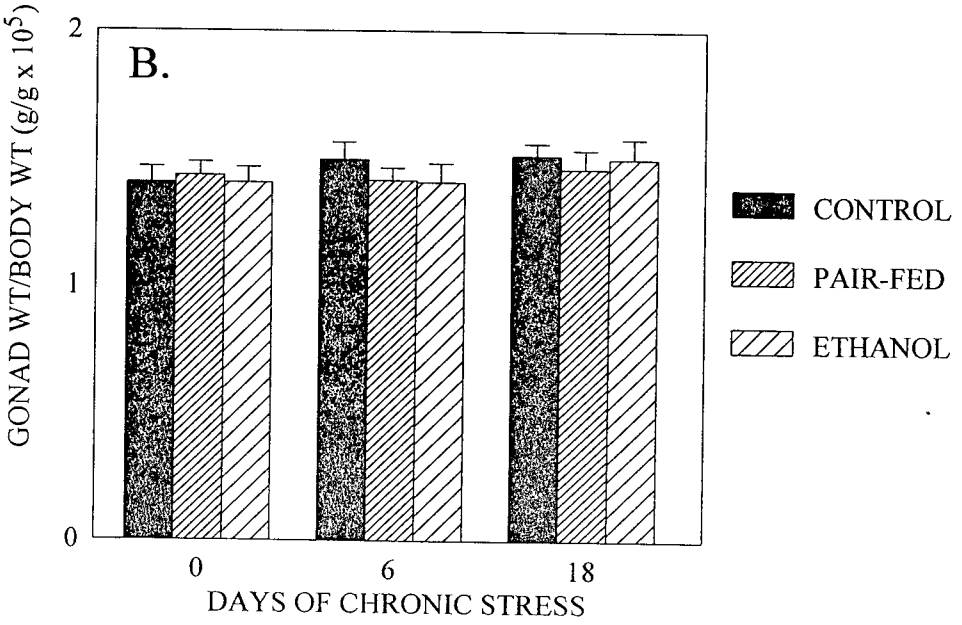
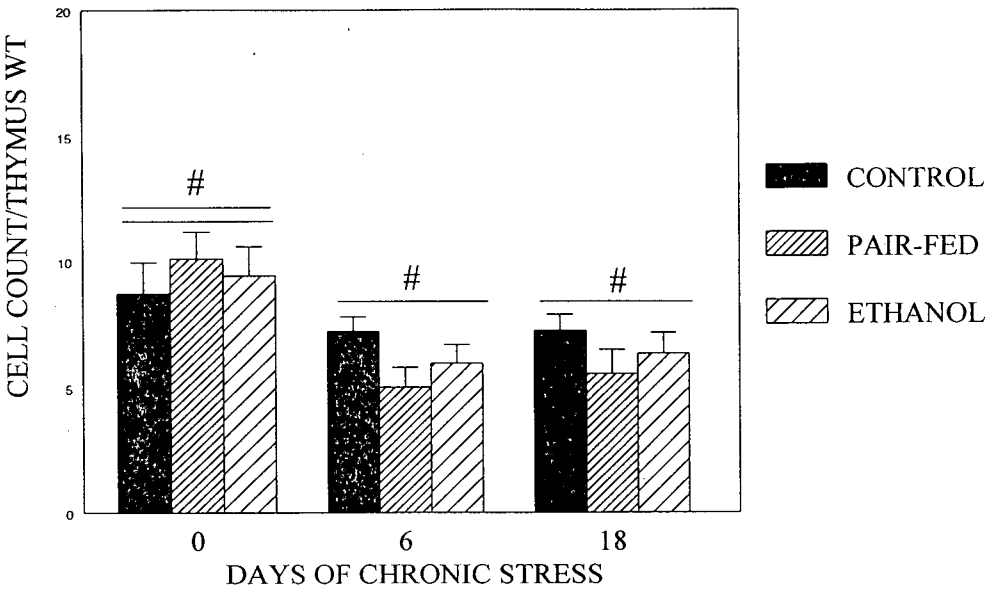


Figure 1.12

THYMUS CELL COUNTS

MALE



FEMALE

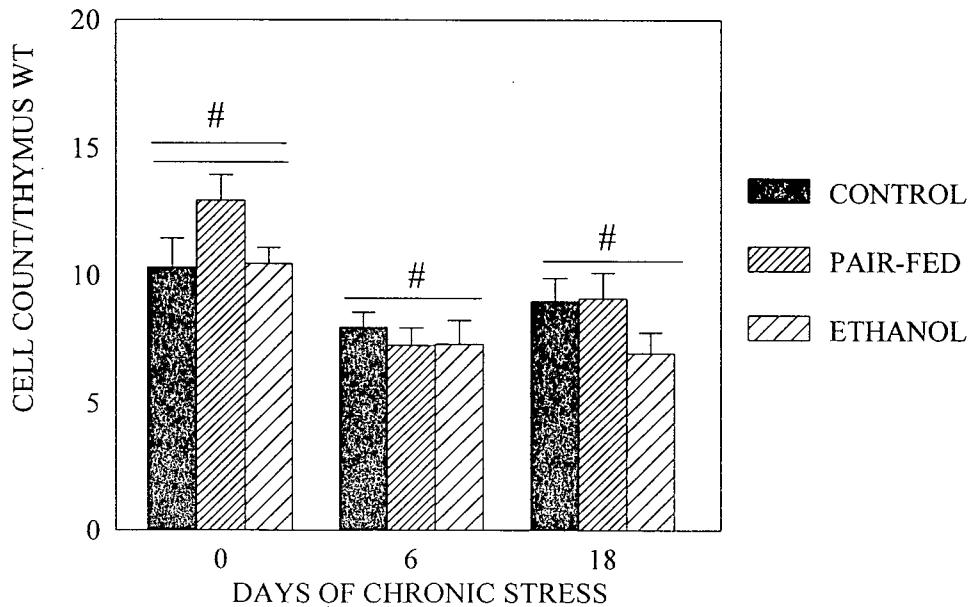
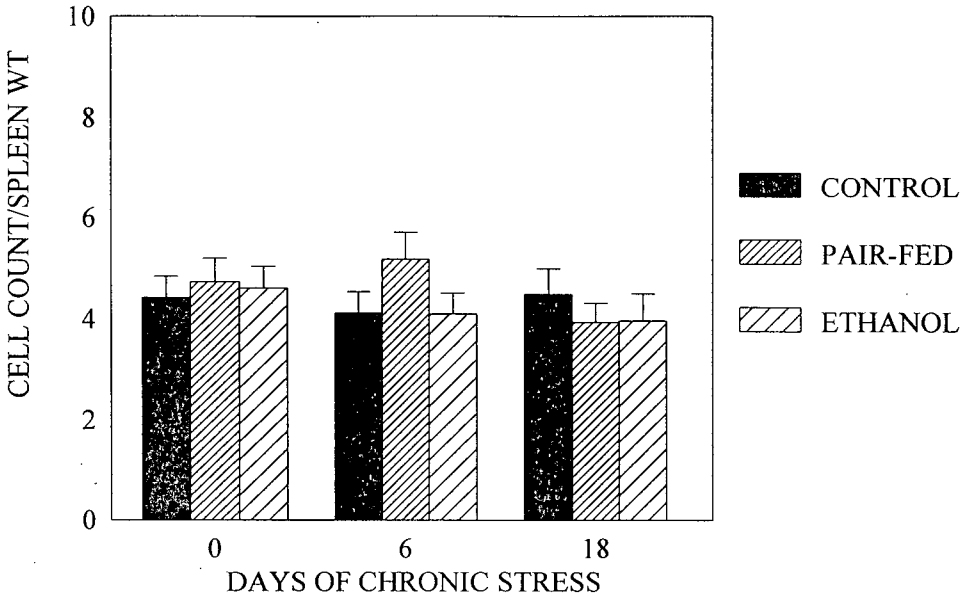


Figure 1.13

SPLEEN CELL COUNTS

MALE



FEMALE

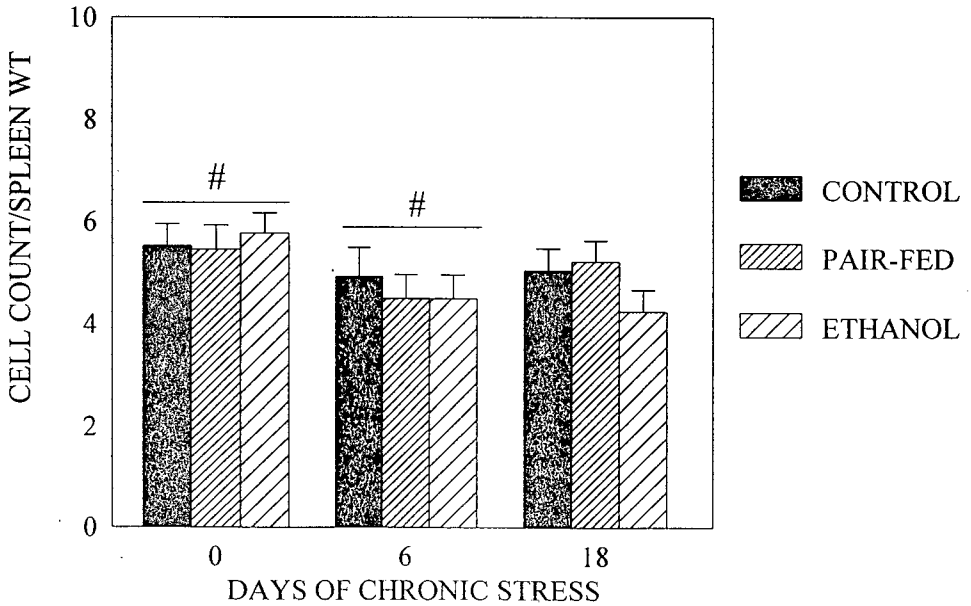


TABLE 2.1. MATERNAL BODY WEIGHTS AND GESTATION LENGTHS
(Mean ± S.E.M.)

Maternal Females									
	Gestational Weights (g)				Gestation Length (days)	Lactational Weights (g)			
	D1	D7	D14	D21		D1	D8	D15	D22
E (14)	251.4 ± 3.0	254.6# ± 3.8	280.3# ± 5.5	337.7# ± 11.8	23.1Ψ ± 0.1	284.1 ± 4.4	316.7 ± 4.5	344.0 ± 6.6	340.4 ± 6.1
PF (15)	255.3 ± 3.4	254.3# ± 3.0	277.7# ± 3.5	345.8# ± 7.6	23.0Ψ ± 0.0	286.0 ± 4.1	315.7 ± 4.6	348.2 ± 4.8	341.8 ± 4.6
C (16)	253.8 ± 5.7	273.9 ± 6.4	303.1 ± 7.5	393.1 ± 10.3	22.6 ± 0.2	293.7 ± 7.1	320.1 ± 6.7	344.1 ± 7.4	330.0 ± 6.8

#E = PF < C, p's < 0.05

ΨE > C, p < 0.05; PF > C, p < 0.07

TABLE 2.2. OFFSPRING BODY WEIGHTS AND NUMBER OF LIVERBORN
(Mean ± S.E.M.)

	Pups									
	Offspring Weight (g) Males				Number of Liveborn Males	Offspring Weight (g) Females				Number of Liveborn Females
	D1	D8	D15	D22		D1	D8	D15	D22	
E	5.7 ± 0.1	14.6 ± 0.4	30.5 ± 0.4	46.4 ± 0.8	7.9 ± 0.8	5.4$^{\otimes}$ ± 0.1	13.7$^{\otimes}$ ± 0.4	29.0 ± 0.6	43.7$^{\otimes}$ ± 0.9	7.1 ± 0.7
PF	5.9 ± 0.1	14.7 ± 0.5	28.7 ± 1.8	47.9 ± 1.0	8.1 ± 0.6	5.5$^{\otimes}$ ± 0.1	13.9$^{\otimes}$ ± 0.4	29.3 ± 0.5	45.3 ± 0.7	7.3 ± 0.7
C	6.3 ± 0.1	16.2 ± 0.5	32.0 ± 0.9	49.2 ± 1.5	7.5 ± 0.8	6.1 ± 0.1	15.7 ± 0.4	31.0 ± 0.7	47.8 ± 1.2	6.6 ± 0.5

$^{\#}$E = PF < C, p's < 0.05

$^{\otimes}$E < C, p < 0.05

FIGURE LEGENDS: EXPERIMENT 2

Figure 2.1. Body weight gain (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d and 21 d chronic stress conditions.

Males and Females. #Effect of chronic stress condition; 21 d < 0 d, p's < 0.001.

Figure 2.2. Relative adrenal weights (mean ± S.E.M.) for E, PF and C males (A) and females (B) in the 0 d and 21 d chronic stress conditions.

Males and Females. #Effect of chronic stress condition; 21 d > 0 d, p's < 0.001.

Figure 2.3. Counts per minute (mean ± S.E.M.) in response to Con A at 0.1 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

Males. ^Effect of prenatal treatment; 21 d chronic stress condition: E > C, $p < 0.05$; E > PF, $p < 0.08$.

Females. #Effect of prenatal treatment; 0 d chronic stress condition: PF > C, $p < 0.005$; E > C, $p < 0.06$.

Figure 2.4. Counts per minute (mean ± S.E.M.) in response to Con A at 1.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

Females. #Effect of prenatal treatment; 21 d chronic stress condition: PF > C, $p < 0.05$.

Figure 2.5. Counts per minute (mean ± S.E.M.) in response to Con A at 10.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

*Effect of sex; **Males** < **Females**, $p < 0.005$.

Figure 2.6. Counts per minute (mean ± S.E.M.) in response to PWM at 0.1 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

*Effect of sex; **Males** < **Females**, $p < 0.001$.

Males. Effect of prenatal treatment; E > C, $p < 0.06$.

Females. #Effect of chronic stress; 21 d > 0 d, $p < 0.05$.

Figure 2.7. Counts per minute (mean ± S.E.M.) in response to PWM at 1.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

*Effect of sex; **Males** < **Females**, $p < 0.01$.

Females. #Effect of prenatal treatment; 21 d chronic stress condition: E > PF, $p < 0.05$.

Figure 2.8. Counts per minute (mean ± S.E.M.) in response to PWM at 10.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

Figure 2.9. Counts per minute (mean ± S.E.M.) in response to LPS at 0.1 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

Females. ^Effect of prenatal treatment; 0 d chronic stress condition: PF > C, $p < 0.05$.

#Effect of prenatal treatment; 0 d chronic stress condition, PF > E, $p < 0.05$.

Figure 2.10. Counts per minute (mean ± S.E.M.) in response to LPS at 1.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

Effect of sex; **Males** < **Females**, $p < 0.095$

Females. ^Effect of prenatal treatment; 0 d chronic stress condition: PF > C, $p < 0.05$.

#Effect of prenatal treatment; 0 d chronic stress condition, PF > E, $p < 0.05$

Figure 2.11. Counts per minute (mean ± S.E.M.) in response to LPS at 10.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

*Effect of sex; **Males** < **Females**, $p < 0.05$

Females. ^Effect of prenatal treatment; 0 d chronic stress condition: PF > C, $p < 0.05$.

#Effect of prenatal treatment; 21 d chronic stress condition, PF > C, $p < 0.05$.

Figure 2.12. Counts per minute (mean ± S.E.M.) in response to LPS at 100.0 μg/ml for E, PF and C males and females in the 0 d and 21 d chronic stress conditions.

*Effect of sex; **Males** < **Females**, $p < 0.002$

Females. #Effect of chronic stress; 21 d > 0 d, $p < 0.05$.

Effect of prenatal treatment; 21 d chronic stress condition: E = PF > C, p's < 0.08.

Figure 2.1

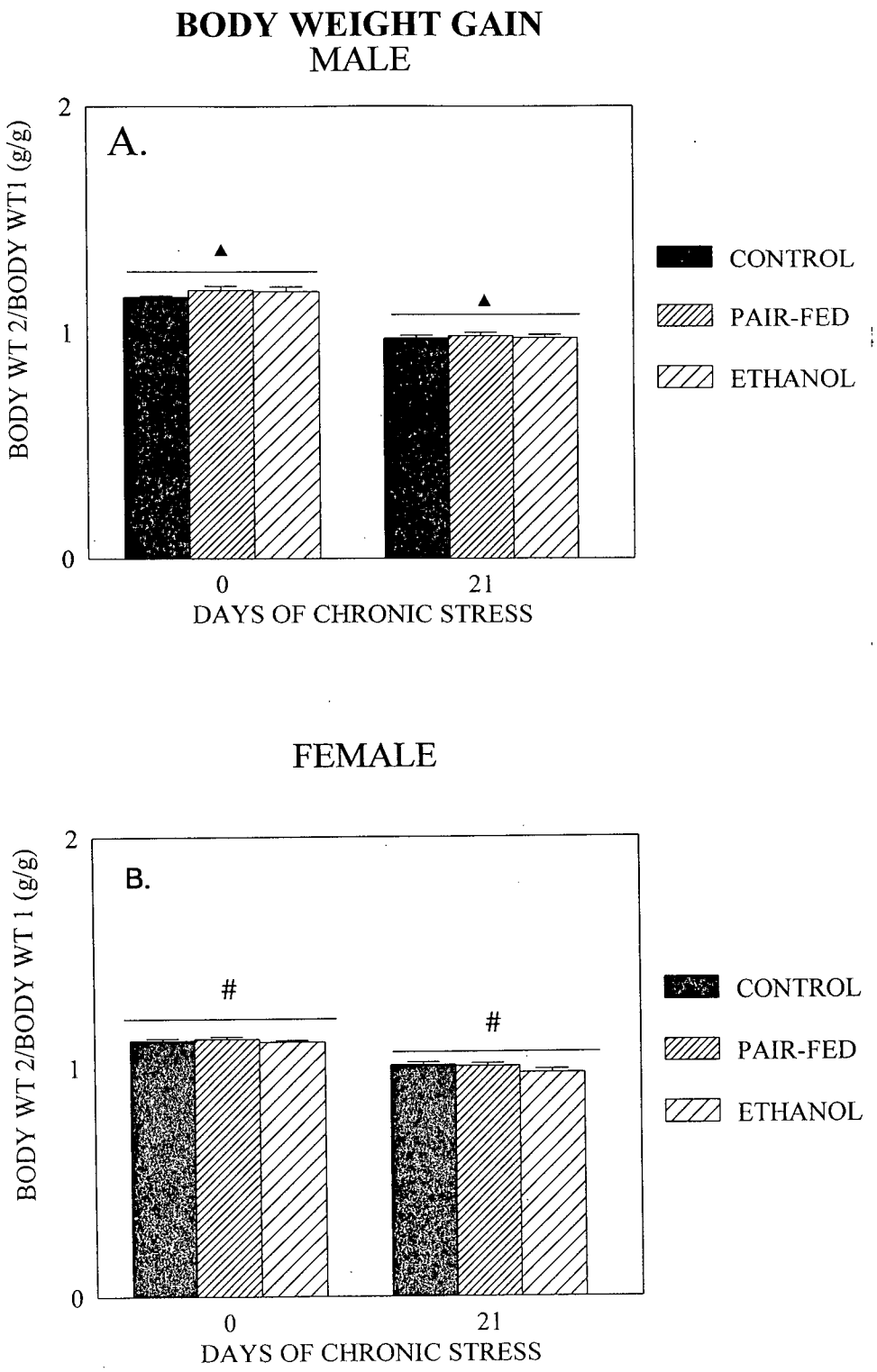

BODY WEIGHT GAIN
MALE
A.
BODY WT 2/BODY WT1 (g/g)
2
1
0
▲
▲
0
21
DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
FEMALE
B.
BODY WT 2/BODY WT 1 (g/g)
2
1
0
#
#
0
21
DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL

Figure 2.2

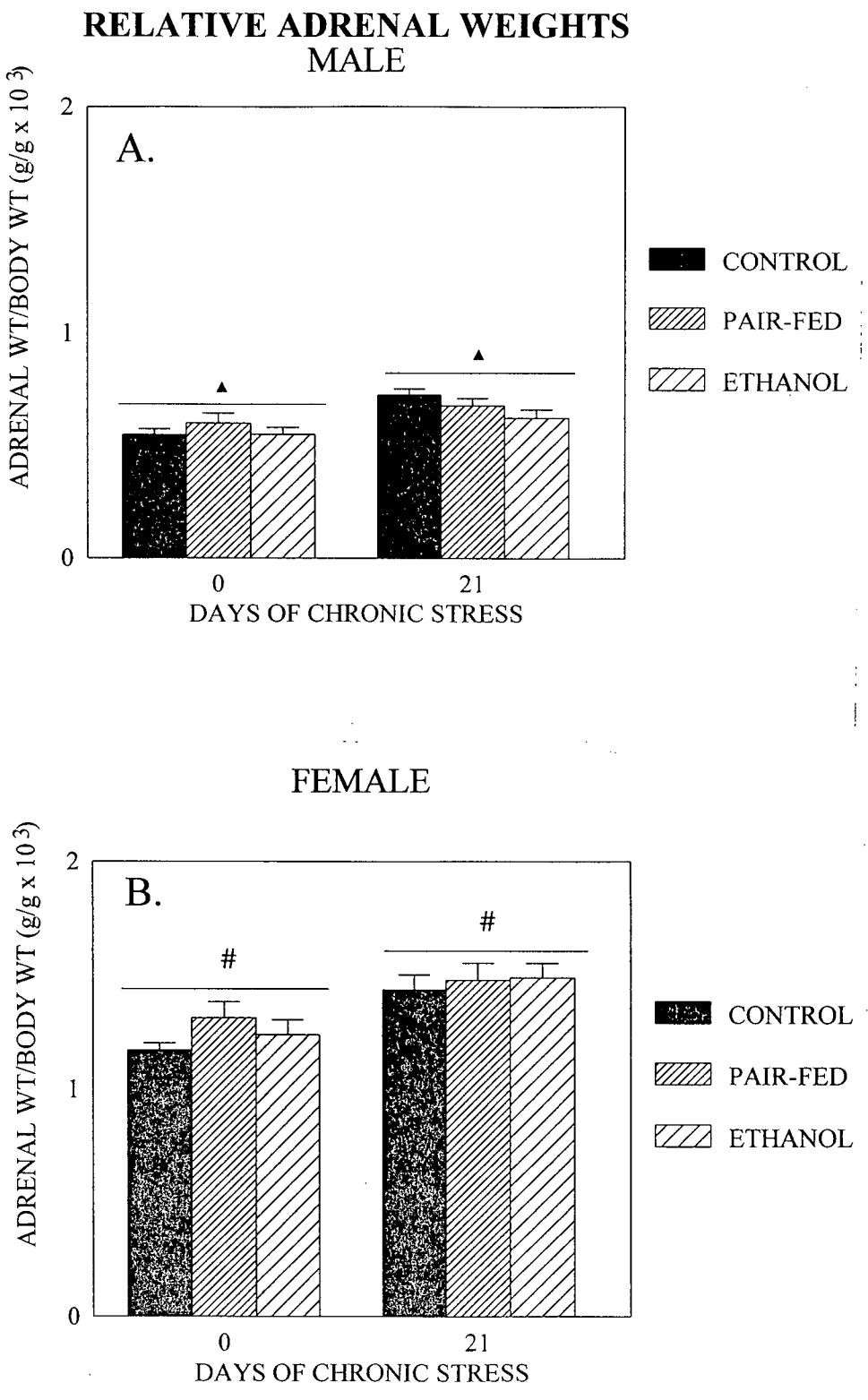

RELATIVE ADRENAL WEIGHTS
MALE
ADRENAL WT/BODY WT (g/g x 10 3)
2
1
0
A.
▲
▲
0
21
DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL
FEMALE
ADRENAL WT/BODY WT (g/g x 10 3)
2
1
0
B.
#
#
0
21
DAYS OF CHRONIC STRESS
CONTROL
PAIR-FED
ETHANOL

Figure 2.3

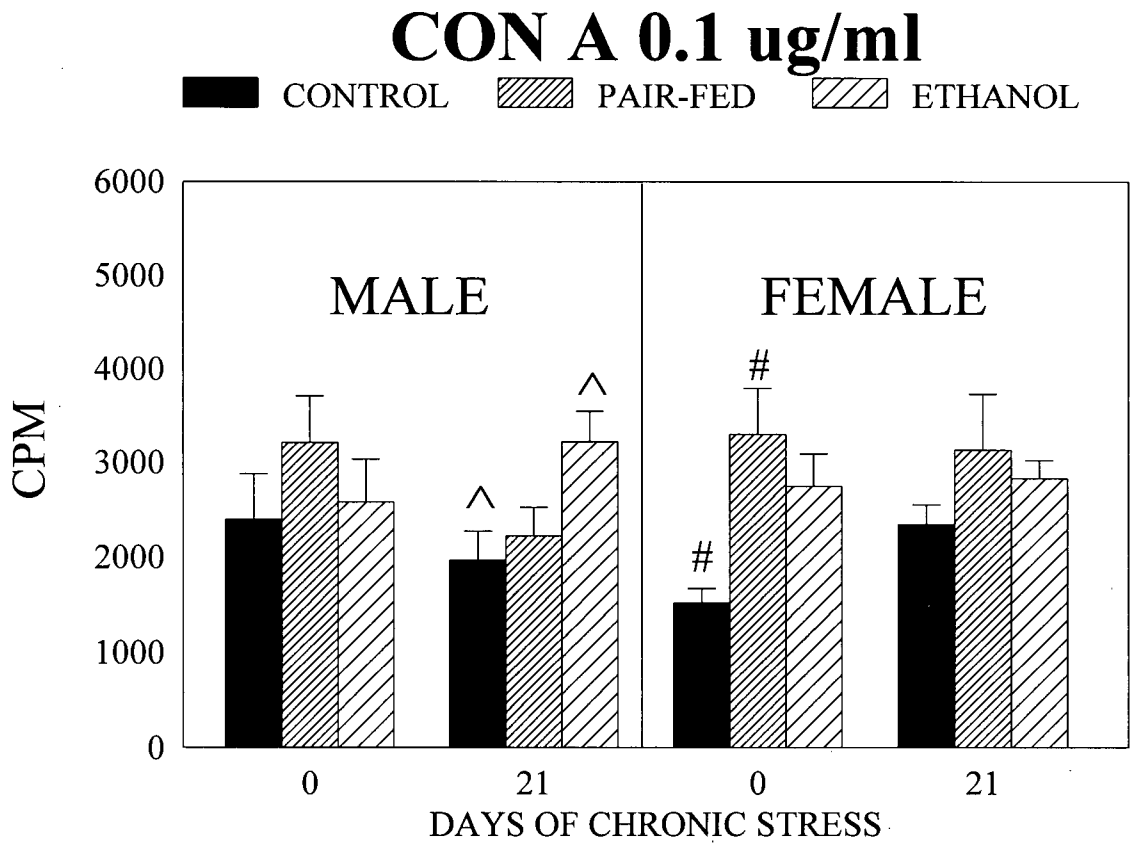

CON A 0.1 ug/ml
CONTROL
PAIR-FED
ETHANOL
6000
5000
4000
3000
2000
1000
0
CPM
MALE
FEMALE
^
^
#
#
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.4

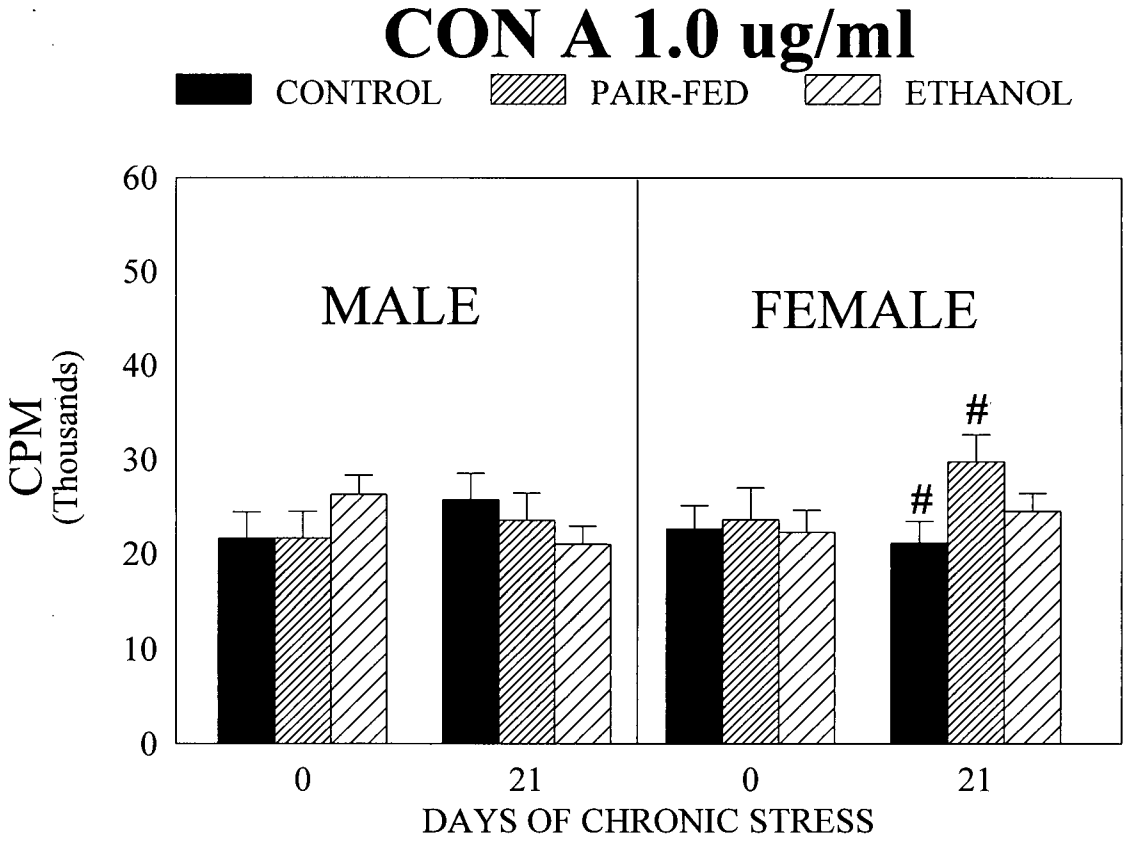

CON A 1.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
CPM
(Thousands)
60
50
40
30
20
10
0
MALE
FEMALE
#
#
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.5

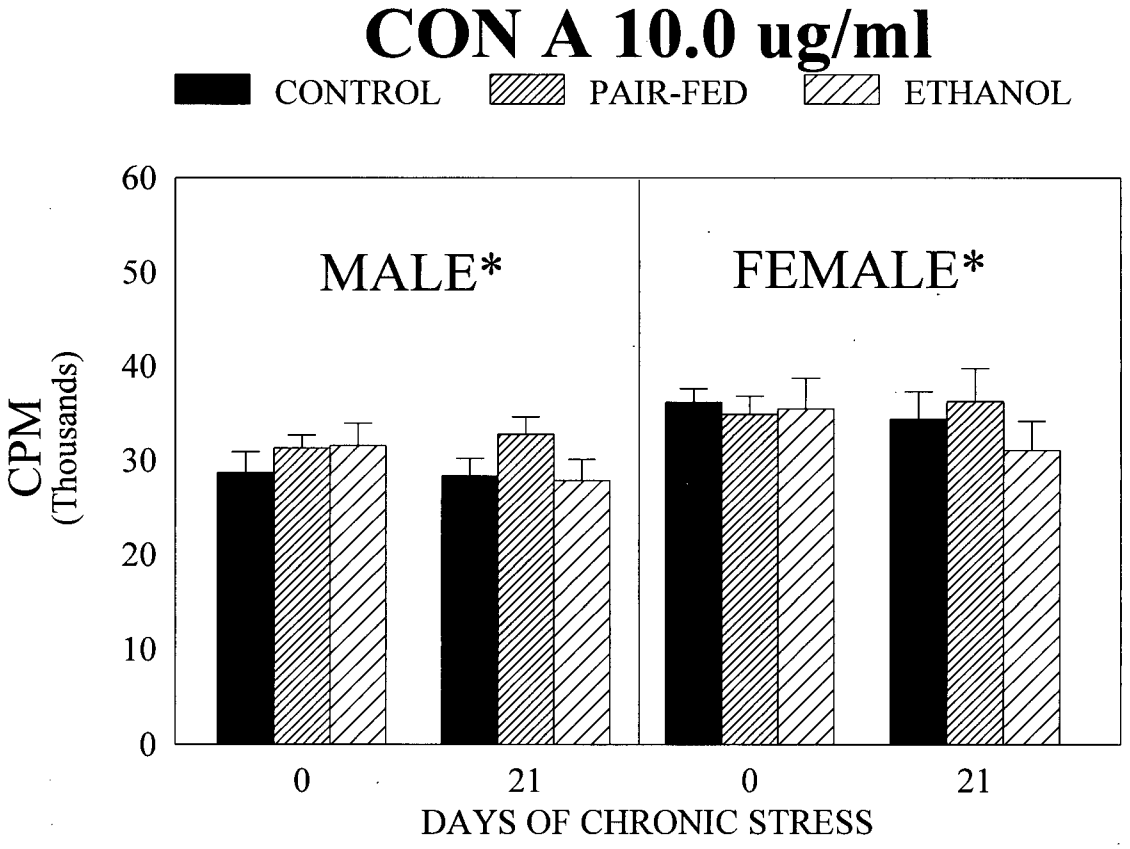

CON A 10.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
MALE*
FEMALE*
CPM
(Thousands)
60
50
40
30
20
10
0
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.6

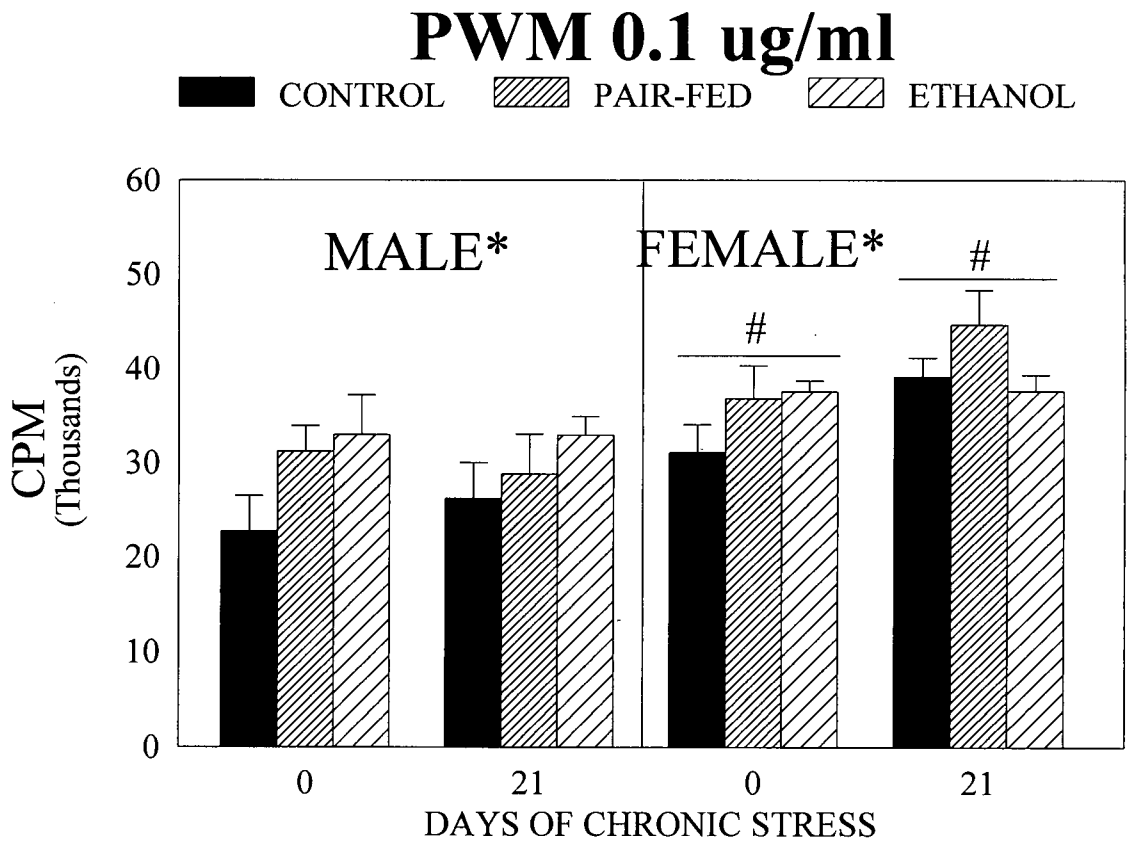

PWM 0.1 ug/ml
CONTROL
PAIR-FED
ETHANOL
MALE*
FEMALE*
#
#
CPM
(Thousands)
60
50
40
30
20
10
0
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.7

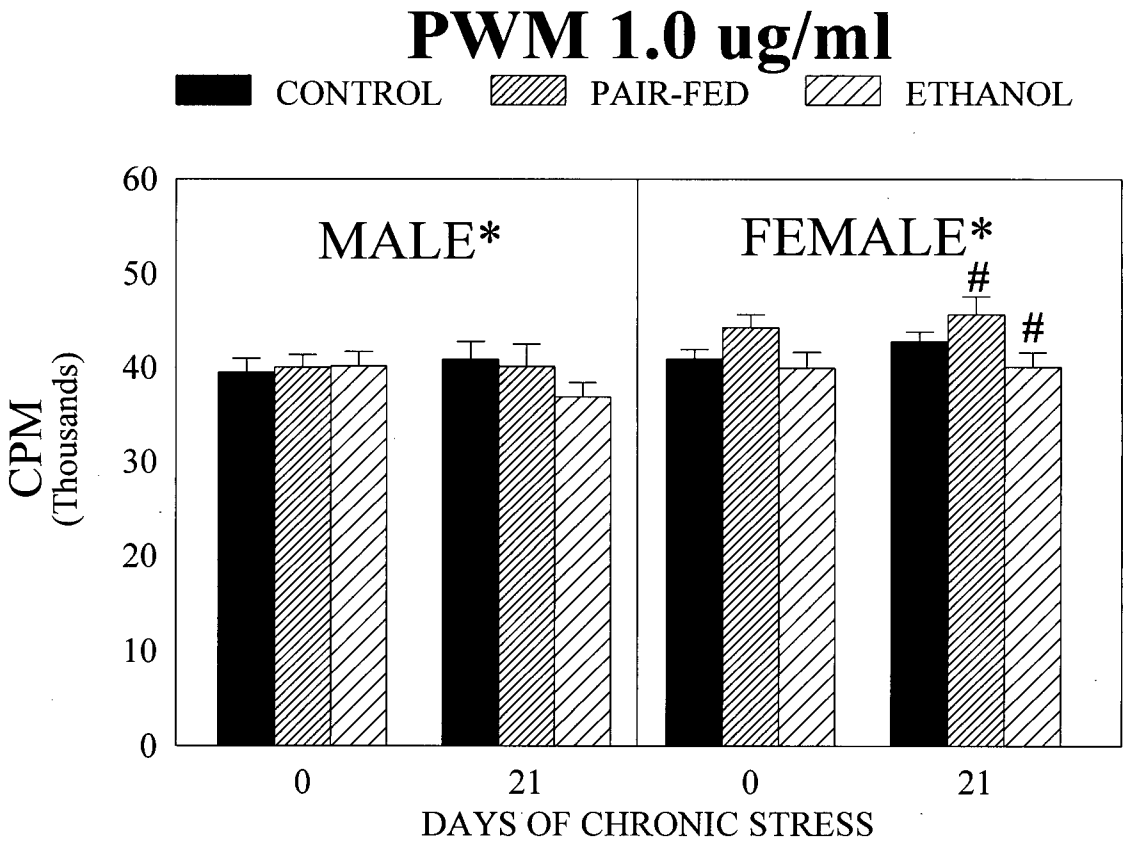

PWM 1.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
MALE*
FEMALE*
#
#
CPM
(Thousands)
60
50
40
30
20
10
0
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.8

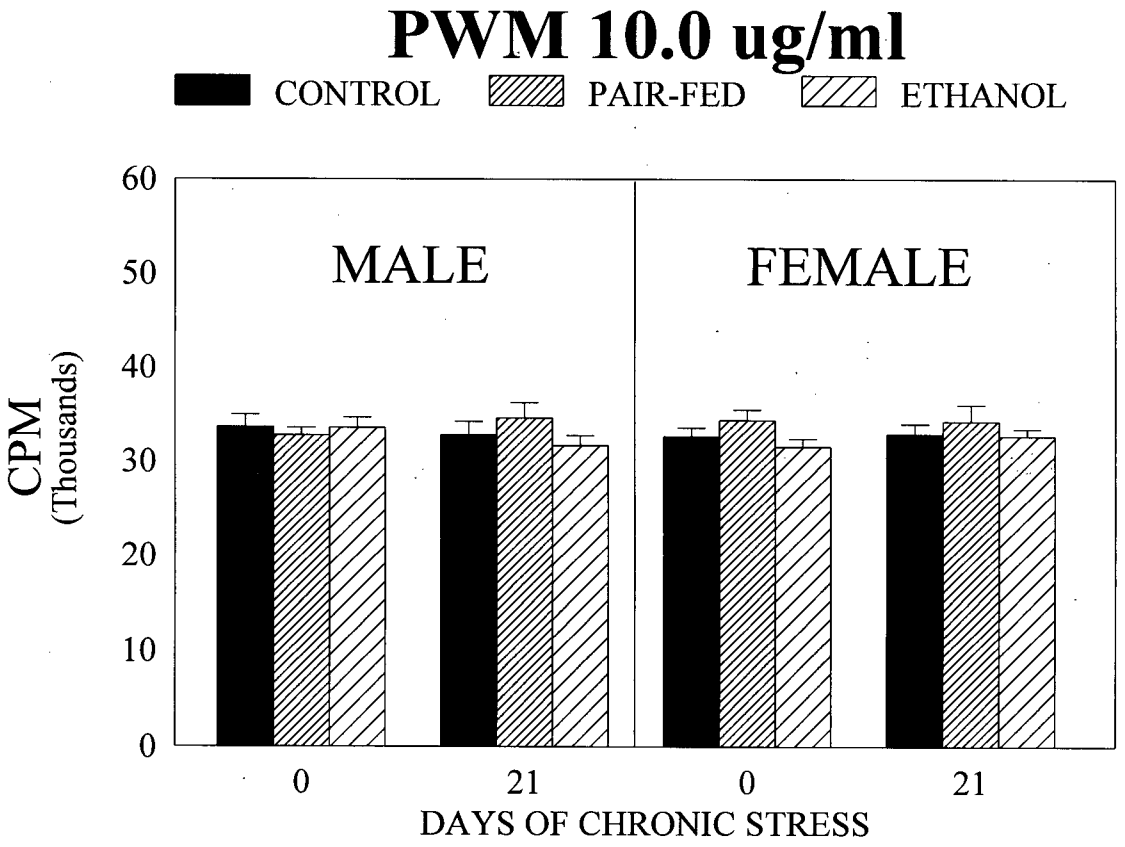

PWM 10.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
MALE
FEMALE
CPM
(Thousands)
60
50
40
30
20
10
0
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.9

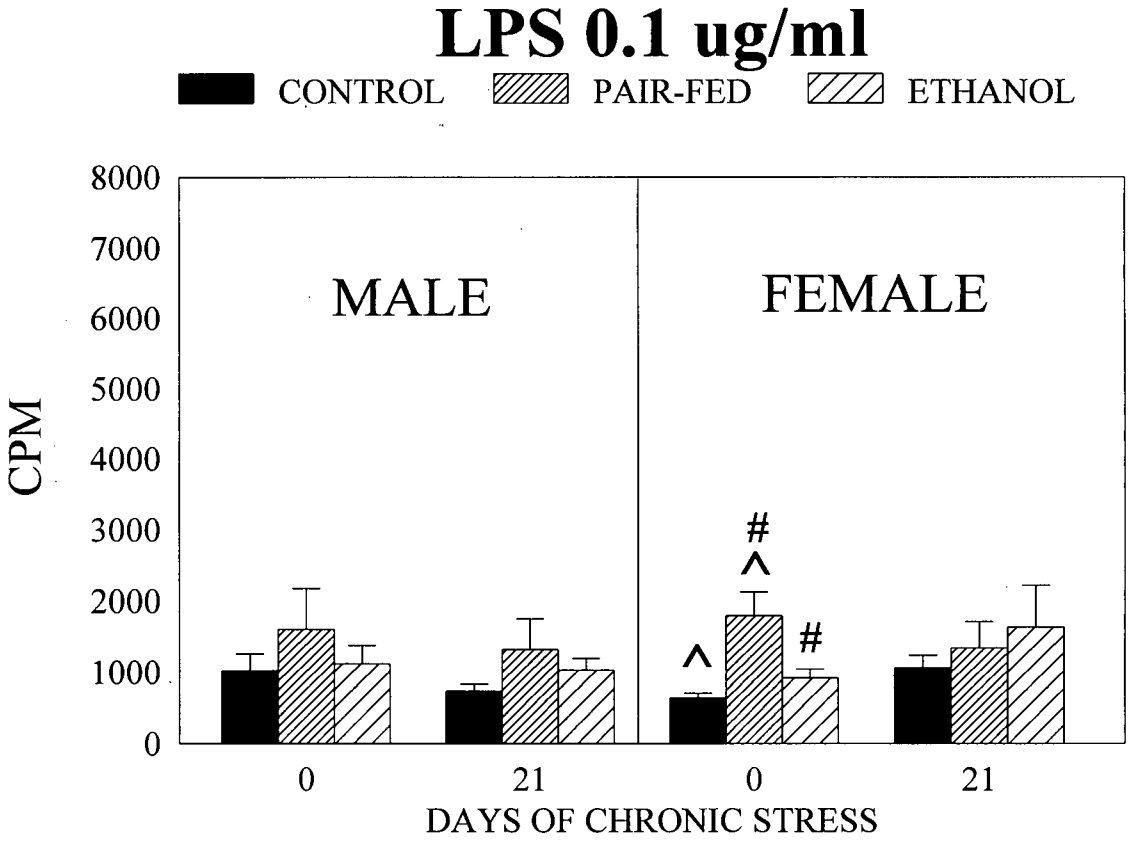

LPS 0.1 ug/ml
CONTROL
PAIR-FED
ETHANOL
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
MALE
FEMALE
#
^
^
#
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.10

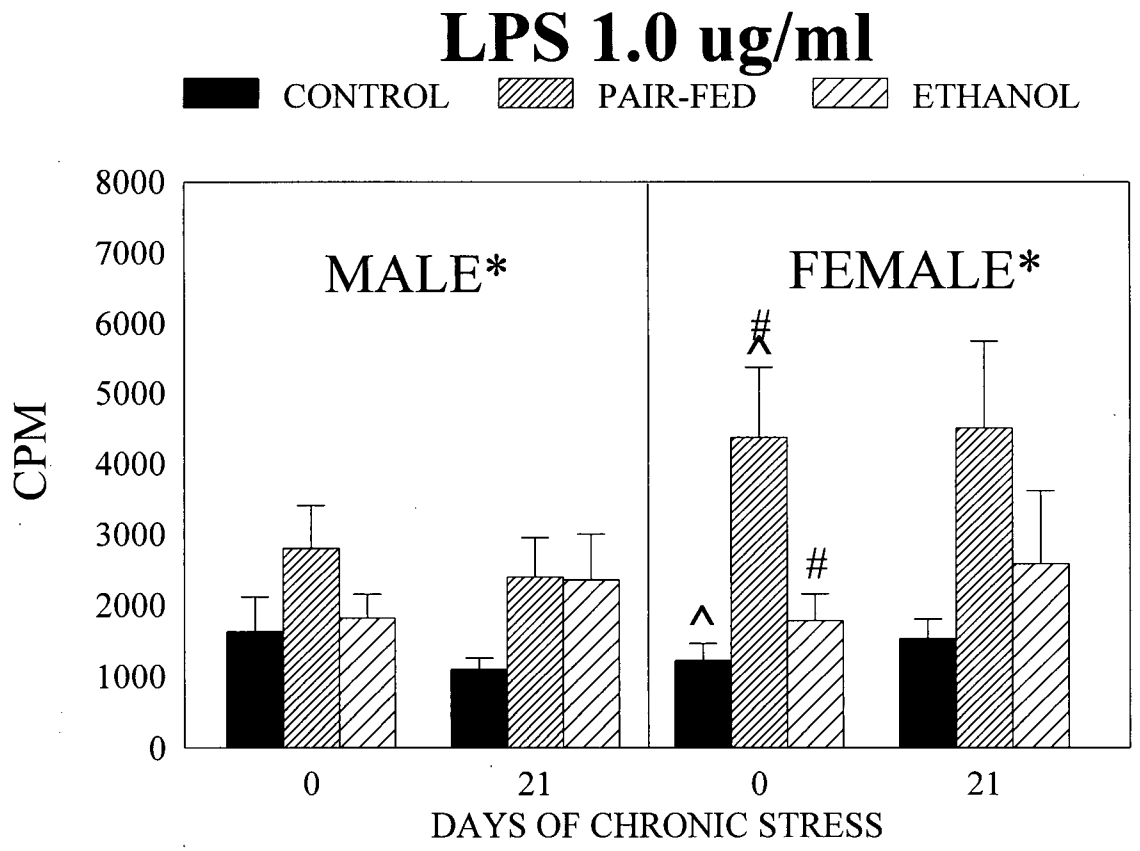

LPS 1.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
MALE*
FEMALE*
#
#
^
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.11

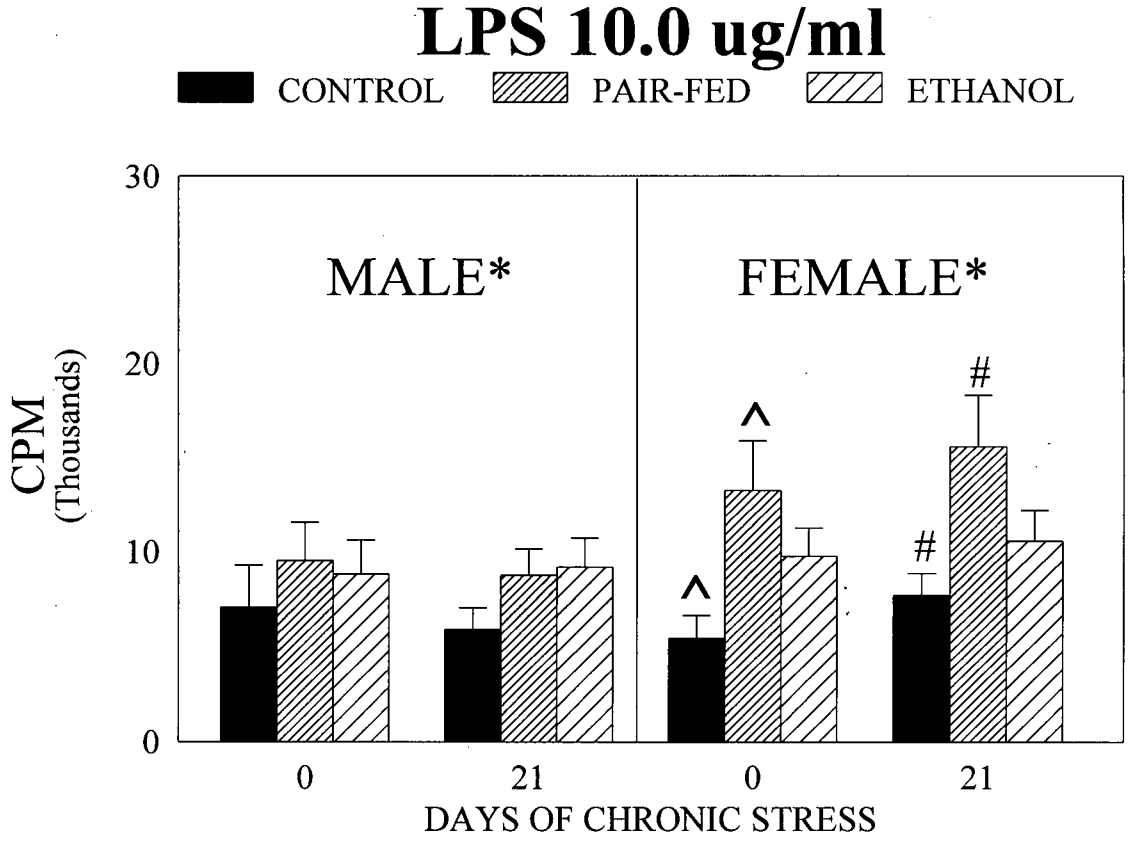
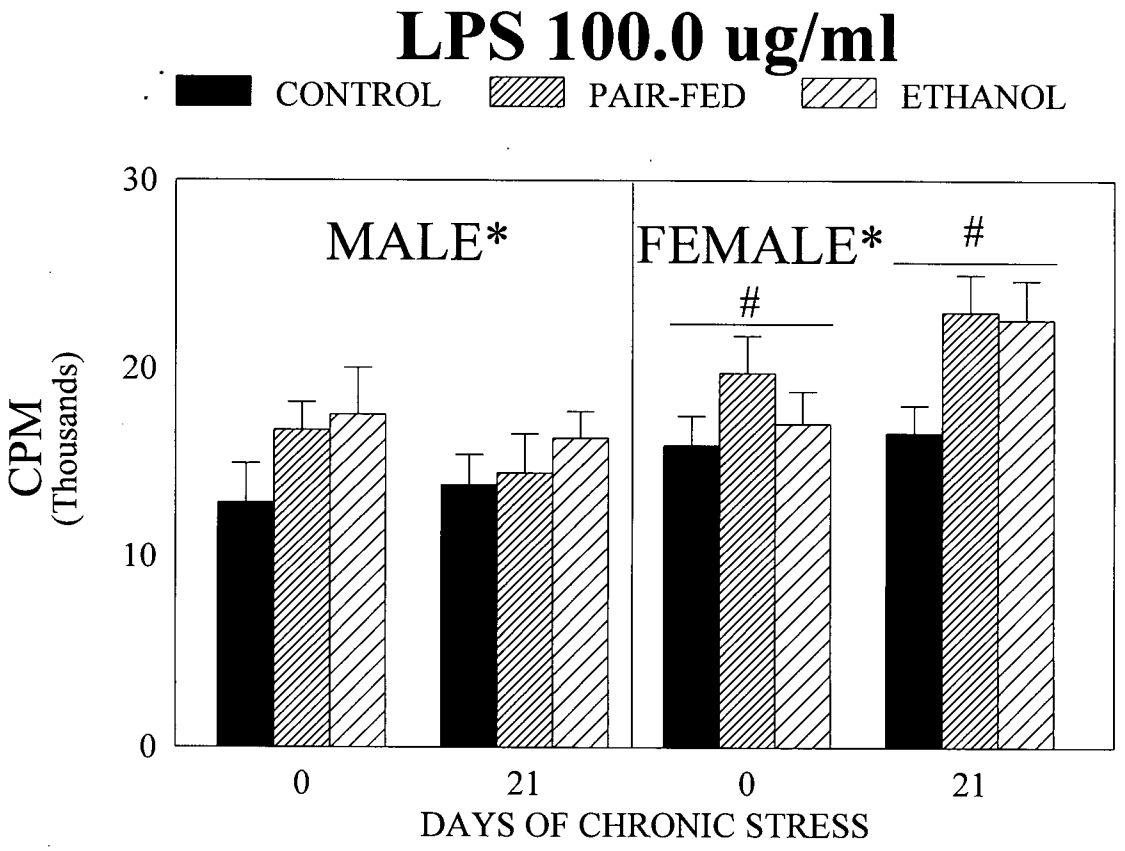

LPS 10.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
30
20
10
0
CPM
(Thousands)
MALE*
FEMALE*
^
^
#
#
0
21
0
21
DAYS OF CHRONIC STRESS

Figure 2.12


LPS 100.0 ug/ml
CONTROL
PAIR-FED
ETHANOL
MALE*
FEMALE*
#
#
CPM
(Thousands)
30
20
10
0
0
21
0
21
DAYS OF CHRONIC STRESS