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Effects of Estrous Stage on Leukocyte Distribution in Response to Restraint Stress in Mice (Mus musculus)

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Effects of Estrous Stage on Leukocyte Distribution in Response to Restraint Stress in Mice (*Mus musculus*)

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Natural Sciences at Carroll College, Helena, Montana.

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April 2, 2004
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4-2-04
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Finally, I would like to thank my family, peers, and professors for their constant support.
Abstract

Research has shown that there are interactions between the stress response and the immune system through the glucocorticoids. It has also been documented that estrogen and progesterone may have immunomodulatory effects and that the stress response may be modulated by the estrous cycle and the cyclic hormone levels associated with it. However, the connections between the stress response, the immune system, and the estrous cycle are not well understood. I examined changes in vaginal cytology and utilized simple leukocyte differential counts to determine if changes in the stress response correspond to the stages of the estrous cycle in *Mus musculus*. My results indicate that the stress treatment became significantly less effective throughout the experiment due to a habituation effect. Therefore, no definitive conclusions regarding the estrous cycle and the immune system could be made.
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Introduction

Interactions between psychological stress and immune response have been studied extensively. For example, Kelley (1985) investigated the effects of restraint stress on the cell-mediated immune response. Stefanski and Engler (1998) also studied the cellular and humoral immune responses using acute and chronic social stress as variables. Others have researched the relationship between stress and the neuroendocrine hormones that play a role in immune reactivity (Golub and Gershwin, 1985; Paavonen, 1987).

Female hormones have also been shown to have immunomodulatory effects. For example, Smart (2001) found that estrogen and progesterone have profound effects on the cellular immune response. In addition, Da Silva (1994) discussed gender differences in immune reactivity, specifically that estrogens promote B-cell immune response and decrease T-cell response, while progesterones and androgens decrease the immune response with respect to both cell types. Finally, Paavonen (1987) noted that reproductive hormones may affect the manifestation of autoimmune disease, as a greater percentage of diagnoses are made in women than men.

Although the hormonal dynamics of the estrous cycle and the effects on stress response and the immune system have been studied previously, knowledge is not extensive. Kryzch et al. (1978) found that the immune response to antigens and mitogens varies with the stages of the estrous cycle. Baron and Brush (1979) determined that acute and chronic stress response is also modulated by the estrous cycle.

This topic has become the subject of recent study as researchers have begun to investigate the interactions between the cyclic hormone levels and variations in the stress
responses of women. Figueiredo et al. (2002) observed that the interactions between the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, and fluctuating levels of female hormones may have made female rats more susceptible to both psychological and physiological stress. In addition, they found that the estrous cycle seemed to modulate the activation of the cerebral cortex and hippocampus. Komesaroff et al. (1998) observed that estrogen lowers and modulates the effects of audiovisual and metabolic stress on the glucocorticoids. These researchers and others suggest that because estrogen and progesterone levels vary throughout the stages of the estrous cycle, immune response may also vary accordingly (Figueiredo et al., 2002; Komesaroff et al., 1998; Kirschbaum et al., 1999).

The connections among the hormonal dynamics of the estrous cycle, stress response, and immune reactivity are not completely understood. Since other data suggest relationships between the cyclic female hormones and immune reactivity, I hypothesized that there would be a difference in stress response corresponding to the changing hormone levels associated with the four stages of the estrous cycle: proestrus, estrus, metestrus, and diestrus.
Literature Review

Immune System

Randall et al. (1997) described three functions of the immune system: identification, marking, and destruction of foreign matter, including pathogens and cells infected by tumors and viruses. Leukocytes (white blood cells), specifically lymphocytes, neutrophils, and monocytes, are key components of the cellular immune response. Cell-mediated immunity is defined as immune system responses that involve direct cell-to-cell interactions and are usually initiated by antigens and mediated by T lymphocytes or natural killer cells. This type of immunity is usually used under physiological conditions to destroy cells that are foreign to the body, including those mutated or altered by viral infections. It can also destroy grafted tissues and cause autoimmune disease. In contrast, humoral immunity is defined as protection against specific pathogens mediated by B lymphocytes that are capable of releasing antibodies into circulation (Martin, 1995).

Lymphocytes are responsible for the recognition of alien substances. Two types of lymphocytes exist—B cells and T cells. T cells are further divided into helper T cells ($T_H$) and cytotoxic T cells ($T_C$). Upon recognition of the antigen (foreign substance), B cells develop into plasma cells. Antibodies that bind specifically to the antigen are produced by the plasma cells, effectively tagging the invading cells as foreign. $T_C$ cells recognize and destroy altered self-cells, like tumors, by becoming cytotoxic T cells. $T_H$ cells produce cytokines, which stimulate the proliferation of B cells, $T_C$ cells, and macrophages and effectively enhance the immune response (Randall et al., 1997).
Neutrophils and monocytes are capable of phagocytizing and destroying the foreign matter. According to Johnson (2003), macrophages produce Interleukin 1 (IL-1), which prompts T cells to produce Interleukin 2 (IL-2). IL-2 stimulates further proliferation of T cells.

**Glucocorticoids**

When the body perceives stressful stimuli, small amounts of corticotrophin-releasing hormone (CRH) are released from the hypothalamus. CRH stimulates the adenohypophysis to release adrenocorticotropic hormone (ACTH), which travels through the bloodstream to its target tissue, the adrenal cortex. In response, the adrenal cortex releases adrenocortical hormones, including cortisol, cortisone, and corticosterone, collectively referred to as glucocorticoids (Randall *et al.*, 1997). The interactions described above act through a system referred to as the hypothalamo-pituitary-adrenal (HPA) axis.

According to Johnson (2003), glucocorticoids affect virtually every tissue of the body, although their precise actions in some tissues are not clearly understood. Some of their well-known actions include the mobilization of amino acids and fatty acids, promotion of gluconeogenesis, maintenance of glomerular filtration rates, anti-inflammation, and suppression of the immune response (Johnson, 2003).

Burchfield (1979) discussed the presence of glucocorticoid receptors on lymphocytes and monocytes circulating throughout the body. Johnson (2003) described the effects of glucocorticoids on the cellular immune response. Glucocorticoids inhibit the production of cytokines by Th cells, which decreases additional B cell and immunoglobulin production. B cells may also be more directly affected by the inhibition
of antibody production and the activation of apoptosis. Glucocorticoids also inhibit T cell proliferation by blocking the production of IL-2. Golub and Gershwin (1985) noted that not only do glucocorticoids inhibit enzyme production, slow antigen processing, and indirectly decrease IL-1 production, but they also cause a dramatic redistribution of leukocytes between the circulatory and lymphatic systems and bone marrow. Specifically, their data show an absolute increase in neutrophils and a decrease in lymphocytes in circulating blood. It has also been documented that glucocorticoids cause a reduction in the mass of lymphoid tissues, specifically the thymus, spleen, and lymph nodes (Johnson, 2003). Golub and Gershwin (1985) noted a “marked reduction [up to 70%] in the size and weight of the thymus,” in rats subjected to stress or direct glucocorticoid treatment. This observation reflects the destruction of lymphocytes that mature within the tissue.

**Stress**

According to Lovallo (1997), a stress response is defined as “the compensatory reaction [physiological or psychological] the body makes to the disturbance caused by the stressor.” Levine (1985) notes that the stress response involves several changes in both neurochemical and metabolic processes. Therefore, when an organism perceives a stressor, the body and brain communicate through the neuroendocrine system and make appropriate adjustments so as to maintain homeostasis.

Research has demonstrated different physiological responses to stress depending on the circumstance or perception of the stressor. Stefanski and Engler (1998) suggest that acute and chronic stress may have different effects on the immune system. They found that following two hours of inescapable social stress, male rats showed a 60%
decrease in lymphocyte numbers, a 65% increase in granulocyte numbers, and an increased T/B cell ratio. Following 48 hours of social stress, lymphocyte counts decreased and granulocyte counts increased, but to a smaller magnitude than that observed after acute stress. The most significant difference was a reversed T/B cell ratio—from 1.72 after acute stress to 0.99 following chronic stress (Stefanski and Engler, 1998).

Levine (1985) asserts that the stress response is dependent on an organism’s perception of the stressful stimuli. He found that the adrenocortical response was dependent on the novelty of the stressor; a more novel stressor produced a more dramatic adrenocortical response and vice versa. In addition, uncertainty contributes to the physiological stress response of an organism. Levine (1985) found that dogs with previous experience with a shock stressor had an adrenocortical response two to three times less than dogs without such experience.

The physiological stress response is largely dependent on the organism’s perception of the stressor as escapable or inescapable (fight or flight vs. coping). If the organism perceives the situation as avoidable or one that can be successfully overcome, the body’s defense relies on the secretion of catecholamines (epinephrine/norepinephrine) from the adrenal medulla (Maule and VanderKooi, 1999). However, if the circumstance is unavoidable/inescapable, the HPA axis is activated and glucocorticoids are released that have effects throughout the body as described previously. For example, Levine (1985) found that animals exposed to footshock had elevated catecholamine levels and low plasma corticosterone levels if they were permitted to act aggressively following the shock. However, animals that were not permitted this action were found to have elevated
corticosterone levels and low catecholamine levels. This observation demonstrates how the body can modulate the physiological response through the neuroendocrine system depending on the character of a stimulus.

There are several methods used to test the stress response in a laboratory setting, including restraint. Kelley (1985) reported a threefold increase in plasma glucocorticoid levels following restraint stress, indicating that it is an effective stressor of laboratory animals. However, Baron and Brush (1979) noted that following "repeated administration of restraint stress the pituitary-adrenal system shows either habituation or sensitization." This trend demonstrates that although restraint is a successful stressor, its effectiveness may decrease following repetitive use.

**Estrogen and Progesterone**

Estrogen and progesterone are female reproductive hormones derived from cholesterol that are produced in the ovaries and in small amounts by the adrenal cortex in both sexes (Randall *et al.*, 1997). These hormones act by binding receptors within cells throughout the body and modifying the expression of genes. The most potent estrogen is estradiol-17β, which acts in the body to promote the development and maintenance of female sex characteristics and behavior, oocyte maturation, and uterine proliferation. Estrogens do not stimulate early differentiation of the female reproductive tract, although they do promote later development of the uterus, ovaries, and vagina. Progesterone maintains uterine secretion and stimulates mammary duct formation (Randall *et al.*, 1997). Both hormones play major roles in the female reproductive cycle.

The female reproductive cycle consists of two main phases, the follicular phase and the luteal phase. Follicle stimulating hormone (FSH) promotes the development of
ovarian follicles at the beginning of the follicular phase. Luteinizing hormone (LH) then causes the follicles to produce and secrete androgens. FSH promotes the production of an enzyme that converts the androgens to estrogens, causing a substantial rise in estrogen levels. High estrogen levels influence the release of more FSH and LH from the hypothalamus and adenohypophysis. One follicle matures and ruptures at the surface of the ovary (ovulation). Rising estrogen levels also trigger the proliferation of the endometrium (Randall et al., 1997).

The luteal phase begins at ovulation, which causes estrogen levels to decrease initially. LH causes the ruptured follicle to become the corpus luteum, which secretes estrogen and progesterone. The rising hormone levels negatively feed back to the hypothalamus to prevent further release of FSH and LH. Progesterone further promotes vascularization of the endometrium in preparation for the implantation of a fertilized ovum. If fertilization does not occur, the corpus luteum degenerates, estrogen and progesterone levels decline, and the cycle starts over (Randall et al., 1997). In most mammals, the female reproductive cycle is called the estrous cycle. In primates, it is called the menstrual cycle, which includes a sloughing off of the vascularized endometrial lining, distinguishing it from the cycle of other mammals.

The estrous cycle of the laboratory mouse can be divided into four stages: proestrus, estrus, metestrus, and diestrus (Kilen and Schwartz, 1999). These stages are centered around the estrus period, which is characterized by mating behavior. Proestrus is the period prior to estrus when follicular growth within the ovary occurs; metestrus follows estrus and is a “recovery” period following ovulation; and diestrus is the period when the corpus luteum secretes estrogen and progesterone (Kilen and Schwartz, 1999).
Estrogen levels are lowest during estrus and metestrus, gradually increasing during diestrus, and peaking during proestrus (Kilen and Schwartz, 1999; Krzych et al., 1978). Mating behavior is induced during proestrus and ovulation follows at the beginning of estrus (Kilen and Schwartz, 1999).

Estrogen seems to play a significant role in the cell-mediated immune response, as demonstrated by Josefsson *et al.* (1992). The researchers found that even low doses of estradiol had significant anti-inflammatory effects on the immune system. In addition, they reported that estrogen caused neutrophil concentrations to decrease significantly in peripheral blood. Schuurs *et al.* (1994) reported the presence of estrogen receptors on several cell types in the body, including thymus and peripheral lymphoid cells of various types. In addition, they note that estradiol appears to promote the humoral immune response and cite a study by Erbach and Bahr (1998) in which cyclic levels of estrogen had a stimulating effect on antibody response. Paavonen (1987) described the presence of both glucocorticoid and estrogen receptors on peripheral lymphocytes suggesting that the immune system may be modulated by the endocrine system. Da Silva (1994) noted that estrogens play a dual role in modulating the immune system, depressing T cell mediated responses and promoting B cell mediated responses.

The cyclic nature of the estrous cycle and the varying hormone levels throughout seem to have important implications for the immune system. Krzych *et al.* (1978) found that splenic lymphocytes of female mice varied throughout the estrous cycle in their capacity to respond to challenges posed by T and B cell mitogens and other antigens. Specifically, they found responsiveness lowest during diestrus and estrus and highest during proestrus and metestrus. Baron and Brush (1979) also found that the magnitude of
the stress response was influenced by the estrous cycle. Their data show that the greatest concentrations of plasma corticosterone were found during estrus for acute stress and both estrus and proestrus for chronic stress.

There is speculation that the interference of reproductive hormones on glucocorticoids may have important consequences (Da Silva, 1994). Da Silva (1994) cites research suggesting that estrogens act on the HPA axis and cause corticosterone release, thereby affecting the inflammatory process. Furthermore, he asserts that estrogens increase the glucocorticoid response and androgens decrease it, suggesting that gender differences in immune reactivity may be influenced by a hormone’s ability to modulate glucocorticoid activity (Da Silva, 1994).

Research has shown that there are significant gender differences in the stress response. Specifically, Rohleder et al. (2001) found that following psychosocial stress, glucocorticoid sensitivity of proinflammatory cytokines in target tissues increased in men and decreased in women in the luteal menstrual phase. In addition, lipopolysaccharide-stimulated production of IL-6 and TNF-α decreased in men and increased in women. These findings suggest that gender may cause a different stress response by altering glucocorticoid sensitivity of cytokine production through the presence of female reproductive hormones (Rohleder et al., 2001). Komesaroff et al. (1998) reported that the hormone fluctuations of the estrous cycle affected both glucocorticoid and catecholamine responses to stress in sheep. Finally, a recent study found that female rats had more stress-related cognitive deficiencies during proestrus than during estrus (Shansky et al., 2003).
Materials and Methods

Animals

Twenty-six female Swiss-Webster mice (*Mus musculus*) were obtained from stock supplies at Simonsen Laboratories in Gilroy, California. The mice were housed 5-6 per cage in a room with 12-hour light-dark cycles, and experimentation was performed during the light phase. All attempts were made to keep the temperature between 65° and 70° Fahrenheit, although considerable fluctuations did occur. Mazuri rodent chow (made by Purina) and water were available *ad libitum*. The mice were about 8 weeks old at the time of study and were handled for 7 days prior to actual experimentation. In addition, vaginal smears were performed for six days prior to testing to establish the presence of regular cycling. Twenty-four mice with regular cycles were used in the study. Twelve mice were designated as the control group, and the other twelve were designated as the experimental group (restraint stress). Three male mice were housed in separate cages but were kept in close proximity to the female mice to ensure regular cycling.

Restraint Procedure

Eleven restraint cages were made from 3 ¾-inch-long sections of 1 3/8-inch-diameter PVC pipe. Holes were drilled about every 2 centimeters to allow for air circulation. The ends of the pipe were closed with rubber stoppers. In addition, one commercially made animal restraint of the same size was used to supplement the number of restraints. The mice were subject to restraint stress for two hours every day at the same time for seven days. The restraint time and method were determined as described by Smart (2001).
Estrous Cycle Determination

According to Allen (1922), changes in vaginal cytology are correlated with the hormonal estrous cycle in mice. Therefore, it is possible to determine the hormonal stage of the estrous cycle by examining vaginal cell types. Estrous cycling in mice typically lasts 4-6 days and consists of four stages: proestrus, estrus, metestrus, and diestrus (Allen, 1922). The vaginal smear procedure was adapted from Hoar and Hickman (1983). Each mouse was tested at the same time daily for eight days following restraint (if applicable) and blood drawing. An eyedropper with a curved end was used to take the samples. Following insertion of the tip of the eyedropper, the vagina was flushed two to three times with 3-4 drops of 0.9% saline solution. The fluid was then transferred to a clean, dry slide and allowed to air dry. The smears were stained according to the procedure below (see Wright Staining section) and examined with a microscope to determine estrous stage. The following guidelines were used: proestrus is characterized predominantly by nucleated epithelial cells; estrus primarily by cornified epithelial cells and a lack of leukocytes; metestrus by cornified clumps of cells, nucleated epithelial cells, and some leukocytes; and diestrus by mainly leukocytes and some nucleated epithelial cells (Allen, 1922; Hoar and Hickman, 1983).

Phlebotomy

Blood was drawn at the same time each day, directly prior to and immediately following restraint (if applicable). Blood was taken from the capillary beds located in the tail. A small nick was made in the tip of the tail to facilitate the drawing of blood. A small drop was placed directly on a slide to perform a leukocyte differential count according to the method of Brosschot et al. (1992).
Wright Staining

The blood smear staining procedure for white blood cell differential counting was adapted from Neal and Kalbus (1983). Staining consisted of thirty seconds in Wright’s stain, three minutes in phosphate buffer (pH 7.2), a brief phosphate buffer rinse, and air-drying. The procedure for staining vaginal smears consisted of thirty seconds in Wright’s stain, three minutes in distilled water, a brief distilled water rinse, and air-drying. This procedure was adapted from Hoar and Hickman (1983).

Data Quantification and Statistical Analysis

To quantify the effect of restraint stress and natural hormone levels during the estrous cycle on peripheral leukocyte distribution, 100 white blood cells were counted on each slide. Each slide was counted twice and the numbers of each cell type per count were averaged. Group data were pooled enabling the production of group means and standard deviations. This method was modified from Brosschot et al. (1992).

A one-way analysis of variance (ANOVA) test was performed to determine the effects of time on the stress effect. A multivariate analysis of covariance (MANCOVA) statistical test was performed to determine the overall effects of restraint stress, estrous cycle stage, and both factors together with time as a covariate. A multivariate analysis of variance (MANOVA) test was performed to determine the overall effects of stress and stage on an additional data set. Statistics were carried out using the Statistica program for PC.
Results

The numbers for statistical analysis and graphing were determined by taking the proportional differences in the leukocyte counts from before and after the two-hour testing period. Using proportional differences allowed me to account for the high variability in the baseline leukocyte numbers of each mouse. (Example: Mouse A may have a baseline lymphocyte count of 65 [per 100] cells and a count of 35 following stress; Mouse B may have a baseline count of 35 and a count of 5 following stress. Absolute values would suggest that both mice had a 30% decrease; however, proportional values show that Mouse A had a 46% decrease [(65-35)/65] while Mouse B had an 86% decrease [(35-5)/35] and, therefore, a greater stress response.)

An *a priori* analysis showed that the effects of stress, stage, or both on leukocyte distribution were not significant. The stress response was measured by analyzing the change in distribution of leukocytes in peripheral blood. There was no significant difference in the stress response of control animals compared to stress animals within each stage of the estrous cycle (Figs. 1a and 1b). Graphical results indicated that the control group had a greater stress response in some estrous stages than the stress group, as demonstrated by a lower percentage of lymphocytes and higher percentage of neutrophils in whole blood (Figs. 1a and 1b). Moreover, the stress group still had less of a stress response than the controls regardless of stage (Figs. 2a and 2b). These findings are opposite of that found in the literature, as acute restraint stress has been shown to cause lymphocytes to decrease and neutrophils to increase (Stefanski and Engler, 1998). It is possible that the application of stress itself may not have been sufficient to elicit a
response. As a result, I decided to examine my experimental design to determine if there were any hidden variables that could potentially affect the data. I suspected that the mice might have acclimated to the stress since each treatment animal was stressed for two hours at the same time every day for a total of eight days. An analysis of variance (ANOVA) test revealed that time indeed had a significant effect on the numbers of both lymphocytes and neutrophils (Table 1). Lymphocyte numbers increased with time and neutrophil numbers decreased with time; thus, the stress treatment became significantly less effective throughout the experiment confirming the suspected habituation effect (Figures 3a and 3b). This observation may account for the lack of differences between the stressed group and controls when the stage variable was removed.

A multiple analysis of covariance (MANCOVA) test enabled me to examine the data with time as a covariate. This test was used to determine if stress, stage, or both factors together had any effect on white blood cell distribution when the time effect was removed. No significant effects could be detected due to these factors (Table 2).

I attempted to eliminate the habituation effect in the analysis by looking only at the first day of testing. Data from controls and treatment animals in only metestrus and diestrus were selected for analysis, as there were greater numbers of animals in those stages at that time. Proportional changes in lymphocyte and neutrophil distributions were analyzed, and no significant difference was found in the stress response of control animals as compared with stress animals in the two stages (Figs. 4a and 4b). The stage of estrous had no statistical effect on the response of the animals to stress (Table 3).
Figure 1a. The Effect of Restraint Stress on Lymphocyte Distribution Within Each Stage of Estrous. The stress response is characterized by a decrease in the number of lymphocytes. The data show that there is no significant difference in the reactions of control animals versus stress animals within the four estrous stages. Numbers were obtained by analyzing proportional change in lymphocyte distribution before and after administration of stress. (n=12 for both groups)

Figure 1b. The Effect of Restraint Stress on Neutrophil Distribution Within Each Stage of Estrous. The stress response is characterized by an increase in the number of neutrophils. The data show that there is not a significant difference in the reactions of control animals versus stress animals within the four estrous stages. Numbers were obtained by analyzing proportional change in neutrophil distribution before and after administration of stress. (n=12 for both groups)
Figure 2a. The Effect of Restraint Stress on Lymphocyte Distribution. The stress response is characterized by a decrease in lymphocyte distribution. The stress group did not have a significant stress response relative to the control group. Numbers were obtained by analyzing proportional change in lymphocyte distribution before and after administration of stress. The data were analyzed without regard to stage of estrous. (n=12 for both groups)

Figure 2b. The Effect of Restraint Stress on Neutrophil Distribution. The stress response is characterized by an increase in neutrophil distribution. The stress group had no significant stress response relative to the control group. Numbers were obtained by analyzing proportional change in neutrophil distribution before and after administration of stress. The data were analyzed without regard to stage of estrous. (n=12 for both groups)
Figure 3a. The Effect of Restraint Stress on Lymphocyte Distribution Over Time. The stress response is characterized by a decrease in lymphocyte distribution. Restraint stress became significantly less effective throughout the experiment. These numbers were determined by analyzing proportional changes in lymphocyte numbers before and after administration of stress. The data were analyzed without regard to stage of estrous. (n=12)

Figure 3b. The Effect of Restraint Stress on Neutrophil Distribution Over Time. The stress response is characterized by an increase in neutrophil distribution. Restraint stress became significantly less effective throughout the experiment. These numbers were determined by analyzing proportional changes in neutrophil numbers before and after administration of stress. The data were analyzed without regard to stage of estrous. (n=12)
**Figure 4a.** Effect of Stress on Lymphocyte Distribution Within Two Stages: Day 1. The stress response is characterized by decreasing numbers of lymphocytes. There is no significant difference in the stress response between animals in metestrus and diestrus. This analysis eliminates the time factor as analysis was performed on data from only the first day of testing. Numbers were obtained by analyzing proportional change in lymphocyte distribution before and after administration of stress. (n=12 for both groups)

**Figure 4b.** Effect of Stress on Neutrophil Distribution Within Two Stages: Day 1. The stress response is characterized by increasing numbers of neutrophils. There is no significant stress response between animals in metestrus and diestrus. The analysis eliminates the time factor as analysis was performed on data from only the first day of testing. Numbers were obtained by analyzing proportional change in neutrophil distribution before and after stress administration. (n=12 for both groups)
Table 1. Univariate analysis (ANOVA) for overall effects of time

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<th>df Error</th>
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<th>P-value</th>
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<td>5.76*</td>
<td>&lt;0.001*</td>
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<td>184*</td>
<td>6.78*</td>
<td>&lt;0.001*</td>
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*Denotes significant difference at P=0.05

Table 2. MANCOVA test for overall results with time as covariate

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Table 3. MANOVA test for overall results for Day 1

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Discussion

The results of my study suggest that there was no significant difference in the stress responses of animals within different stages of the estrous cycle. The data are contrary to other research that suggests that the estrous cycle may affect the immune system. Krzych et al. (1978) reported estrous cycle modulation of the immune system through the reaction of splenic lymphocytes to mitogens and antigens in mice and rats. In addition, Komesaroff et al. (1998) found that the hormonal changes of the estrous cycle modulated the response of the glucocorticoids to metabolic and audiovisual stress in sheep. Baron and Brush (1979) asserted that the magnitude of the stress response varied with the estrous cycle.

The effects of stress on the immune system have been well documented. Golub and Gershwin (1985) discussed how glucocorticoids affect the immune system through receptors on circulating lymphocytes. Inescapable, unavoidable stress (like restraint) has been shown to elicit the action of the glucocorticoids, which produces marked changes in blood cell distribution, as suggested by Stefanski and Engler (1998). They found a 65% increase in granulocyte (neutrophil) numbers and a 60% decrease in lymphocyte numbers following the administration of social stress. Levine (1985) documented graded glucocorticoid actions depending on the novelty and uncertainty of stressful stimuli.

My research indicated an unusual stress response in which lymphocyte distribution increased and neutrophil distribution decreased (Figs. 2a and 2b). This finding warranted further study, so the effect of time on leukocyte distribution was examined. It is evident that the mice in this study were able to acclimate to the daily
stress treatment. Baron and Brush (1979) noted that either habituation or sensitization has been seen to occur in response to “repeated administration of restraint stress.” Levine (1985) also discussed that novel stress elicits a different physiological response than repetitive stress. My data show that habituation did indeed occur after a period of about five days of daily administration of restraint stress. This habituation effect is revealed by the significant effect of time on leukocyte distribution (Figs. 3a and 3b, Table 1). The atypical response of the stress group is most likely explained by this phenomenon.

I attempted to eliminate the habituation effect by examining only day one of the experimental period. No significant difference was found in the stress response of animals within either metestrus or diestrus. This finding suggests that either the estrous cycle has no effect on stress response or that the variation in the data is too great to detect any subtle changes.

Laboratory conditions were unstable throughout the duration of the experiment, which could have had an effect on the variance in the data. The experiment was performed in a chemistry lab where alarms went off spontaneously. In addition, temperatures in the lab fluctuated throughout the experimental period, although every attempt was made to stabilize them. These factors may have contributed to an elevated baseline stress level of the experimental animals.

Future work on this project would merit changing the protocol in order to decrease variance and increase the ability to detect subtle changes. The use of hundreds of animals would be necessary in order to minimize the effects of individual variation. It would also be necessary to randomize the study to eliminate the habituation effect.
Finally, enzyme-linked immunosorbent assay tests could be used to assess estrogen levels in the blood in order to determine estrous stage more accurately.
Literature Cited


