Influence of Estrogen on IL-6 Response to Prolonged Treadmill Running in Elite Female Runners

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ABSTRACT

ASHLEY KALLMAN: Influence of Estrogen on IL-6 Response to Prolonged Treadmill Running in Elite Female Runners
(Under the direction of Anthony C. Hackney, PhD, DSc)

This study assessed the influence of estrogen (E₂) on IL-6 and IL-1β responses to prolonged aerobic exercise. Eumenorrheic women (n = 10) completed 90-minute treadmill running bouts at 67% VO₂peak during low E₂ (LE) and high E₂ (HE) hormonal conditions. Resting, immediate, 24-hour, and 72-hour post blood samples were collected to determine changes in cytokines. Resting blood samples for E₂ confirmed appropriate hormonal status for both conditions. Overall IL-6 concentration was significantly higher in LE (main effect; \( p = 0.011 \)); however, relative exercise IL-6 percent change was not different between hormonal conditions. Interleukin-1β was unobtainable due to procedural challenges. In conclusion, the influence of hormonal condition on IL-6 and IL-1β in response to exercise is still unclear; therefore, it cannot be suggested that highly trained female runners should alter their training program with regards to hormonal status. Additional research is essential to add to the limited literature on this topic.
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per Minute</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrates</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>$E_2$</td>
<td>Estradiol β-17</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>HE</td>
<td>High Estrogen</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Axis</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LE</td>
<td>Low Estrogen</td>
</tr>
<tr>
<td>MF</td>
<td>Midfollicular – Low Estrogen</td>
</tr>
<tr>
<td>ML</td>
<td>Midluteal – High Estrogen</td>
</tr>
<tr>
<td>RPE</td>
<td>Ratings of Perceived Exertion</td>
</tr>
<tr>
<td>$VO_2$</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>$VO_2\text{max}$</td>
<td>Maximal Oxygen Consumption</td>
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<tr>
<td>$VO_2\text{peak}$</td>
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CHAPTER I

Introduction

Cytokines are the first response of the immune system to stress, infection, or damage to muscle (Corwin, 2000). Cytokine secretion is stimulated by growth, illness, injury, inflammation, or stress (Corwin, 2000). Cytokines exist in both pro- and anti-inflammatory forms. In general, pro-inflammatory cytokines act as messengers by binding to damaged tissue and signaling other immune cells to contain and eradicate the damaged tissue, promoting healing and recovery (Corwin, 2000; Pederson, Ostrowski, Rohde, & Bruunsgaard, 1998). Anti-inflammatory cytokines are released in response to elevated pro-inflammatory cytokine levels; their intent is to mitigate and inhibit the actions and further secretion of pro-inflammatory cytokines (Corwin, 2000; Pederson et al., 1998).

One of the prominent pro-inflammatory cytokines is interleukin-1β (IL-1β) (Corwin, 2000). Interleukin-1β stimulates the secretion of interleukin-6 (IL-6) via activation of macrophages and monocytes in response to tissue damage (Corwin, 2000). Interleukin-6 has pro- as well as some anti-inflammatory characteristics. Interleukin-6 stimulates acute phase protein secretion from the liver, which helps to increase recognition and destruction of foreign molecules (Abbas & Lichtman, 2003). Receptors for IL-6 are housed on both the anterior pituitary and the adrenal gland (Fischer, 2006). Interleukin-6 will stimulate the release of cortisol via activation of the hypothalamic-pituitary-adrenal regulatory axis and direct stimulation of the adrenal cortex (Fischer, 2006; Nieman et al., 1998). Increases in the concentration of cortisol will feedback to inhibit further secretion of IL-6 and IL-1β (Nieman
et al., 1998). Additionally, elevated IL-6 concentrations increase expression of the anti-inflammatory cytokine interleukin-1 receptor alpha (IL-1ra), which inhibits the release of IL-1β, subsequently reducing IL-6 release (Corwin, 2000; Pederson et al., 1998). This system of checks and balances between pro-inflammatory and anti-inflammatory cytokines is purportedly in place to avoid over-activation of the immune system. Such checks and balances are critical because a non-regulated immune system can lead to detrimental physiological effects; the most serve being an autoimmune disease (Corwin, 2000).

An acute bout of exercise is known to increase levels of IL-6 and IL-1β immediately post-exercise and for hours into recovery from exercise. The magnitude of the IL-6 and IL-1β response to exercise is dependent on exercise mode, intensity, and duration (Pederson et al., 1998). Aerobic exercise bouts greater than 60 minutes above 60% maximal aerobic capacity (VO₂max) elicit a significant increase in IL-6 (Brenner et al., 1999; Ostrowski, Rohde, Asp, Schjerling, & Pederson, 1999; Nieman et al., 1998; Nieman et al., 2001) and IL-1β concentrations in the blood (Ostrowski et al., 1999; Nieman et al., 2001). Furthermore, the IL-6 and IL-1β responses are significantly more elevated from eccentric exercise compared to concentric exercise (Bruunsgaard et al., 1997; Nieman et al., 1998). Regrettably, however, the research literature examining the cytokine response to exercise is primarily limited primarily to male populations and few studies have examined female subjects despite the large number of women participating in exercise and sport. Nieman et al. (1998; 2001) did, however, incorporate female subjects; nonetheless, reported no apparent significant differences between the genders with respect to cytokine responses. Conversely, Edwards, Burns, Ring, and Carroll (2006) reported a significant difference between genders in cytokine responses; however, these investigators did not control for the menstrual phase cycle
fluctuations in estrogens (E$_2$) of the female subjects. This last point, control of the menstrual cycle, is of key importance as many hormonal responses are related to immune responses, such as E$_2$, and are known to fluctuate across the cycle (Angstwurm, Gartner, & Ziegler-Heitbrock, 1997; Bouman, Moes, Heineman, de Leij, & Faas, 2001; Lynch, Dinarello, & Cannon, 1994; Willis, Morris, Danis, & Gallery, 2003); one such hormone being E$_2$. High E2 (HE) concentrations are observed at ovulation as well as during the luteal phase, while low E$_2$ (LE) concentrations are present during menses and the follicular phase of the menstrual cycle (Williams & Larsen, 2003).

Studies evaluating the relationship between basal resting E$_2$ and IL-6 are conflicting. Some have suggested non-significant fluctuations in IL-6 between varying concentrations of E$_2$ (Al-Haarthi et al., 2000). Others support elevated IL-6 concentrations or elevated immune constituents known to stimulate secretion of IL-6 during HE compared to LE hormonal conditions (Bouman et al., 2001; Willis et al., 2003). Conversely, Angstwurm et al. (1997) found an inverse relationship between concentrations of E$_2$ and IL-6; an elevated concentration of E$_2$ was associated with reduced IL-6 while a lower concentration of E$_2$ was associated with increased IL-6. Literature evaluating resting IL-1$\beta$ and E$_2$ is also divergent. Some research shows higher IL-1$\beta$ concentration during periods of LE (Lynch et al., 1994) while others suggest a significantly greater IL-1$\beta$ concentration during the HE phase of the menstrual cycle (Willis et al., 2003). These demonstrated fluctuations in basal, resting IL-6 and IL-1$\beta$ to varying levels of E$_2$ beg the question - Is there an altered cytokine response following exercise at different levels of E$_2$? Researchers have not apparently directly assessed this question in a clear and systemic fashion.
Researchers have, however, indirectly looked at aspects of the above question by examining exercise induced muscular damage in females (i.e., muscle damage can provoke cytokine responses). Tiidus et al. (2001) observed a decreased neutrophil concentration in response to reduced creatine kinase levels [CK] (i.e. biological marker of muscle damage) following one hour of inclined running in ovariectomized rats given estrogen supplementation compared to non-supplemented ovariectomized rats. Neutrophils attract monocytes, a major producer and secretory cell for IL-1β and IL-6, to the area of damage (Corwin, 2000; Pederson et al., 1998). Research has shown that E₂ acts as an antioxidant, thereby reducing reactive oxygen species produced by neutrophil activity and can stabilize muscle membrane further reducing the potential for damage (Kendall & Eston, 2002). If E₂ supplementation reduced the neutrophil response, one would expect to see a reduction in cytokine response as well.

Another study using human subjects observed a significantly greater increase in CK in women after eccentric exercise in LE compared to HE was observed (Carter, Dobridge, & Hackney, 2001). The authors speculated the lower CK observed in HE suggest E₂ acts as a shield against muscle degradation and abated the inflammatory response which others have also proposed (Tiidus et al., 2001). Research has shown a positive correlation between CK levels and select cytokines and that muscle damage does cause local secretion of pro-inflammatory cytokines (Pederson et al., 1998). Therefore, if higher E₂ concentrations reduce muscle damage and the response of other immune cells known to stimulate cytokine release following exercise, one would expect to see a reduced pro-inflammatory cytokine response during periods when E₂ is elevated.
*Purpose of the Study:*

The primary purpose of this study was to evaluate the IL-6 and IL-1β responses when resting E₂ concentrations were elevated and reduced, (as manipulated by menstrual cycle phase), following prolonged moderate intensity treadmill running in females. On separate days, subjects performed an exercise test on the treadmill to determine their VO₂ max, two 90-minute treadmill running bouts at 70% of their VO₂ max, and had blood drawn before, immediately post, 24-hours post, and 72-hours post the 90-minute running bouts. The 90-minute running bouts and subsequent blood draws were performed at the two specified hormonal conditions: low E₂ (LE) and high E₂ (HE) concentrations.

*Research Questions:*

1. Is there a significant interaction effect between IL-6 concentration and hormonal condition?
   a. Is there a significant difference in IL-6 concentration at rest between HE and LE conditions?
   b. Is there a significant difference in IL-6 concentration immediately post exercise between HE and LE conditions?
   c. Is there a significant difference in IL-6 concentration 24 hours post-exercise between HE and LE conditions?
   d. Is there a significant difference in IL-6 concentration 72 hours post-exercise between HE and LE conditions?

2. Is there a significant interaction effect between IL-1β and hormonal condition?
   a. Is there a significant difference in IL-1β concentration at rest between HE and LE conditions?
b. Is there a significant difference in IL-1β concentration immediately post exercise between HE and LE conditions?

c. Is there a significant difference in IL-1β concentration 24 hours post-exercise between HE and LE conditions?

d. Is there a significance difference in IL-1β concentration 72 hours post exercise between HE and LE conditions?

Research Hypotheses:

1. A significant interaction effect will exist between IL-6 and hormonal condition.

   a. Interleukin-6 concentration will be significantly greater at rest in women during LE condition compared to HE.

   b. The magnitude of change from rest in IL-6 will be significantly greater immediately post exercise in women during LE condition compared to HE.

   c. The magnitude of change from rest in IL-6 will be significantly greater 24 hours post-exercise in women during LE condition compared to HE.

   d. The magnitude of change from rest in IL-6 will be significantly greater 72 hours post exercise in women during LE condition compared to HE.

2. A significant interaction effect will exist between IL-1β and hormonal condition.

   a. Interleukin-1β concentration will be significantly greater at rest in women during LE condition compared to HE.

   b. The magnitude of change from rest in IL-1β will be significantly greater immediately post exercise in women during LE condition compared to HE.

   c. The magnitude of change from rest in IL-1β will be significantly greater 24 hours post-exercise in women during LE condition compared to HE.
d. The magnitude of change from rest in IL-1β will be significantly greater 72 hours post-exercise in women during LE condition compared to HE.

*Definition of Terms:*

Estrogen (E$_2$): Estrogen is the primary sex hormone for females. Estrogen is produced and secreted from the ovaries and exist in several forms (estrodial β-17 being the major). The level of circulating E$_2$ is known to fluctuate throughout the menstrual cycle. In addition to its role in female reproductive development, E$_2$ is believed to influence non-reproductive tissues (Ruggiero & Likis, 2002).

Cytokine: Cytokines are small regulatory proteins in the innate immune system of the human body. Cytokines release is stimulated by stress, infection, illness, and inflammation (Corwin, 2000). Cytokines act to signal additional immune responses to contain and remove foreign or damaged substances (Corwin, 2000; Pederson et al., 1998). Cytokines can be either pro-inflammatory or anti-inflammatory. Predominant pro-inflammatory cytokines include IL-1β and IL-6. Interleukin-6 also acts as an anti-inflammatory cytokine in conjunction with IL-1ra to regulate the innate immune response.

Prolonged running bout: Exercise that is performed on a treadmill at 70% of the subject’s VO$_2$ max at 0% grade for 90-minutes.

Maximal Oxygen Consumption (VO$_2$ max): Maximal oxygen uptake is the maximal amount of oxygen that can be delivered and utilized by active muscles; it is a variable primarily used to assess the efficiency of the cardiorespiratory system (Plowman & Smith, 2003).
Assumptions

1. Subjects are healthy and free of infections. Injury or illness are known to elicit an immune response independent of E$_2$.

2. Subjects are not knowingly pregnant or trying to become pregnant. Pregnancy is accompanied by altered hormonal response and absence of menstruation, potentially altering cytokine response.

3. The physical and emotional stress level of the subject is minimal. Stress is known to elicit an inflammatory response.

4. Subjects refrain from no more than 30-minutes light exercise during the 72-hour window from the end of the 90-minute running bout and the 72-hour post blood draw. Additional exercise outside of daily living activities can elicit an additional inflammatory response.

Delimitations

1. Healthy, non-smoking, highly trained women runners between the ages of 18-30.

2. The treadmill running bout is set at 70% VO$_2$max for 90 minutes.

3. Subjects had to complete a half marathon or equivalent distance within the last year, under 2 hours 30 minutes, with no associated injuries (Nieman et al., 2001).

4. A minimum VO$_2$max of approximately 45 ml/kg/min is required.

5. Individuals must be currently training with a minimum training volume of 3 - 5 days a week, 45 - 120 minutes per session of aerobic activity.

Limitations

1. The inclusion criteria and study protocol led to a small sample size.
2. Only eumenorrheic women and oral contraceptive users were studied; therefore, the results are not applicable to men and may not be applicable to premenopausal, postmenopausal, or amenorrheic women.

3. Only runners were tested; therefore, results may only be applicable to exercisers who run.

*Significance of Study*

The results of this study will add to the limited body of knowledge available on the influence of $E_2$ on cytokine expression in response to exercise within women. Additionally, IL-6 and IL-1β exert effects not only on damaged tissue and foreign microbes but have receptors on other tissues too including vascular, cardiac, bone, and nerve. The impact of IL-6 and IL-1β on these tissues is reduced vasoreactivity, reduced heart contractility, and increased muscle and bone degradation (Moldoveanu, Shephard, & Shek, 2001). Elevated concentrations of IL-6 and IL-1β will only exacerbate the aforementioned effects leading to potentially a maladaptation within select physiological processes - these detrimental changes could potentially alter proper functioning and compromise physical performance. Most endurance training programs involve a tremendous volume of work, requiring consecutive days of prolonged and intense exercise. The results of this study could also provide insight for women on the necessity of perhaps altering their training programs for optimal adaptation and minimal risk during different $E_2$ concentrations based on IL-6 and IL-1β response potentially being greater in one hormonal condition compared to the other.
CHAPTER II

Review of Literature

This review of literature is organized in the following manner: with a basic overview of general cytokine physiology and immunology. Next is a discussion on the cytokine response, namely IL-6 and IL-1β, to exercise in primarily male populations followed by the influence of gender on such responses. Exercise studies with only male cohorts will be organized based on exercise duration starting with the shortest duration (30 minutes) and ending with the longest (3.5 hours). Gender studies will be organized on whether or not they separated men and women, starting with not separating followed by separating. The gender studies using women, however, did not account for E₂ fluctuations across the menstrual cycle. Therefore, E₂ physiology and the influence of E₂ on inflammatory markers, such as IL-6 and IL-1β, at rest are also examined in this review. This section will be arranged based on studies with similar outcomes being grouped together. Next, this review will discuss the influence of E₂ on muscle damage following exercise as muscle damage is strongly correlated to cytokine response. Studies utilizing animal models will be reviewed first, followed by studies utilizing human models. The review chapter will end with a look at studies directly measuring the influence of E₂ on IL-6 and IL-1β in response to exercise. The review will conclude with a brief summary.

Cytokine Physiology

Cytokines are 6 - 70 kilodalton-sized glycoproteins secreted from both immune and non-immune cells in response to stress, growth, infection, trauma, and exercise (Corwin,
In general, cytokines act as messengers, signaling further immune activation or inhibition (Corwin, 2000; Pederson et al., 1998). Cytokines exert their effect by binding to surface receptors on target tissues triggering a cascade of enzymes in the cell cytoplasm to bring about the desired response (Corwin, 2000). Two groups of cytokines exist: pro- and anti-inflammatory.

Immediately following infection, trauma, or certain forms of exercise, pro-inflammatory cytokines such as IL-1 and IL-6 are released (Corwin, 2000; Moldoveanu et al., 2001). These cytokines initiate the inflammatory process. Inflammation is a protective mechanism utilized to contain and destroy foreign pathogens, remove damaged tissue, and stimulate repair and regeneration (Plowman & Smith, 2003). This is accomplished by signaling additional immune cells to the area of damage. The inflammatory response is typically robust. To regulate the inflammatory response, anti-inflammatory cytokines such as IL-1ra and IL-10 are released shortly after the aforementioned pro-inflammatory cytokines. The anti-inflammatory cytokines act to suppress the inflammatory response by inhibiting further secretion of pro-inflammatory mediators and reducing the activity of pro-inflammatory mediators by blocking receptors with increased antagonist expression (Corwin, 2000; Moldoveanu et al., 2001). As stated previously, regulation of the immune system between pro- and anti-inflammatory cytokines is necessary. A non-regulated immune system can lead to a compromised or excessive immune response resulting in increased infection or an autoimmune disease.
### Table 1: Common immune cells - characteristics and function

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>What it is</th>
<th>Function</th>
</tr>
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| T-lymphocyte       | White blood cells matured in the thymus. Part of cell mediated immunity -   | • Activated when foreign antigens bind to recognition site on the cell  
| (T-cell)           | primarily exerts its actions through immune cells.                          | • Cytotoxic T-cells: directly destroy foreign pathogens via break down of cell membrane and nuclear material  
|                    |                                                                             | • Helper T-cells: enhance immune response through production and secretion of cytokines                                                                                                                  |
| B-Lymphocyte       | White blood cells matured in bone marrow. Part of humoral immunity -       | • Activated when foreign antigens bind to recognition site on cell  
| (B-cell)           | primarily exerts its actions in bodily fluids, e.g. blood and lymph.       | • Produce antibodies – protein that identifies antigens and marks it for destruction  
|                    |                                                                             | • Block antigen binding site inhibiting its attachment to host cells  
|                    |                                                                             | • Coat antigen attracting additional immune mediators (monocytes) to the area for containment and eradication of foreign material.                                                                         |
| Monocyte           | Phagocytic white blood cell that circulates in lymph and blood.             | • Ingests foreign material and destroys it via destruction of cell membrane and nuclear material  
|                    |                                                                             | • Presents fragments of the antigen to T-cells for immune cell proliferation  
|                    |                                                                             | • Produces and secretes cytokines                                                                                                                                                                     |
| Macrophage         | A monocyte that moved from blood or lymph to tissues and has become fixed  | • Ingests foreign material and destroys it via destruction of cell membrane and nuclear material  
|                    | within tissues.                                                           | • Presents fragments of the antigen to T-cells for immune cell proliferation  
|                    |                                                                             | • Produces and secretes cytokines                                                                                                                                                                     |
| Neutrophil         | The most prominent phagocytic white blood cell that circulates in the blood.| • Attracted to area of tissue damage or infection by chemoattractants.  
|                    | One of the first responding immune cells to foreign material.              | • Ingests the foreign material and destroys it via enzymes housed in granules within the cytoplasm, neutrophils generally die during the process  
|                    |                                                                             | • Produces reactive oxygen species during the breakdown of foreign material  
|                    |                                                                             | • Attracts monocytes to the area of damage                                                                                                                                                    |

Interleukin-1β has been characterized as a potent pro-inflammatory cytokine, exerting its effects on other immune cells, vasculature, heart tissue, kidneys, pancreas, muscle, and bone (Corwin, 2000; Moldoveanu et al., 2001). Interleukin-1β perpetuates the pro-inflammatory response by activating T and B cells, increasing cell adhesion molecule expression, stimulating the acute phase response of the immune system, and increasing further production of itself and other pro-inflammatory cytokines such as IL-2 and IL-6 (Corwin, 2000; Moldoveanu et al., 2001). Furthermore, IL-1β increases the production of both prostaglandins and nitric oxide both of which are strong vasodilators (Corwin, 2000; Moldoveanu et al., 2001). Vasodilators repress the ability of smooth muscle to contract thereby reducing vasoreactivity of the vasculature. At the heart, IL-1β reduces the sensitivity of beta-receptors affecting the heart’s ability to forcefully contract (i.e. contractility) decreasing the amount of blood pumped out to the body (Moldoveanu et al., 2001). The combination of vasodilation and reduced contractility leads to a hypotensive state (Moldoveanu et al., 2001), potentially affecting the body’s ability to deliver oxygenated blood to organs. Interleukin-1β also causes the breakdown of bone by activating osteoclasts and muscle via stimulation of protein catabolism and degradation (Moldoveanu et al., 2001).

Interleukin-6 is another prominent cytokine produced and secreted by almost all leukocytes as well as smooth muscle cells and adipocytes (Corwin, 2000; Moldoveanu et al., 2001). Receptors for IL-6 are housed on other immune cells, liver, bone, and bone marrow (Moldoveanu et al., 2001). Interleukin-6 stimulates T-cell activation, anti-body formation, and the release of acute phase proteins from the liver (Corwin, 2000; Moldoveanu et al., 2001). Acute phase proteins further amplify the inflammatory response by increasing neutrophil and monocyte mobilization to the area of damage and initiating repair of damaged
tissue (Plowman & Smith, 2003). Interleukin-6 also increases osteoclast activation perpetuating accelerated bone breakdown (Moldoveanu et al., 2001). At bone marrow, IL-6 stimulates platelet formation increasing the potential for clot formation and this in turn increases the risk of blockages within the vasculature (Moldoveanu et al., 2001). Along with pro-inflammatory characteristics, paradoxically IL-6 exhibits some anti-inflammatory actions too [e.g. activation of the HPA-axis thereby increasing cortisol secretion – cortisol inhibits IL-6 release and increase expression of the anti-inflammatory cytokine IL-1ra] (Corwin, 2000; Fischer, 2006; Nieman et al., 1998; Pederson et al., 1998).

The mechanisms behind why a cytokine response occurs following exercise are not fully known or understood. Some suggest cytokines are released in response to muscle damage (Bruunsgaard et al., 1998). Muscle damage creates cell debris – a type of trauma, thereby stimulating a rush of cytokines to the area to remove and repair – i.e., inflammatory response (Moldoveanu et al., 2001). Others postulate endotoxemia – leakages of endotoxins from the gut as a result of blood flow redistribution away from the splanchnic region are a means that mediates the cytokine response (Pederson & Hoffman-Goetz, 2000). Another possibility is the increased reactive oxygen species as a result of neutrophil bursts encountered during exercise (Kendall & Eston, 2002). Finally, it is highly possible that all of these means and mechanism work in some degree of a synergistic fashion to provoke a post-exercise cytokine response.

**IL-6 and IL-1β Response to Exercise**

The influence of various exercise protocols on IL-6 and IL-1β responses has been extensively studied and an exhaustive review of these works is beyond the scope of this chapter. Thus, only key studies will be discussed below.
Bruunsgaard et al. (1997) compared the influence of concentric versus eccentric cycling on blood IL-6 concentration. Nine trained (VO\(_{2\text{max}}\) ~ 51 ml/kg/min) male subjects completed 2 separate exercise sessions: 30 minutes of normal cycling (concentric) and 30 minutes of reverse braked cycling (eccentric) at approximately 60% VO\(_{2\text{max}}\). Blood samples were obtained pre, 20 minutes, 30 minutes, 2 hours, 2, 4, and 7 days post exercise. Results showed a significant elevation in IL-6 concentration during and following eccentric exercise compared to a non-significant increase during and following concentric exercise. The IL-6 concentration was 0.78 pg/ml prior to starting the eccentric bout. Interleukin-6 peaked 2 hours post exercise with a 5.74 fold increase above pre-exercise values (~ 4.5 pg/ml). While not statistically significant, IL-6 remained elevated above resting values 7 days post the eccentric exercise. The results of the study suggest that the mode of exercise muscular contraction performed influences the pro-inflammatory immune response.

Brenner et al. (1999) compared the IL-6 response following three different forms of exercise. Eight male subjects of average aerobic fitness (VO\(_{2\text{max}}\) ~ 43 ml/kg/min) completed all exercise sessions. The three exercise sessions were: five minutes of cycling at 95% VO\(_{2\text{max}}\), a resistance circuit routine completing 3 rounds of 5 different exercises incorporating major muscles at an intensity level indicative of 50% VO\(_{2\text{max}}\), and a two-hour cycling bout at 63% VO\(_{2\text{max}}\). A control, rest session of sitting for 5 hours was also conducted. Blood samples were taken pre, immediately post, 3 hours, 24 hours, and 72 hours post the sessions. The results showed only a significant elevation in IL-6 concentration following the two-hour cycling bout. Pre-exercise IL-6 concentration was 1.14 ± 0.32 pg/ml. Interleukin-6 peaked 3 hours post exercise at 6.06 ± 1.95 pg/ml, a 5.32 - fold increase. By 24 - hours post exercise, IL-6 had returned to pre-exercise concentrations.
Suzuki et al. (2003) investigated the impact of a marathon race (26.2 miles) on cytokine secretion and its role in development of post exercise health status. Ten male subjects, training an average of 43 - 86 miles of running a week, competing in the 1999 Beppu marathon were recruited from the study. Blood and urine samples were obtained 24 hours prior to the start of the race and within 10 minutes of completion of the race. The average finishing time was 2:37 (hrs:min). A non-significant increase in blood IL-1β was observed following the marathon. The lack of elevation in IL-1β may be the result of accelerated clearance rate, blood sample timing, or their assay sensitivity. Furthermore, a significant increase in blood IL-6 was observed following the race (baseline: 1.27 ± 1.19 pg/ml vs. post race: 101.4 ± 50.34 pg/ml). These results are in agreement with those of Brenner et al. (1999) who conducted a similar study observing changes following cycling instead of running.

Nieman et al. (1998) compared the influence of carbohydrate (CHO) supplementation on cytokine response following 2.5 hours of cycling versus running at 75% VO$_2$max. Ten highly trained subjects (VO$_2$max ~ 55.6 ml/kg/min) subjects, two of which were women, completed four exercise conditions each: running with CHO solution, running with placebo (P) solution, cycling with CHO solution, and cycling with P solution. Blood samples were obtained pre exercise, immediately post, 1.5, 3, and 6 hours post exercise. Pre-exercise concentration was approximately 1 pg/ml for all conditions. All conditions saw a significant increase in IL-6 immediately following exercise; however, the greatest increase in IL-6 was the P running group (~ 51.6 pg/ml), while the least responsive condition was the CHO cycling group (~ 10.7 pg/ml). By the 6$^{\text{th}}$ hour of recovery all conditions were at or slightly above baseline values. The results show that both mode of exercise and supplementation
significantly influence IL-6 production. Running had a greater IL-6 response compared to cycling. The authors suggest running has a greater eccentric component compared to cycling, which has been shown to elicit a greater pro-inflammatory reaction. The P group had a greater IL-6 response compared to the CHO group. Carbohydrate supplementation minimizes plasma glucose fluctuations, reducing secretion of stress hormones, thereby reducing stress on the body – a mediator of pro-inflammatory activation (Nieman et al., 1998).

Ostrowski et al. (1999) studied the effect of a marathon race on IL-1β and IL-6 levels in the blood. Ten highly trained (VO2max ~ 61.2 ml/kg/min) male subjects competing in the 1997 Copenhagen marathon were recruited for this study. Blood samples were collected one week prior to the race, within 10 minutes of race completion, and every 30 minutes for the first 4 hours post race. The median finish time for the marathon was 3:27 (hrs:min). Interleukin-1β was significantly elevated immediately post (~ 1.75 pg/ml) and for the first 30 minutes of recovery (~ 1.50 pg/ml). Within two hours of recovery IL-1β had returned to pre-race concentration (~ 1.0 pg/ml). Interleukin-6 significantly increased following the marathon, peaking at 75 pg/ml immediately post-race, and was still significantly elevated at 4 hours post-race (~ 10 pg/ml). The results of this study are in line with those previously reported studies above.

The studies reviewed in this section offer support for an elevated cytokine response following exercise. A limitation to the studies just noted is they employed primarily male populations; this is despite many women participating in regular strenuous exercise.

Influence of Gender on Exercise Immune Response

The numbers of studies investigating the influence of gender on cytokine responses to exercise are minimal. In 2001, Nieman et al. conducted a study exploring the influence of
age, gender, and CHO supplementation on immune response following a marathon. Ninety-eight subjects, 12 were women, competing in the 1999 Charlotte or 2000 Boone marathon were divided into two groups: CHO or P. Subjects ingested 650 ml of their assigned solution 30 minutes before the start of the race and 500 ml every half hour throughout the race. Blood samples were collected prior to the start of the race, within 5 minutes after completion of the race, and 1.5 hours post-race. The average intensity during the race was 78% of the subject’s maximal heart rate. The average finishing time was 4:28 (hrs:min). The authors indicated no significant differences were observed for age and gender grouping with respect to IL-1β and IL-6 following the race. For both the P and CHO groups plasma IL-1β was significantly elevated immediately following the event, but back to baseline values by 1.5 hours post race. Despite statistical significance, the magnitude of change from baseline to immediately post was minimal (0.04 - 0.15 pg/ml). Interleukin-6 was significantly elevated immediately and 1.5 hours post exercise for both groups. While the CHO group saw a greater absolute increase in IL-6 concentration compared to the P group (CHO: 59.4 ± 8.0 pg/ml vs. P: 50.5 ± 5.0 pg/ml), the magnitude of change was greatest for the P group (P: 42 - fold increase vs. CHO: 40 - fold increase from baseline values respectively). These results are in line with the earlier study Nieman et al. (1998) that demonstrated CHO supplementation attenuates the cytokine response to exercise. Additionally, the minimal increase in IL-1β and increase IL-6 following prolonged exercise observed in this study is in agreement with the previous studies discussed (Brenner et al., 1999; Bruunsgaard et al., 1997; Nieman et al., 1998, Ostrowski et al., 1999; Suzuki et al., 2003).

Edwards et al. (2006) compared the IL-6 response in men and women following two different types of exercise and a control, rest session. Twenty-four non-competitive active
subjects were recruited, 12 males and 12 females. Of the women, nine were on some form of birth control. The two exercise sessions were: a maximal cycle ergometer test followed by a steady state ride at 55% max power (Wmax) for a total of 45 minutes of work and 20 minutes of cycling at a defined workload (~77% Wmax for women, ~81% Wmax for men) followed by 25 minutes at 55% Wmax. The control session was sitting on the cycle ergometer for 45 minutes with no exercise. Blood samples were drawn pre-exercise, immediately post, 30, and 60 minute post. The pre-exercise IL-6 concentrations were <0.5 pg/ml for all conditions. As a collective group, IL-6 increased significantly following both the maximal and submaximal sessions and displayed an upward trend at the last blood sample 1-hour post (maximal: ~1.95 pg/ml; submaximal: ~1.75 pg/ml). When comparing men and women following the maximal session, the trend of IL-6 remained the same between genders immediately post and at 30 minutes of recovery. However after 30 minutes of recovery, IL-6 decreased in men while continuing to increase significantly in women (men: ~1.1 pg/ml vs. women: ~2.95 pg/ml). The results of this study suggest gender does influence the pro-inflammatory cytokine response following high intensity exercise. The authors postulated that the divergent cytokine response was related to the different hormonal profiles of men and women.

The articles reviewed in this section present divergent findings on gender and cytokine response following exercise. Within these studies hormonal status of the women subjects was not controlled.

**Estrogen Physiology**

Estrogen physiology has been thoroughly studied and a comprehensive review is outside the scope of this chapter. Therefore, the following section will give a brief overview
of estrogen metabolism, mechanism of action, and influences on non-reproductive related tissues.

Estrogen, the primary sex hormone for women, is an 18-carbon steroid hormone produced via de novo lipogenesis of low-density lipoproteins primarily in the ovary, with small amounts produced in the adrenal cortex and adipose tissue (Enns & Tiidus, 2010; Williams & Larsen, 2003). Three main forms of estrogen exist: estrone (E1), estradiol-β17 (E2), and estriol (E3) (Ruggiero & Likis, 2002). The dominance of one form of estrogen over another is dependent on reproductive stage (Ruggiero & Likis, 2002). Estrone is prominent following menopause, estriol is dominant during pregnancy, and estradiol is the dominant estrogen for non-pregnant females throughout the reproductive years (Ruggiero & Likis, 2002). The primary focus of this thesis is the effects of estradiol on the immune response; therefore, the use of the word E2 throughout this document will refer to estradiol specifically despite estrogen encompassing all forms.

Estrogen metabolism is regulated by a complex system of feed forward and feedback loops within the hypothalamic-pituitary-gonadal axis (Williams & Larsen, 2003). Estrogen levels fluctuate throughout the menstrual cycle (Bouman et al., 2005; Williams & Larsen, 2003). This fluctuation is primarily dependent on the stage of follicular development from growth, differentiation, ovulation, and its eventual release if not fertilized (Constantini & Dubnov, 2005; Williams & Larsen, 2003). At the start of the cycle, denoted as the onset of menses, estrogen is low. Low E2 stimulates the hypothalamus to release gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone triggers the anterior pituitary to release follicle-stimulating hormone (FSH). Follicle-stimulating hormone has its impact on the ovaries to recruit and develop follicles. Growth of a follicle includes an increase in the
number of granulosa cells – these cells primarily produce and secrete E$_2$. One of the follicles will dominate over the others and continue to grow and differentiate into an immature ovum (egg). Estrogen will continue to increase, feedback to the hypothalamus, and stimulate the release of leutinizing hormone (LH) from the anterior pituitary. Release of LH further stimulates production and secretion of E$_2$ and increases secretion of progesterone. With elevated LH and E$_2$, the ovum (egg) is released from the follicle – marking ovulation. When the ovum is released, the follicle changes in structure and function to the corpus luteum, secreting primarily progesterone. The corpus luteum continues secreting E$_2$ and progesterone. If the egg is not fertilized, the elevated E$_2$ and progesterone levels feedback to the hypothalamus and inhibit further secretion of GnRH. Without FSH and LH the corpus luteum regresses, reducing E$_2$ and progesterone levels eventually leading to menses and the beginning of a new cycle. Theoretical E$_2$ fluctuation throughout the menstrual cycle is present in Figure 1.

Once E$_2$ is secreted, it travels throughout the blood on transport proteins (Ruggiero & Likis, 2002; Williams & Larsen, 2003). The low molecular weight and carbon backbone of E$_2$ allow it to easily move across a cell membrane (Ruggiero & Likis, 2002; Williams & Larsen, 2003). In the cytoplasm, E$_2$ binds to a receptor. Once bound, the E$_2$ receptor complex moves toward the nucleus where it will stimulate DNA transcription of a particular gene sequence to elicit a desired response (Ruggiero & Likis, 2002). Additionally, some cells house E$_2$ receptors on the cell membrane thereby allowing E$_2$ to bind at the surface and trigger cascade of signaling pathways to provoke the appropriate response (Levin, 2002). The magnitude of these responses is dependent of the bioavailability of the hormone and the affinity of the receptor (Ruggiero & Likis, 2002).
Two main forms of receptors exist: alpha (α) and beta (β) receptors (Enns & Tiidus, 2010; Ruggiero & Likis, 2002). Both receptors are present on reproductive tissues, vasculature, muscle, brain, and bone (Ruggiero & Likis, 2002). Alpha-receptors are also housed on the liver and kidneys, while β-receptors are located within the gastrointestinal and urinary tract (Ruggiero & Likis, 2002). Estrogen has a multitude of actions other than female reproductive development; Table 2 highlights some of the non-reproductive actions of E$_2$.

Additionally, E$_2$ receptors have been found on the surface of immune cells including T and B lymphocytes, monocytes, and macrophages – all of which perpetuate pro-inflammatory cytokine production (Bouman et al., 2005; Bird, Karavitis, & Kovacs, 2008). It is believed that E$_2$ exerts its influence on the transcription factor associated with immune cell gene expression, nuclear factor kappa B. (NF-$\kappa$B) (McKay & Cidlowski, 1999). When stimulated, NF-$\kappa$B promotes transcription of gene sequences known to stimulate growth,
development, propagation of immune cells, including IL-1β and IL-6 (McKay & Cidlowski, 1999).

Table 2: Non-reproductive actions of E₂

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Action</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>Inhibits osteoclasts by modulating IL-6 production and secretion</td>
<td>Reduces break down of bone - preserving bone mineral density</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Increase nitric oxide production</td>
<td>Improves vasoreactivity of blood vessels and reduces prevalence of atherosclerosis by reduced platelet aggregation and leukocyte adhesion</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Increases HDL through suppression of hepatic lipoprotein lipase at liver, adrenal gland, testes, and ovary</td>
<td>Removes excess lipid on and around arterial walls, carrying the lipid to the liver for excretion or to the adrenal gland, testes, or ovary for production of steroid hormones</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Decreases LDL through up-regulation of hepatic LDL receptors</td>
<td>Reduces the amount of fat deposit within the arterial wall thereby perpetuate atherosclerosis</td>
</tr>
<tr>
<td>Muscle</td>
<td>Inhibits gene expression of myostatin which regulates muscle growth and development</td>
<td>Suppresses muscle wasting effects of myostatin, thereby preserving the integrity of muscle tissue</td>
</tr>
<tr>
<td>Muscle</td>
<td>Stabilizes cell membrane – reducing calcium influx thereby inhibiting stimulation of proteases, enzymes associated with muscle break down</td>
<td>Reduces likelihood of muscle damage and degradation</td>
</tr>
<tr>
<td>Muscle</td>
<td>Shares similar chemical structure to antioxidants – decreasing oxidation of LDLs</td>
<td>Protects cells from damage</td>
</tr>
<tr>
<td>Muscle</td>
<td>Increases activation and maturation of satellite cells post exercise</td>
<td>Promotes muscle growth and repair</td>
</tr>
</tbody>
</table>

Enns & Tiidus, 2010; Gueda et al., 1996; Horowitz, 1993; Jones, Schmidt, Pickard, Foxworthy, & Eacho, 2002; Kameda et al., 1997; Mikkola & Clarkson, 2002; Ruggiero & Likis, 2002; Walsh et al., 1991; Zhu et al., 2002

Influence of Estrogen on Cytokines at Rest

Estrogen receptors are known to be present on cytokine producing cells; however, the research on the influence of menstrual cycle phase, as determined by E₂ levels, on cytokine production is conflicting. Some studies suggest elevated cytokine production with elevated
E₂, others say E₂ inhibits production, and a few have reported no change in production across the menstrual cycle. These studies are discussed below.

The following section will be organized into two subsections on E₂’s influence: IL-1β and IL-6. Within each of these subsections studies with similar outcomes will be discussed collectively. Additionally, many studies generally refer to menstrual cycle phases, not hormonal conditions. For ease of comparison in the following sections, hormonal concentration status will be used: low E₂ hormonal condition (LE) refers to the midfollicular phase, while high E₂ hormonal condition (HE) refers to the midluteal phase of the menstrual cycle.

**Interleukin-1β**

A majority of studies suggest elevated resting IL-1β levels during the LE condition. Cannon and Dinarello (1985) assessed IL-1β in five women during both the LE and HE hormonal conditions. The results revealed an approximate 36% increase in IL-1β during the HE compared to LE.

Bouman et al. (2001) studied the immune response across the menstrual cycle in fifteen eumenorrheic women. The average age was 30.7 years and cycle length ranged from 26 - 32 days. Blood samples were taken at the LE and HE points within the cycle. Low E₂ condition was deemed 6-9 days after the onset of menses and HE condition was approximately 19 - 25 days after the onset of menses. Estrogen levels were significantly different between conditions (LE: 0.57 ± 0.07 vs. HE: 27 ± 0.03 nM, p < 0.05). The percentage of immune cells that produce IL-1β were found to be significantly elevated in the HE compared to the LE (~77% vs. ~67%), suggesting IL-1β levels are amplified during periods of high E₂.
Willis et al. (2003) reported similar findings compared to Bouman et al. (2001) in ten eumenorrheic women aged 24 - 44 years. The average 28-day cycle was divided into two phases: pre-ovulation, low E₂, (days 8 - 12 from onset of menses) and post-ovulation, high E₂ (days 18 - 22 from onset of menses). The authors found no significant difference between conditions for E₂ levels (pre: 382 ± 11 vs. post: 399 ± 44 pmol/L); however, they did find a significant elevation in IL-1β post ovulation (HE) compared to pre-ovation (LE) (2.0 ± 0.9 vs. 0.8 ± 0.3 ng/10⁶ cells, p < 0.05).

Some studies found no change in IL-1β across varying E₂ levels. Al-Haarthi et al. (2000) measured the cytokine levels in blood, vaginal cells, and saliva across changing E₂ concentrations. Biological specimens were obtained weekly across two cycles. The LE phase was defined as more than 14 days before the next menses and HE phase was deemed 0 - 14 days before the onset of menses. Blood IL-1β remained the same between hormonal phases at less than 1 pg/ml.

Stock, Coderre, McDonald, and Rosenwasser (1989) studied IL-1 in eleven post-menopausal women, age range 25 – 61 years, following 3 - 9 weeks of oral ingestion of 0.625 mg E₂ daily. Prior to consumption, IL-1 release was 11 ± 4.2 % of the maximum release based on monocyte number. Following consumption IL-1 release was 7.8 ± 1.6 % of the maximum release based on monocyte number. While the change was non-significant, the results trend in a direction suggesting that E₂ reduces IL-1 release. A limitation to this study is that multiple isoforms of IL-1 exist, some more bioactive than others, and some are the antagonist to IL-1β.

On the contrary, some researchers have observed IL-1β to be depressed in the HE condition compared to LE. Lynch et al. (1994) observed IL-1 changes in eleven women - six
oral contraceptive users and five eumenorrheic. Blood samples were drawn 7 - 10 days (LE) and 18 - 24 days (HE) after the onset of menses. During sampling day, bloods were obtained at 4 time points: 8 AM, 12, 4, and 8 PM. Interleukin-1β secretion remained constant throughout the time points assessed in the HE state, ~200 pg/10^6 cells. Conversely, IL-1β secretion was significantly elevated in the LE state and increased during the first three time points, peaking at 4 PM ~1000 pg/10^6 cells, returning to baseline values ~500 pg/10^6 cells at 8 PM.

Morshita, Miyagi, and Iwamoto (1999) found similar results to Lynch despite not measuring IL-1β during two distinct hormonal conditions. Twenty-two individuals with an average age 23.6 years were recruited, eleven of which were women. Blood was analyzed for all isoforms of IL-1 as well as E_2 and progesterone. Results showed with increasing amounts of E_2 a corresponding decrease in IL-1β production with a threshold E_2 concentration of 0.04 ng/ml necessary to warrant a significant reduction. Normal concentration of E_2 in a HE state is significantly greater than the 0.04 ng/ml seen in Morshita’s study to inhibit IL-1β; suggesting E_2, in fact, suppresses IL-1β.

**Interleukin-6**

A majority of studies have found attenuated IL-6 levels during periods of HE. For example, Angstwurm et al. (1997) studied five women throughout two menstrual cycles to determine if cytokines fluctuated with E_2 and progesterone. Their age ranged from 22 - 34 years and the average cycle length was 28 days. Bloods were sampled seven times throughout one cycle: 3, 4, 5, 10, 14, 21, and 28 days after the onset of menses. Interleukin-6 was significantly elevated in the LE compared to HE phase (LE: 1050 fg/ml vs. HE: 600 fg/ml, p < 0.009).
Berg et al. (2002) found comparable results in post-menopausal women. With menopause, E_2 production significantly declines. Seventeen post-menopausal women, aged 52 - 57 years, were divided into two groups: E_2 treatment (n = 10) and placebo (n = 7). The treatment group received 50 µg of E_2 transdermally for 12 weeks. The results indicated IL-6 was significantly reduced in the treatment group at 12 weeks with no change in the placebo group. These results further support the claim IL-6 is inhibited by E_2.

Some have found no significant changes in IL-6 to varying levels of E_2 throughout the menstrual cycle; however, the results of these studies are not entirely conclusive. Chiu et al. (2000) studied the influence of parathyroid hormone and E_2 on IL-6 production with respect to bone resorption in 20 premenopausal women aged 20 - 40 years. Bloods were tested three days a week through an entire cycle starting on day three after the onset of menses, cycle length ranged from 24 - 34 days. Estrogen concentration was significantly different between hormonal conditions (HE: 100 pg/ml vs. LE: 50 pg/ml). Interleukin-6 concentration was slightly lower at 1.49 pg/ml in the HE compared to 1.66 pg/ml in the LE. Additionally, an inverse correlation between parathyroid hormone and E_2 was observed (r = -0.292, p = 0.002) – parathyroid hormone is positively correlated with IL-6 (r = 0.063, p = 0.027), implying E_2 could have a negative correlation with IL-6. The author’s proposed the mechanism for E_2 to exert its influence is on the promoter region of the IL-6 gene sequence, inhibiting stimulation of the promoter.

Schwartz, Schafer, and Bode (2000) compared the relationship between E_2 and cytokine production in men, eumenorrheic women, and postmenopausal women not receiving hormone replacement therapy. For the eumenorrheic women, IL-6 was assessed 2-6 days after the last day of menses (LE) and 2-6 days before the onset of the next menses
While an insignificant reduction in IL-6 was observed between conditions (LE: 24.2 ± 4.6 vs. HE: 17.9 ± 3.2 pg/1000 adherent cells), a significant reduction in IL-6 was found in the eumenorrheic women during the HE state compared to men and post-menopausal women. Both men and post-menopausal women produce less E2 compared to eumenorrheic women. The authors implied their findings support the concept that E2 may inhibit IL-6 production. Further support for E2 anti-inflammatory nature was seen from the other findings in this study, as there was a significant reduction in TNF-α, a pro-inflammatory cytokine known to stimulate IL-6 secretion, during HE periods.

Jilma et al. (1997) hypothesized the IL-6 response would mirror that of IL-1β throughout the menstrual cycle since IL-1β stimulates IL-6 secretion and production. Eighteen eumenorrheic women, average age 26 ± 3 years, were studied. The average menstrual cycle length was 29 days. The LE condition blood samples were taken 7 days after the start of menses. Subjects were given an at home ovulation kit. Nine days following a positive ovulation test another blood sample was taken, marking the HE condition – approximately 5 days before the onset of menses. Estrogen concentration nearly tripled from the LE to HE (LE: 145 pmol/L vs. 392 pmol/L); however, IL-6 concentration remained steady at 0.68 pg/ml throughout both hormonal conditions.

Al-Haarthi et al. (2000) had similar findings in ten eumenorrheic women. The purpose of their research was to measure immune constituents throughout the menstrual cycle within different specimens including blood, vaginal cells, and saliva. Biological specimens were obtained weekly across two cycles. The LE phase was defined as more than 14 days before the next menses and HE phase was deemed 0 - 14 days before the onset of menses. Interleukin-6 concentration did not change between the phases (<1 pg/ml).
Willis et al. (2003) reported opposite findings compared to Chiu et al. (2000) and Schwartz et al. (2000). Interleukin-6 was assessed both pre (LE) and post (HE) ovulation (pre: days 8 - 12; post: days 18 - 22 from onset of menses) in ten eumenorrheic women aged 24 – 44 years (Willis et al., 2003). While IL-6 was lower pre-ovulation compared to post-ovulation (pre: 18.1 ± 5.7 vs. post: 35.1 ± 11.0 ng/10⁶ cells) the difference was not statistically significant.

Konecna et al. (2000) studied the fluctuation of IL-6 across a normal menstrual, 26 - 28 days, in five non-oral contraceptive using women (average age = 31 years). Estrogen concentration followed the theoretical physiological model throughout the cycle: increase to ovulation, slight drop, followed by a second surge, and another drop to initiate menses. The authors found IL-6 significantly elevated during ovulation and late luteal (1.9 pg/ml) compared to follicular (1.5 pg/ml). This conflicting result, compared to previous research, could be a function of hormonal condition determination. Konecna et al. (2000) observed elevated IL-6 levels during ovulation and late luteal phase of the menstrual cycle. Late luteal is generally marked by a reduction in E₂ in order to stimulate menses (Williams & Larsen, 2003); thereby if E₂ levels are lower at this point it would actually by in line with previous research (Angstwurm et al., 1997; Berg et al., 2002). Konecna et al. (2000) reported E₂ concentration during late luteal was ~ 300 pmol/L, more than two times lower than the peak E₂ concentration observed (~ 700 pmol/L), Additionally, within Konecna’s study, IL-6 fluctuates dramatically post ovulation and throughout the luteal phase, making it difficult to find a definitive point of reference.

In summary, basal E₂ has an influence on resting IL-1β and IL-6 across varying E₂ levels; however, the nature of the relationship is inconsistent. Despite the inconsistency, this
fluctuation observed in IL-1β and IL-6 at rest when E2 changes warrant the question of whether or not a similar response is present during and following exercise since exercise influences both E2 and cytokines.

**Estrogen and Muscle Damage**

As previously stated, muscle damage is a form of tissue trauma and this tissue trauma can provoke an immune response. The following section reviews studies looking at the relationship between E2 and biological markers of muscle damage in response to exercise.

Tiidus et al. (2001) studied the influence of E2 on membrane damage in animals following exercise. Forty-eight rats were ovariectomized and randomly assigned to one of four treatment groups: unexercised + placebo (UP), unexercised + 25 mg E2 (UE), 60-minutes exercised + placebo (EP), 60-minutes exercised + 25 mg E2 (EE). Estrogen concentration was 10 - 20 times higher in the E2 supplemented group comparatively. Creatine kinase (CK) was significantly lower in UE versus EE. Following exercise, CK levels in EP were almost twice that of EE. These results suggest E2 protects against muscle damage. Additionally, neutrophil count, myeloperoxidase activity, and calpain activity in the muscle were significantly lower in EE compared to EP. All three of these measures are associated with destruction of tissue; therefore, these findings support that E2 appears to reduce the likeliness of muscle damage.

Kerksick et al. (2008) analyzed the effect of E2’s effect on muscle injury in recreationally active individuals following eccentric exercise. Eight men and women, aged 21 years, were recruited for the study. Women performed the exercise bout during a HE phase. The HE time point revealed the greatest difference in E2 (men: 9.5 vs. women: 113.8 pg/ml). The treatment exercise treatment was 7 sets, 10 repetitions on an isotonic resistance
knee extension machine focusing on the eccentric contraction. Markers of muscle injury was recorded before, 6-hours post, and 24-hours post exercise. Results showed the bax/bcl-2 ratio was reduced in women compared to men following exercise, possibly the result of women having a significant increase in bcl-2. The bax/bcl-2 ratio explains the regulation of cell apoptosis; bax protein promotes cell death, while bcl-2 protein inhibits cell death. Additionally, cell death was significantly greater in men at all time points and gradually increased from resting values, while cell death did not change in women. Cell death occurs in response to trauma in an attempt to clear away damaged or infected tissue; the authors concluded these results suggest less injury occurs in women as a result of E₂ protecting cells against insult.

Carter et al. (2001) examined the influence of E₂ on muscle damage following 30 minutes of downhill running. Twenty-seven fit women (VO₂max ~ 43 ml/kg/min) were recruited to participate; ten were non-oral contraceptive users tested during their LE phase (6 – 10 days after start of menses) and seventeen were oral-contraceptive users tested during their HE phase (22 – 25 days after start of menses). Estrogen levels were significantly different between groups (LE: 112.4 ± 56.9 vs. HE: 269.8 ± 62 pg/ml). Creatine kinase activity was measured before, immediately post, 24-hours, 48-hours, and 72-hours following exercise. Twenty-four hours following exercise, CK was higher in the LE compared to HE; by 72-hours the difference was significant.

Collectively, the studies discussed above support the notion that exercise induces muscle damage. Furthermore, the magnitude of damage appears to be attenuated by the presence of E₂, indicating a protective role of E₂.
Estrogen and Immune Response to Exercise

Few studies have directly looked at the influence of menstrual cycle on cytokines following exercise. Timmons, Hamadeh, Devries, and Tarnopolsky (2005) conducted a study observing the effect of gender and menstrual cycle phase on immune parameters following prolonged exercise. Twelve women (6 oral contraceptive users) and eleven men were recruited for the study. The age of the subjects ranged from 19 – 25 years. Subjects completed a VO₂max test on a cycle ergometer; VO₂max averaged 54 ml/kg fat free mass/min and 56 ml/kg fat free mass/min for women and men, respectively. The treatment protocol was a 90-minute cycling bout at 65% VO₂max during both the LE and HE hormonal condition (approximately 9 and 20 days after onset of menses). Blood samples were obtained before the start of exercise and immediately post exercise. Estrogen was higher in HE compared to LE (361 ± 263 vs. 195 ± 188 pmol/l, comparatively); however the difference was not statistically significant, possibly the result of large variance. Resting IL-6 levels were similar across both genders and hormonal conditions (~ 2.0 pg/ml). Immediately following exercise, IL-6 was slightly higher in both absolute values and percent change in the LE state compared to men and the HE condition, but again the difference was not statistically significant. Interleukin-6 increased by 285% in the LE compared to 235% in men and 215% in the HE. These results imply that E₂ is not a significant modulator of the cytokine response to exercise.

Conversely, Northoff et al. (2008) found that pro-inflammatory cytokines genes were up-regulated during the HE phase of the cycle compared to the LE phase. The authors were studying gene expression of immune cells with respect to gender and varying E₂ concentration across the menstrual cycle. Twelve men and nine non-oral contraceptive using
women were recruited. Age ranged from 25.4 - 36.4 years. Men were significantly more physically fit compared to women; training volume and running speed at anaerobic threshold were significantly higher for men compared to women (61 vs. 38 kilometers/week; 13.9 vs. 11.7 kilometers per hour, respectively). Subjects completed two 60-minute running trials at 93% of their anaerobic threshold running speed: for women, one during the LE condition and the other during the HE condition (day 10 and 25 after the start of menses). Blood was measured one hour prior to the start and immediately following cessation of exercise. Estrogen was non-detectable in men and non-significantly higher in the HE compared to the LE (491 vs. 370 pmol/l) in the women. Baseline IL-6, classified as anti-inflammatory according to the authors, genes were elevated in the HE state and suppressed in the LE. Following exercise, IL-6 along with IL-1ra gene expression were significantly downregulated in the HE phase and trending toward being significantly upregulated during the LE phase, suggesting a pro-inflammatory response following exercise during times of HE. The authors suggest that these results may be confounded by the fact that baseline IL-6 were not similar between conditions, e.g. IL-6 gene expression was suppressed in LE condition and enhanced in the HE. Additionally, only gene expression was observed. Alteration of gene expression does not necessarily translate to an increase in circulating cytokine levels.

The findings of these two studies suggest IL-6 is upregulated during the LE condition. However, Timmons et al. (2005) did not reach significance. Additionally, Northoff et al. (2008) classified IL-6 as an anti-inflammatory cytokine and saw additional anti-inflammatory genes down-regulated in the HE condition, suggesting HE is more pro-inflammatory in
nature. These limited number of studies suggest additional research in this area are necessary to clearly define the relationship between E₂ and cytokines.

Summary

In summary, cytokines are messenger proteins released from immune and non-immune related cells (Corwin, 2000; Moldoveanu et al., 2001). These messengers are released in response to stress, infection, trauma, and exercise – stimulating further responses to either upregulate (pro-inflammatory) or suppress (anti-inflammatory) immune action (Corwin, 2000; Moldoveanu et al., 2001; Pederson et al., 1998). Generally, pro-inflammatory cytokines such as IL-6 and IL-1β are released immediately following infection and trauma (Corwin, 2000; Moldoveanu et al., 2001). Along with upregulating cells associated with humoral immunity (e.g. B-cells, anti-body formation, acute phase protein production), IL-1β and IL-6 influence cardiovascular tissue, muscle, and bone as well (Abbas & Litchman, 2003; Diehl & Rincon, 2000; Faist, Schinkel, & Zimmer, 1996; Smith, 2004; Corwin, 2000; Moldoveanu et al., 2001). Shortly following, anti-inflammatory cytokines are secreted in an attempt to maintain immune reaction within a narrow window (Corwin, 2000; Moldoveanu et al., 2001). Unregulated immune responses can lead to autoimmune diseases.

Previous research has shown elevated IL-1β and IL-6 levels following exercise. Mode, duration, and intensity of exercise influence the elicited response (Pederson et al., 1998). Eccentric exercise appears to elicit a greater increase in pro-inflammatory cytokines compared to concentric exercise (Bruunsgaard et al., 1997; Nieman et al., 1998). It also appears there is a duration dose response with respect to the magnitude of the pro-inflammatory response. That is, the longer the duration of the exercise session the more pronounced the response (Brenner et al., 1999; Ostrowski et al., 1999; Nieman et al., 1998;
Nieman et al., 2001). The mechanism behind this increase is not entirely understood. Some possible mechanisms include increased muscle damage, endotoxemia, and or increased production of reactive oxygen species (Bruunsgaard et al., 1998; Pederson & Hoffman-Goetz, 2000; Kendall & Eston, 2002).

A limitation to the aforementioned studies is the population is primarily males. Nieman et al. (2001) studied both males and females following exercise and found no influence of gender on cytokine response. Conversely, Edwards et al. (2003) reported a significant difference between men and women following intense exercise. Unfortunately, Nieman et al. and Edwards et al. did not take hormonal status into consideration.

In studies measuring cytokines across varying E2 levels, basal E2 appears to have an influence on resting IL-1β and IL-6; however, the nature of the relationship is inconsistent (Angstwurm et al., 1997; Berg et al., 2002; Chiu et al., 2000; Konecna et al., 2000; Schwartz et al., 2000). The variance in conclusions could be the result of small sample sizes, improper phase determination, timing of blood samples, or assay sensitivity. Despite the conflicting reports, an influence of E2 on the immune system does exist at rest.

Few have examined the relationship following exercise. Timmons et al. (2005) observed a greater increase in IL-6 during the LE condition compared to HE; however, the result was not statistically significant. Conversely, Northoff et al. (2008) saw an upregulation of IL-6 genes in the LE following exercise; however, IL-6 in their study was classified as an anti-inflammatory. These are the only two known studies to assess the influence of changing E2 levels on immune response to exercise. The lack of available information suggest additional studies should be carried out to further our understanding of the relationship.
CHAPTER III

Methodology

The study required seven visits for each subject. On separate days subjects performed an exercise test on a treadmill to determine VO$_2$max, two 90-minute running bouts at approximately 70% of their VO$_2$max, and had blood drawn before, immediately post, 24-hours post, and 72-hours post exercise for each 90-minute running bout. The 90-minute running bout and subsequent blood draws occurred at two specific hormonal conditions: low E$_2$ (LE) and high E$_2$ (HE). The blood samples were used to determine E$_2$, IL-6 and IL-1β concentration, and CK activity.

Subjects

Sample size was determined using expected responses of the IL-6 cytokine to 90-minute bouts of exercise based from previous research data. A $\alpha$ level of 0.05 required a total of 10 healthy, highly trained women for a statistical power of 0.8. Women between the ages of 18-30 were recruited. Subjects had maintained eumenorrheic status, defined as a menstrual cycle consistently lasting 24-35 days. A minimum VO$_2$max of approximately 45 ml/kg/min was necessary to participate in the study. Completion of at least one half-marathon, or a race of an equivalent distance under 2 hours 30 minutes was required for participation. No major injuries that could impact performance or the ability to participate in physical activity within 6 months prior to testing were necessary. If an injury was reported within 6 months prior to testing, the subject had to be completely recovered as noted by her physician. Subjects had a
current minimum training volume of 3-5 days a week, 45-120 minutes per session of aerobic activity for inclusion.

Women who were knowingly pregnant or became pregnant during the study were excluded. Amenorrhoeic (absence of menstruation) or Oligomenorrhoeic (irregular cycle) women were excluded. Participants who used substances known to alter immune response (e.g. NSAIDS) the week before each 90-minute exercise session or became ill with immune responding conditions (i.e., colds, respiratory infections...etc.) were excluded. If any contraindications to exercise highlighted by ACSM discovered during the medical history interview and physical examination or abnormal rhythms were found on their 12-lead electrocardiogram the subject was excluded and encouraged to consult her physician.

Instrumentation:

Subject height was measured using a stadiometer (Perspective Enterprises, Portage, MI). Mass was measured using a mechanical scale (Detecto, Webb City, MO). A Lange skinfold caliper (Model 68902, Cambridge Scientific industries, Inc., Cambridge, MA) was used to assess body composition. A refractometer (Milton Roy, Rochester, NY) was used to assess hydration status (urine specific gravity) prior to the prolonged treadmill running bout. The running bout was performed on a Quinton MODEL # Q65 treadmill (Cardiac Science Corporation Bothell, WA). A Polar heart rate monitor was used to monitor heart rate throughout the exercise bout (Polar Electro Inc., Lake Success, NY). Respiratory gas exchanges were measured using Parvo Medics TrueMax® 2400 metabolic System (Parvo Medics, Salt Lake City, UT, USA). The Borg scale was used to determine ratings of perceived exertion [RPE] (American College of Sports Medicine, 2006). Hematocrit was determined using microcapillary tubes (Fisher Scientific International Inc., Hampton, NH),
cortisone (Krakeler Scientific Inc., Albany, NY), Adams MCHT II microhematocrit centrifuge (Beckton Dickinson, Franklin Lakes, NJ), and an International Microcapillary Reader (International Equipment Company, Needham Heights, MA). Hemoglobin was analyzed using the stat-site WT-9” hemoglobin meter (Stanbio Laboratory, Boerne, TX). Blood separation consisted of centrifuging blood at 3000 $X$ g for 15 minutes in an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA). The plasma was aliquoted and frozen at -80 °C. Estrogen levels were determined using an enzyme-linked immosorbent assay [ELISA] (Abnova, Walnut, CA). Creatine kinase levels were analyzed using a spectrophotometric assay (Stanbio Laboratory, Boerne, TX). The cytokines, IL-6 and IL-1β, were determined using an ELISA (Biolegend, San Diego, CA). All ELISA plates were analyzed via Finstruments microplate reader (Model 310, MTX Lab System Inc., Vienna, VA) and Spectra Software (MTX Lab System Inc., Vienna, VA).

Protocol

Orientation/Familiarization Session

A prescreening questionnaire concerning menstrual status and training status was administered over the phone to determine if a potential subject met the inclusion criteria (see Appendix A). If so, the subject was then scheduled to come to the Applied Physiology Laboratory at University of North Carolina at Chapel Hill. Explanation of the study protocol, schedule, inherent benefits, and potential risks were discussed with subject, followed by their signing an informed consent statement (Appendix B). The subject completed a medical history questionnaire and underwent a physical examination and 12-lead electrocardiogram (Appendices C & D). Height and mass were obtained and the subject outfitted with a heart rate monitor. Next, the subject rested for 10 minutes to obtain a resting heart rate. A modified
Astrand protocol was administered to determine VO$_2$max. Heart rate and respiratory gases were monitored continuously throughout the test. Heart rate was recorded at the end of each minute of the test. Rating of perceived exertion was recorded at the end of each stage. Participants were provided verbal encouragement throughout the test. At the completion of the test, subjects were permitted to recover actively or passively. Once heart rate was less than 100 bpm and no adverse post-exercise responses observed, the subject was allowed to leave the lab.

Successful attainment of VO$_2$max was defined as: a decrease, plateau, or less than 0.15L/min increase in VO$_2$ with subsequent increase in workload, a respiratory exchange ratio greater than 1.1, and RPE score greater than 18. If all criteria were not met the test was deemed a VO$_2$peak test. The maximal VO$_2$ attained was used to determine running speed for the specific menstrual phase running bout for each individual subject. Estimated running speed to require 70% of VO$_2$max was determined through a linear regression equation plotting steady state VO$_2$ against subsequent running speed.

_Hormonal Condition Determination_

The subject informed the researcher at the start of menses, denoted as day 1. Approximately 7 ± 2 days after the start of menses marked LE (Williams & Larsen, 2003). Approximately 22 ± 2 days after the start of menses marked HE (Williams & Larsen, 2003). The above was based on an average 28-day cycle. For shorter or longer menstrual cycles (as determined from phone screening), hormonal states were calculated based on the proportionality of the average 28-day cycle (e.g. HE phase is approximately day 22 of a 28-day cycle [22/28=0.79], for a 32-day cycle HE would be approximately day 25 [32 x 0.79] of the menstrual cycle). Scheduling of the prolonged treadmill running bout corresponded with
the subject’s LE and HE hormonal conditions (± 2 days). Desired hormonal status was confirmed later with E2 measurements.

**Prolonged, Treadmill Running Bout**

Twenty-four hours prior to the prolonged running bout session, the subject was instructed to refrain from exercise, consume plenty of water, and eat high carbohydrate foods. The subject reported to the laboratory at the same time of day during the specified phase of the menstrual cycle. Upon arrival, a urine sample was collected from the subject to determine hydration status. If a subject’s specific gravity was below 1.030 cc³ she was deemed dehydrated, did not complete the prolonged running bout, was encouraged to consume plenty of fluids, and rescheduled for a later date. If the specific gravity was above 1.030 cc³ the subject was weighed, fitted with a HR monitor, and asked to rest in the supine position for 10 minutes. At the completion of the 10-minute rest period, resting HR was recorded and a blood sample was drawn via venipuncture by a nationally certified phlebotomist, placed in K3-EDTA blood collection and SST sterile tubes, and immediately put on ice. The blood sample was used to confirm menstrual cycle status and resting IL-6, IL-1β, E2, and CK levels. Next, the subject was moved to the treadmill and fitted for a mouthpiece. The subject sat quietly for 4 minutes as respiratory gases were collected to determine resting VO2. The subject warmed up for 10 minutes: 5 minutes of easy walking on the treadmill followed by 5 minutes of stretching. During the warm-up, the subject practiced putting the mouthpiece in and out of their mouth while moving, as she had to do so periodically during the 90-minute running bout. Following the warm-up, the subject ran on a treadmill at a speed (0% grade) to elicit 70% VO2max as estimated from the VO2max test. At 6 minutes, 26 minutes, 56, and 86 minutes the subject returned to the mouthpiece and
respiratory gases were recorded for four minutes. The purpose of gas collection was to ensure appropriate intensity and make adjustments in running speed if necessary. Heart rate and RPE were recorded during the last 10 seconds of minute 9, 29, 59, and 89 of the running bout. Throughout the running bout the subject drank water *ad libitum*, listened to music, and had a fan to keep cool.

At the completion of exercise, a blood sample was drawn following the same procedure, placed in K$_3$-EDTA blood collection and SST sterile tubes, and immediately placed on ice. This blood sample was analyzed for IL-6 and IL-1β concentrations. After blood collection the subject cooled down. Once HR was below 100 bpm the subject was released from the laboratory.

*Follow-up Blood Draws*

At 24 and 72 hours post running bout the subject reported to the laboratory for additional blood draws. The subject rested in the supine position for 10 minutes. Blood samples were obtained following the same blood draw procedures. These blood samples were analyzed for IL-6 and IL-1β concentrations and CK activity. During the 72 hours of recovery from the running bout subjects were asked to refrain from performing no more than 30 minutes of light physical activity. The subject repeated the aforementioned protocol during both their LE and HE phase.
Figure 2: Prolonged treadmill running bout timeline.
Blood Analysis

Hematocrit

Resting and immediate post exercise hematocrit values were determined for each running bout. Whole blood was drawn into a heparin treated microcapillary tube, sealed, and placed in a microhematocrit centrifuge for 3 minutes at 10,000 rpm. Next, the microcapillary tube was placed on a hematocrit wheel; the proportion of red blood cells within blood was determined. Measures were performed in triplicates and averaged for each blood sample to be used in plasma volume shift determinations.

Hemoglobin

Resting and immediate post exercise hemoglobin values were determined for each running bout using a handheld analyzer. Twenty-five microliters of whole blood was pipetted onto on a slide and analyzed instantly. Measures were performed in duplicates and averaged for each blood sample to be used in plasma volume shift determinations.

Plasma Volume

To account for changes in plasma volume that occur as a result of exercise, plasma volume shifts were calculated with hemoglobin and hematocrit levels using the Dill and Costill method (Dill & Costill, 1974).

Estrogen, Cytokines, Creatine Kinase

The blood samples were centrifuged at 3000 X g for 15 minutes at 4°C to separate the plasma. The plasma and serum were aliquoted, placed in cryofreeze tubes, and frozen at -80°C until later analysis.

To confirm menstrual phase status, E2 concentrations were measured using an enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assays were
also used to determine IL-6 and IL-1β concentrations. A spectrophotometric assay was used to determine CK activity. Details of these assay procedures are in the Appendix F. Triplicate determinations were executed for standards. Duplicate determinations were performed for all of the above assays.

Statistical Analysis:

SPSS version 18 (Chicago, IL) was used to analyze data. The alpha level was set at $p < 0.05$ a priori. Values are reported as means ± standard deviations (SD).

To examine $E_2$ concentration between hormonal conditions a one way repeated measures analysis of variance (ANOVA) was performed. The analysis was used to determine whether or not the appropriate difference in $E_2$ between LE and HE was elicited.

To ensure the subjects were in the same physiological state prior to each prolonged running bout, pre-exercise measurements (e.g. specific gravity, weight, CHO consumption, resting HR, and resting VO$_2$) were evaluated using separate repeated measures ANOVA.

Cardiorespiratory measurements (i.e., HR, VO$_2$, and RPE) recorded during the running bout were assessed using separate one-way repeated measures ANOVA. A Bonferroni procedure was administered for post hoc testing if a significant F-ratio was found in an ANOVA. These analyses were performed to establish if subjects were working at a steady state throughout each of the prolonged bouts and if the intensity were the same between the two trials.

To determine if any muscle damage was amassed as a result of the prolonged running bout CK activity was assessed using a 2 x 3 (menstrual cycle phase x blood sample time: pre-exercise, 24-hr post, 72-hr post) within-subjects ANOVA followed by bonferroni adjusted post hoc testing for any significant F-ratio found. To determine whether or not the magnitude
of change in CK activity was different between phases, percent change scores were calculated. Percent changes scores were calculated for the change in CK from rest to 24-hours post exercise and rest to 72-hours post exercise. The percent change scores were calculated for both menstrual cycle phases for a total of four values. All changes scores were calculated using the following equation: percent change = \([\frac{(post\ exercise - pre\-exercise)}{pre\-exercise}] \times 100\). A 2 x 2 repeated measures ANOVA was conducted with bonferroni post hoc testing for any significant F-ratio found in the ANOVA.

To assess whether or not fluctuations in E\(_2\) modulate the IL-6 and IL-1\(\beta\) response during exercise, separate 2 x 4 (menstrual cycle phase x blood sampling time: pre-exercise, immediate post, 24-hr post, 72-hr post) within-subjects analysis of variance (ANOVA) were performed (research questions 1 and 2). A Bonferroni procedure was administered for post hoc testing if a significant F-ratio was found in an ANOVA.

To evaluate if the magnitude of change in cytokine concentration following exercise was influenced by hormonal condition, three percent change scores were calculated for IL-6 and IL-1\(\beta\) for each hormonal condition for a total of six percent change scores. The percent change scores calculated for each cytokine were as follows: change in concentration from rest to immediately post exercise, rest to 24-hours post exercise, and rest to 72-hours post exercise. A 2 x 3 repeated measures ANOVA was conducted to evaluate the influence of menstrual cycle phase on the magnitude of change in IL-6. A Bonferroni procedure was used for post hoc testing if a significant F-ratio was found in the ANOVA.
CHAPTER IV

Results

Originally, the goal of the study protocol was to enroll only eumenhorreic women; however, given unforeseen challenges in recruitment not enough of these women who met all other inclusion criteria could be found. As a result, the study also recruited women currently using oral contraceptives (OC) who met the other inclusion criteria. Five eumenhorreic women and five OC women completed all aspects of the study protocol, except OC women completed only one of the 90-minute running bouts during what was hormonally considered LE. For the remainder of the document, the phrase “eumenhorreic women” will refer to the collective group of both OC and eumenhorreic subjects recruited into the study.

Additionally, the study protocol was highly involved requiring repeated trips to the laboratory for follow-up blood draws. In some instances, subjects were unable to make it to the laboratory resulting in a missed blood sample. In order to maintain the integrity of the repeated measures design, SPSS (version 18.0) missing variables analysis function was employed to estimate missing data values. This method for using estimated missing values in the statistical analysis follows the suggestions published by Linton and Gallo (1975).

Subject Characteristics

Twelve highly trained female runners were recruited for this study. Ten of the twelve completed the study protocol while two subjects withdrew for personal reasons. All participants met the inclusion criteria: maintained normal menstrual cycle, completed a half marathon under 2 hours 30 minutes within the last year, no major injuries with the last six
months, a minimum VO₂ peak of 45 ml/kg/min, and trained a minimum of 3-5 days per week, 45 to 120 minutes per session aerobically. Participant physical characteristics are reported in Table 3.

Table 3: Participant physical characteristics (n=10)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>25 ± 4</td>
<td>20 – 29</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.8 ± 3.8</td>
<td>158.0 – 171.0</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>58.0 ± 3.6</td>
<td>52.0 – 63.9</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>24.5 ± 4.7</td>
<td>19.4 – 35.1</td>
</tr>
</tbody>
</table>

**VO₂ peak Testing**

All maximal oxygen uptake tests are collectively referred to as VO₂ peak tests as subjects did not unanimously meet all VO₂ max criteria set forth in the methodology chapter. The absolute VO₂ peak (mean ± SD) measured was 3.20 ± 0.40 L/min (range 2.73 – 3.89 L/min) which converts to a relative VO₂ peak of 55.7 ± 3.4 ml/kg/min (range 50.1 – 60.9 ml/kg/min). The peak RPE obtained was 18 ± 2 Borg units (range 15 – 20 Borg units) while the peak HR was 188 ± 7 bpm (range 174 - 197 bpm). The mean running speed at the VO₂ peak response was 15.7 ± 1.0 km·h⁻¹ (range 14.4 – 17.3 km·h⁻¹). The mean time to reach VO₂ peak during testing was 18.27 ± 1.78 minutes (range 16.07 – 22.00 minutes).

**Hormonal Condition Determination**

Menstrual cycle length from the previous four months was collected for each subject. The menstrual cycle length (mean ± SD) was 29 ± 2 days (range 24 - 34 days). Individual menstrual cycle phases were determined using a proportion of the average 28-day menstrual cycle as detailed in the methodology chapter. The midfollicular (LE) phase was 8 ± 1 days
(range 6 – 9 days) after the onset of menses. The midluteal (HE) phase was 23 ± 3 days (range 20 – 26 days) after the onset of menses. The actual days on which the subjects performed their prolonged running bouts (90 minutes) was 9 ± 2 days (range 7 – 10 days) for LE and 23 ± 3 days (range 19 – 27 days) for HE.

Hormonal analysis of resting blood samples for 17β-estradiol (E2) confirmed the appropriate menstrual phase and hormonal status (i.e., treatment effect) of the subjects. The LE E2 concentration was 53.35 ± 13.33 pg/ml, while the HE E2 concentration was 106.16 ± 20.55 pg/ml. These concentrations were significantly different \([F (1, 9) = 133.907, p < 0.001]\).

**Prolonged Treadmill Running Bout**

All subjects followed and complied with pre-session guidelines (see Chapter 3). The mean specific gravity was well below the 1.030 cc\(^2\) for both LE and HE prolonged running bouts, suggesting all subjects were in a euhydrated state prior to running. There was a significantly higher specific gravity at LE (1.013 ± 0.0049 cc\(^2\)) compared to HE (1.004 ± 0.0065 cc\(^2\)) \([F (1, 9) = 8.034, p = 0.02]\), but both values were normal. A diet moderately high in carbohydrates (CHO) was consumed 24-hours prior to both LE and HE prolonged running sessions. The percent of daily calories from CHO was 53 ± 6% and 57 ± 5% for LE and HE, respectively and was not different \([F (1, 9) = 3.586, p = 0.091]\). Mean body mass at the time of the prolonged running bouts at LE and HE were 57.63 ± 3.52 kg and 58.66 ± 2.37 kg and did not differ \([F (1, 9) = 0.655, p = 0.439]\).

The prolonged running bouts were to be run for 90 minutes at 70% of individual subjects VO\(_{2}\)peak. All subjects were able to complete each of their 90-minute sessions, and the actual running speed was 11.2 ± 0.6 km·h\(^{-1}\), corresponding to an overall VO\(_{2}\) of 2.20 ±
0.05 L/min or approximately 67.0 ± 1.6 % VO2peak and was identical for LE and HE sessions. No significant differences between estimated and actual VO2 (L/min) were found \([F (2, 18) = 2.11, p = 0.150]\).

While the exercise VO2 responses were alike between hormonal conditions, the absolute VO2 values obtained throughout the prolonged running bouts showed a slight increase in effort over time \([F (3, 27) = 30.739, p < 0.005]\) (i.e. main effect; see Table 4). Post Hoc analyses revealed a significantly higher absolute VO2 at 30, 60, and 90 minutes compared to 10 minutes during the exercise \((p = 0.001)\). Analyses also showed a significantly higher mean absolute VO2 at 90 minutes compared to 30 minutes \((p = 0.030)\).

Resting HR was slightly, but significantly, lower at LE compared to HE \([t (9) = -2.428, p = 0.038; \text{see Table 4}]\). Heart rate responses over the running bout were similar between hormonal conditions. Heart rate, however, did gradually increase over the course of the run in both HE and LE \([F (3, 27) = 58.543, p < 0.005]\) (i.e. main effect; Table 6). Post Hoc analyses showed HR was significantly higher at 30, 60, and 90 minutes compared to 10 minutes during the exercise \((p < 0.001)\). Additionally, results revealed mean HR was significantly higher at 60 and 90 minutes compared 30 minutes \((p = 0.016; p < 0.001)\). Lastly, mean HR was significantly higher at 90 minutes compared to 60 minutes \((p < 0.001)\).

Ratings of perceived exertion measures obtained suggest the perception of effort was not the same between hormonal conditions (Table 4). That is, the ANOVA results for RPE throughout the prolonged running bouts presented a significant interaction effect for hormonal condition and time \([F (3, 27) = 9.959, p < 0.001]\). Post hoc analyses indicated at 10 minutes of running RPE was significantly higher in LE compared to HE \((p = 0.013)\). At 30 and 60 minutes, however, RPE was significantly higher in HE compared to LE \((p = 0.045, p < 0.001)\).
Additionally, perceived effort gradually increased over time for both LE and HE conditions \([F (3, 27) = 57.756, p < 0.001]\). Post hoc analyses showed RPE were significantly higher at 30, 60, and 90 minutes compared to 10 minutes during the exercise \((p < 0.001)\). Also, the RPE was significantly higher at minutes 60 and 90 compared to minute 30 \((p = 0.003, p = 0.004)\).

Plasma volume decreased over the entire 90-minute running bout. Mean plasma volume decreased by -12.3 ± 5.8 % and -9.3 ± 4.6 % at LE and HE, respectfully; conversely, these plasma volume shifts observed were not significantly different between hormonal conditions \([F (1, 9) = 2.570, p = 0.143]\).

**Blood Responses to Prolonged Running Bout**

Resting, 24-hour, and 72-hour CK activity are reported in Table 5. The statistical analysis indicated a significant interaction effect for hormonal condition and time \([F (2, 18) = 4.310, p = 0.030]\). Post hoc revealed resting CK activity was not significantly different between LE and HE \((p = 0.573)\). Twenty-four hours following the prolonged running bout, CK activity tended to be higher in LE than HE \((p = 0.079)\); and by 72-hour, CK responses were significantly higher in LE comparative to HE \((p = 0.026)\). Conversely, when assessing the percent changes from rest in CK only a main effect for hormonal condition was found \([F (1, 9) = 7.361, p = 0.024; \text{ see Table 5}]\). The percent change for CK from rest following exercise was significantly higher for the LE compared to HE.

Interleukin-6 responses to the prolonged running bout are presented in Table 6. Statistical analysis indicated no interaction effect for IL-6 concentration; however, significant main effects for hormonal condition \([F (1, 9) = 10.344, p = 0.011]\) and for time existed \([F (3, 27) = 12.029, p = 0.002]\). Relative to hormonal status, IL-6 concentration was significantly
higher for the LE session compared to the HE session. Relative to time, post hoc analyses revealed IL-6 was significantly elevated from rest immediately (IP) following exercise ($p = 0.016$). Moreover, IL-6 concentration was significantly lower at 72-hour post exercise compared to IP ($p = 0.012$).

Concomitantly, analysis for the percent changes from rest in IL-6 revealed only a significant main effect for time [$F (2, 18) = 13.043, p = 0.001$; see Table 6]. Post hoc analyses indicated the change (increase) in IL-6 observed at IP following the prolonged running bout was greater compared to the change from rest observed at 72-hour post exercise ($p < 0.001$).

Due to procedural problems and assay complications, detectable levels of IL-1β were unobtainable for the blood samples collected in this study. Therefore, no IL-1β values are reported.
Table 4: Mean (±SD) VO$_2$, HR, and RPE for prolonged running bouts for LE and HE hormonal conditions.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Measure</th>
<th>HR (bpm)</th>
<th>RPE</th>
<th>VO$_2$ (L/min)</th>
<th>HR (bpm)</th>
<th>RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Estrogen (LE)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>0.28±0.02 *</td>
<td>60±8足协</td>
<td>X</td>
<td>2.12±0.14</td>
<td>156±10足协</td>
<td>X</td>
</tr>
<tr>
<td>30</td>
<td>2.22±0.15</td>
<td>101±7足协</td>
<td>12±1 #</td>
<td>2.19±0.15</td>
<td>165±6足协</td>
<td>14±2</td>
</tr>
<tr>
<td>60</td>
<td>2.22±0.15</td>
<td>169±6足协</td>
<td>14±2</td>
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<td>169±6足协</td>
<td>14±2</td>
</tr>
<tr>
<td>90</td>
<td>2.22±0.15</td>
<td>169±6足协</td>
<td>14±2</td>
<td>2.22±0.15</td>
<td>169±6足协</td>
<td>14±2</td>
</tr>
<tr>
<td></td>
<td>High Estrogen (HE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.30±0.05 *</td>
<td>67±6足协</td>
<td>X</td>
<td>2.13±0.10</td>
<td>153±5足协</td>
<td>X</td>
</tr>
<tr>
<td>30</td>
<td>2.21±0.11</td>
<td>159±5足协</td>
<td>13±1 ¶</td>
<td>2.21±0.11</td>
<td>162±4足协</td>
<td>13±1 ¶</td>
</tr>
<tr>
<td>60</td>
<td>2.21±0.11</td>
<td>159±5足协</td>
<td>13±1 ¶</td>
<td>2.21±0.11</td>
<td>162±4足协</td>
<td>13±1 ¶</td>
</tr>
<tr>
<td>90</td>
<td>2.21±0.11</td>
<td>159±5足协</td>
<td>13±1 ¶</td>
<td>2.21±0.11</td>
<td>162±4足协</td>
<td>13±1 ¶</td>
</tr>
</tbody>
</table>

An “x” indicates no value was recorded for the particular time point. * Indicates a significantly lower VO$_2$ compared to 10, 30, 60, and 90 minutes ($p < 0.001$). ▶ Indicates significantly lower VO$_2$ compared to 30, 60, and 90 minutes ($p = 0.001$). § Indicates a significantly lower VO2 compared to 90 minutes ($p < 0.05$). ¶ Indicates a significantly lower HR compared to HE. ¶ Indicates significantly lower HR compared to 10, 30, 60, and 90 minutes ($p < 0.001$). $ Indicates significantly lower HR compared to 30 minutes ($p < 0.001$). ¶ Indicates significantly lower HR compared to 60 minutes ($p < 0.001$). £ Indicates significantly lower HR compared to 90 minutes ($p < 0.001$). ♪ Indicates a significantly lower RPE compared LE ($p < 0.05$). ¥ Indicates a significantly lower RPE compared to HE ($p < 0.05$). ^ Indicates a significantly lower RPE compared to 30, 60, and 90 minutes ($p < 0.001$). # Indicates a significantly lower RPE compared to 60 and 90 minutes ($p < 0.005$).
Table 5: Mean (±SD) resting, 24-hour, and 72-hour CK activity and percent change (relative to rest) in CK during the prolonged running bouts for the LE and HE hormonal conditions.

<table>
<thead>
<tr>
<th>Hormonal Condition</th>
<th>24-hour change (%)</th>
<th>72-hour change (%)</th>
<th>Rest (U/L)</th>
<th>24-hour (U/L)</th>
<th>72-hour (U/L)</th>
<th>Prolonged 24-hour</th>
<th>Prolonged 72-hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE</td>
<td>89.7 ± 16.7 *</td>
<td>451.2 ± 386.1</td>
<td>92.6 ± 9.6 *</td>
<td>504.8 ± 399.9</td>
<td>422.7 ± 269.7</td>
<td>119.1 ± 29.6 £</td>
<td>201.2 ± 25.4 S £</td>
</tr>
<tr>
<td>HE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$ Indicates significantly lower CK compared to LE ($p < 0.05$). * Indicates significantly lower CK compared to 24 and 72 hours post running bout [main effect; $F(1.279, 11.513) = 17.931$, $p \leq 0.005$]. Significant lower CK in HE [main effect; $F (1, 9) = 5.442$, $p = 0.045$]. £ Indicates significantly lower percent change in CK compared to LE (main effect; $p < 0.05$).
Table 6: Mean (±SD) IL-6 response and percent change (relative to rest) during the prolonged running bout for LE and HE hormonal conditions.

<table>
<thead>
<tr>
<th>Hormonal Condition</th>
<th>Rest (pg/ml)</th>
<th>24-hour change (%)</th>
<th>72-hour change (%)</th>
<th>LE (pg/ml)</th>
<th>24-hour change (%)</th>
<th>72-hour change (%)</th>
<th>HE (pg/ml)</th>
<th>24-hour change (%)</th>
<th>72-hour change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.4 ± 5.9</td>
<td>23.6 ± 18.2 *</td>
<td>3.3 ± 3.4</td>
<td>646.8 ± 751.1</td>
<td>143.4 ± 422.8</td>
<td>-32.2 ± 57.6 *</td>
<td>839.2 ± 1451.7</td>
<td>0.8 ± 0.3</td>
<td>-21.1 ± 23.6 *</td>
</tr>
<tr>
<td></td>
<td>13.5 ± 6.3 *</td>
<td>1384.0 ± 928.7</td>
<td>-21.1 ± 23.6 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Indicates significantly higher IL-6 compared to resting values (main effect; \( p < 0.05 \)). £ Indicates significantly lower IL-6 compared to IP values (main effect; \( p < 0.05 \)). Significant lower IL-6 in HE (main effect; \( p = 0.011 \)). ¥ Indicates significantly smaller percent change compared to IP (main effect; \( p < 0.001 \)).
CHAPTER V

Discussion

The purpose of the present investigation was to determine if fluctuations in E\textsubscript{2}, as manipulated by menstrual cycle phase, influence IL-6 and IL-1β response to prolonged aerobic exercise. It was hypothesized that the magnitude of change from rest for both IL-6 and IL-1β would be significantly greater at all measurement time points (rest, immediately post, 24-hour, and 72-hour post exercise) measured during LE compared to HE. These hypotheses were evaluated via examination of the response of 10 highly trained female runners who completed a 90-minute treadmill running bout at 67% of their VO\textsubscript{2peak} during both hormonal conditions, and returned 24 and 72-hours later for follow up blood collection.

Hormonal Condition Determination

In the present study a marked difference in E\textsubscript{2} was observed between hormonal conditions. Estrogen concentration was approximately two times higher for the HE compared to the LE (106.16 ± 20.55 vs. 53.35 ± 13.33 pg/ml, respectively). These results fell within the expected norms for the desired hormonal condition according to the ELISA kit used for analysis; midfollicular phase (LE) have levels ranging from 30 – 100 pg/ml, while midluteal (HE) have levels varying from 60 – 400 pg/ml (Human E\textsubscript{2}, Abnova, Walnut, CA). Furthermore, the difference in E\textsubscript{2} concentration between hormonal conditions (~ 2 fold change) obtained in this study is similar to the E\textsubscript{2} differences reported in previous research. Specifically, a majority of studies have reported a 1.3 – 4 fold increase in E\textsubscript{2} between LE and HE conditions (Chiu et al., 2000; Hackney et al., 1991; Jilma et al., 1997; Konecna et al.,
2000; Northoff et al., 2008; Timmons et al., 2005). However, Bouman et al. (2001) observed a 47-fold increase from LE to HE in women. It is unclear why the Bouman study order of magnitude is so different from others.

**Prolonged Treadmill Running Bout**

The running speed of the subjects across both 90-minute running bouts corresponded to an average VO\(_2\) of 67.0 ± 1.6% VO\(_2\)peak, slightly lower than the target intensity of 70% VO\(_2\)peak. This difference between estimated and actual VO\(_2\) (L/min) was not significantly different, suggesting the appropriate treatment effect for intensity was obtained. Mean absolute VO\(_2\) responses between hormonal conditions were alike too; however, despite the running speed remaining relatively constant, VO\(_2\) gradually increased over each 90-minute period of exercise. The slight elevation in VO\(_2\) over time suggests the effort to maintain exercise intensity increased slightly, this was an expected outcome as a result of the cardiac drift phenomenon [see below] (Hausswirth & Lehenaff, 2001).

Exercise heart rate response between hormonal conditions was similar and displayed a gradual increase over the course of the 90-minutes. At each subsequent time point, HR was significantly higher than the previous. This increase in HR is not unusual with prolonged exercise. Despite exercising in a thermoneutral environment, exercise for a prolonged duration increases body temperature (Hausswirth & Lehenaff, 2001). To abate the thermal load, cutaneous vasodilatation redirects blood to the skin and water within blood plasma is released from sweat glands for evaporative cooling (Hausswirth & Lehenaff, 2001; Plowman & Smith, 2003). These two actions reduce plasma volume and venous return; in order to maintain blood flow to active tissues subsequently maintaining exercise intensity, HR must increase - the cardiac drift phenomenon (Hausswirth & Lehenaff, 2001; Plowman & Smith,
This concept is further supported in the current study by the reduced plasma volume observed following both prolonged running bouts.

Despite identical exercise workloads between the two bouts, RPE measures were significantly different between hormonal conditions. Ratings of perceived exertion were significantly higher in LE compared to HE at 10 minutes; however, at 30 and 60 minutes, RPE was significantly higher for the HE. While the difference in RPE early in the run is statistically significant, the differences in RPE later in the exercise are more clinically relevant. This difference in perception of effort is not atypical; previous research has reported similar findings between different hormonal conditions (Hackney et al., 1991). The mechanism behind the discrepancy is unclear; however, the divergence could be the result of physiological differences between menstrual cycle phases. Research has shown during the HE periods, women generally retain water, resulting in weight gain (Constantini et al., 2005). This idea is supported in the present study by the slightly higher weight and lower specific gravity for the HE condition. Moreover, it is believed during HE, concurrent to the water retention, women have elevated basal core temperature (Constantini et al., 2005). Increased basal core temperature reduces the capacity to effectively thermoregulate translating to increased perception of effort (Constantini et al., 2005). Regrettably, core temperature was not measured in the present study.

Furthermore, in both hormonal conditions, RPE gradually increased over the course time. The increase in perceived effort can be explained by the prolonged nature of the exercise. The ability to exercise for a prolonged period of time is highly dependent on motivation, environment, and hydration and nutrition status (Plowman & Smith, 2003). In this particular instance, subjects did not consume carbohydrates (CHO) during the run but
drank water *ad libitum*. The lack of CHO supplementation could have led to glycogen depletion potentially causing fatigue and a gradual increase in effort perception.

In general the VO₂, HR, and RPE response observed during the 90-minute run followed the expected physiological outcome and were extremely similar between hormonal conditions. This suggests any differences in blood measure responses to exercise, such as CK and IL-6, were the result of differences in hormonal condition.

**Blood Responses to Prolonged Treadmill Running Bout**

**Creatine Kinase**

A significant interaction effect of hormonal condition by time was observed for CK activity. Further analyses showed that CK levels were trending toward significantly higher 24-hours post and were significantly higher 72-hours post exercise for the LE condition comparatively. These results are in line with previous research and support the notion that more muscle damage is accrued following exercise in LE periods compared to HE periods (Carter et al., 2001; Tiidus et al., 2001). Also, the levels of CK found following exercise in each condition are in line with what has previously been reported following exercise (Carter et al., 2001; Tiidus et al., 2001). Carter et al., (2001) observed slightly higher CK levels 24-hours following exercise in both LE and HE conditions and saw a continued increased in CK for the LE condition at 72-hours, comparatively. This disparity at 24 and 72-hours could be related to the mode of exercise; the subjects in the study of Carter et al. ran at a -10% grade, while in the current investigation subjects ran on a flat surface. The downhill running elicits a greater eccentric component to the activity, known to induce more rapid and greater muscle damage (Bar et al., 1988). Conversely, while Tiidus et al. (2001) observed a significantly reduced CK response in E₂ supplemented rats versus non-supplemented, the overall CK
response following the 60-minute run was slightly lower in both hormonal conditions compared to the present study. Creatine kinase levels were only measured once, one hour following exercise. In general, CK levels increase slowly, peaking somewhere between 24 and 96 hours following exercise depending on the mode, duration, and intensity of the exercise bout (Carter et al., 2001; Bruunsgaard et al., 1997; Kendall & Eston, 2002). Given only one time point measurement shortly after exercise in the Tiddus et al. study, the maximum amount of CK in the blood may not have been present, resulting in lower overall CK values. The present investigation measured CK at 24 and 72-hours following exercise, with levels peaking at 24-hours in both hormonal conditions.

The mechanism behind the difference in CK response phenomenon to varying hormonal conditions is not entirely understood, but believed to be the result of E$_2$’s lipophilic nature and anti-oxidant capabilities (Enns & Tiidus et al., 2010; Kendall & Easton, 2002). Estrogen is able to easily implant itself within the cellular bilayer via its lipid backbone while the anti-oxidant nature reduces production of reactive oxygen species from membrane lipids and proteins resulting in a more rigid membrane (Enns & Tiidus et al., 2010; Kendall & Easton, 2002). The more stable and rigid membrane attenuates damage thereby potentially reducing CK release from muscle (Bar et al., 1988; Carter et al., 2001).

*Interleukin-6*

While no interaction effect of hormonal condition and time was observed for absolute concentration, IL-6 did significantly increase following exercise during both hormonal conditions and was significantly elevated in the LE session comparatively. Resting IL-6 at LE was slightly higher in the present investigation compared to others (Brenner et al., 1999; Bruusnsgaard et al., 1997; Chiu et al., 2000; Jilma et al., 1997; Konecna et al., 2000). These
studies reported resting IL-6 values ranging from 0.5 - 1.5 pg/ml, approximately 4 times lower than the present investigation. It is unclear why LE rest values were slightly higher presently compared to other studies. This could be a function of; a) assay technique differences between studies, b) small sample size adding to variability, c) timing of the resting blood draw. Future studies are necessary to elucidate why this occurred.

Immediate post exercise IL-6 was 4.9 times higher compared to rest (i.e. main effect); similar to the findings reported by Bruunsgaard et al. (1997) and Brenner et al. (1999). However, others have reported significantly greater increases in IL-6 following exercise. Ostrowski et al. (1999) saw a 75-fold increase in IL-6 following a marathon (3 hrs and 27 min). Nieman et al. (1998) observed a 51-fold increase in IL-6 following a 2.5 hour run at 75% VO_{2}max. The disparity in results could be related to differences in the intensity and duration of the exercise bout used in the studies.

Subjects were either self-directed or entirely restricted from ingesting CHO during these experiments. Without adequate CHO consumption, muscle glycogen depletion can occur 1 to 3 hours into exercise depending on intensity (Coyle, 1995). This reduction in glycogen is believed to trigger IL-6 release from muscle (Febbraio & Pederson, 2002). Interleukin-6 stimulates glucose production and secretion by direct activation of gluconeogenic pathways at the liver and indirectly by lipolysis at adipose tissue and increased release of cortisol via HPA axis (Febbraio & Pederson, 2002). This increase in glucose production and secretion will help to maintain plasma glucose levels (compensating for the low glycogen) subsequently maintaining exercise intensity (Coyle, 1995). The subjects in the Ostrowski et al. and Nieman et al. studies, due to the intensity-duration of their efforts, were probably in a hypoglycemic state, increasing greatly IL-6 secretion. While
subjects did not ingest CHO during the run in the present study, exercise did cease at 90-minutes, thereby potentially reducing the magnitude of the IL-6 response and muscle glycogen depletion occurrence.

By 24-hours, IL-6 had returned to near baseline values (i.e. main effect). Few studies have assessed IL-6 concentration further than 24-hours post exercise; however, those studies that have, reported parallel findings with the present investigation (Brenner et al., 1999; Bruunsgaard et al., 1999).

Interleukin-6 concentration at 72-hours post exercise was similar to those observed at rest. Additionally, IL-6 was significantly lower at 72-hours post exercise compared to IP. This finding is not unusual considering IP presented peak IL-6 concentrations, creating the largest difference between two time points.

It is important to note the references cited above used primarily male cohorts in their design (Bruunsgaard et al., 1997; Brenner et al., 1999; Nieman et al., 1998; Ostrowski et al., 1999). Given the known hormonal divergence between men and women, the differences in the magnitude of IL-6 responses observed may be related somewhat to gender. Women generally have less muscle mass, reducing the amount of tissue susceptible to muscle damage and oxidative stress (Janssen et al., 2000; Plowman & Smith, 2003). Less oxidative stress reduces production of reactive oxygen species resulting in a smaller pro-inflammatory cytokine response (Kendall & Eston, 2002). Additionally, E₂ is known to modulate metabolic pathways; for example, women are better able to utilize fat for energy compared to men (Constantini et al., 2005). With increased fat utilization, plasma glucose levels are spared, further reducing likelihood of glycogen depletion during exercise subsequently decreasing IL-6 response (Constantini et al., 2005; Plowman & Smith, 2003).
Despite a main effect of hormonal condition for IL-6 concentration, no main effect of hormonal condition was observed for relative IL-6 values (change scores). It was hypothesized that the magnitude of change IP, 24-hours, and 72-hours post-exercise would be of a greater extent for the LE session, respectfully. This was not the case in the present study; the percent change from rest in IL-6 was not of a greater magnitude for the LE compared to HE condition. This finding is in agreement with those previously reported (Timmons et al., 2005), suggesting E₂ does not play as great a role in cytokine response following exercise as previously thought.

The lack of interaction effects for absolute and relative IL-6 values may be the result of limitations in the current study design. It is possible the duration and intensity of the exercise session was not great enough to elicit significant differences in IL-6 between phases. Exercise lasting more than 2.5 hours at high intensities had upwards of 50-fold increases in IL-6 (Nieman et al., 2001; Ostrowski et al., 1999). If not enough damage or stress was accrued during the session it may mask the influence of E₂. Additionally, the sample size was too small. Original power calculations required 10 subjects to complete two exercise sessions at different hormonal conditions; however, only five completed both exercise sessions, while five completed one prolonged exercise session thereby limiting the statistical strength of the design.

The greater IL-6 concentration observed for the LE condition compared to HE (i.e. main effect) in the present investigation may be explained by E₂’s protective effect on muscle (Enns & Tiidus et al., 2010; Kendall & Easton, 2002). A single bout of exercise has been shown to cause acute muscle damage, as determined from changes in CK levels in the blood (Bruunsgaard et al., 1997; Bar et al., 1988; Carter et al., 2001). Muscle damage is a form of
trauma; this tissue trauma is known to stimulate the immune system, specifically the innate branch (Bruunsgaard et al., 1998; Moldoveanu et al., 2001; Pederson et al., 1998). The innate response consists of direct release of pro-inflammatory cytokines (e.g. IL-6) and migration of neutrophils to the site of damage via chemoattractants (Abbas et al., 2000; Pederson et al., 1998). Neutrophils will further draw monocytes to the area (Nathan, 2006; Pederson et al., 1998). Monocytes will stimulate additional production and secretion of IL-6 (Corwin, 2000; Moldoveanu et al., 2001). This augmentation in pro-inflammatory cytokines further up-regulates the adaptive branch of the immune system (Abbas et al., 2000; Corwin, 2000; Smith, 2004). Consequently, not all lines of the adaptive immune system are activated the same; during tissue trauma only the humoral branch is activated, while the cell-mediated branch is suppressed (Diehl & Rincon, 2000; Faist et al., 1996; Smith, 2004). The cell-mediated branch is necessary for protection against bacterial and viral infections [see Figure 3 below] (Abbas et al., 2000; Smith, 2004). Contraction of a virus may alter not only the nature of a training session but the ability to train at all, consequently affecting performance. Hence, periods of high E₂ may provide protection against muscle damage, thereby attenuating CK release and reducing susceptibility to overt inflammatory reactions. This idea is purely speculative given the lack of a significant interaction effect for hormonal condition and time within the present study.
Figure 3: Typical immune response to trauma, stress, infection, and inflammation (adapted from Hackney, [2011]).

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**Interleukin-1β**

In the present investigation levels of IL-1β were unobtainable due to technical problems. In particular, the standard curve for the assay was invalid. Without a standard curve, no reference point exists to convert unknown sample values to concentrations. This technical error was not observed with the IL-6 assay.

It was hypothesized that the change in IL-1β would be of a significantly greater magnitude at all time point measurements in the LE compared to the HE based upon previous research (Lynch et al., 2001; Morshita et al., 1999; Nieman et al, 2001; Ostrowski et al., 1997). Northoff et al., (2008) indirectly assessed IL-1β by measuring gene expression of IL-1ra in eumenhorreic women following 60 minutes of running at 93% of individual anaerobic threshold (∼ 60 – 70% VO₂max). Interleukin-1 receptor alpha (IL-1ra) is the anti-inflammatory protein that inhibits IL-1β action. In Northoff et al.’s study, IL-1ra expression was significantly downregulated following exercise in the HE condition, while expression
was trending towards a significant upregulation in the LE condition. These results suggest a pro-inflammatory state during periods of high E₂. This is the only known study to assess E₂ influence on IL-1β following exercise.

Literature on the influence of E₂ on IL-1β at rest is conflicting. Some report higher IL-1β concentration in HE compared to LE (Cannon & Dinarello, 1985; Bouman et al., 2001; Willis et al., 2003). While others suggest just the opposite, lower IL-1β concentration in HE comparatively (Lynch et al., 1994; Morshita et al., 1999). With such limited research available on the influence of E₂ on IL-1β at rest and in response to exercise, additional studies must be conducted to clarify the relationship. The IL-1β results of the present study would have significantly added to the limited literature. It is regrettable the data could not be obtained.

Conclusion:

In conclusion, IL-6 does in fact increase in both LE and HE conditions immediately following exercise compared to rest, returning to near baseline levels by 24-hours. However, the influence of hormonal condition on cytokine response to exercise is not quite as clear. Interleukin-6 concentration was significantly higher (main effect) in the LE session compared to the HE session; however, the magnitude of change from rest to IP, 24-hours, and 72-hours following exercise was not different between the hormonal conditions. Due to the lack of statistical significance for changes in IL-6 (interaction), it cannot be completely suggested that highly trained female runners should alter their training program – decreasing training volume during periods of low E₂. Although the CK findings do suggest that a slightly greater degree of muscle damage will occur following exercise in a LE condition.
Given the limited research present on the influence of $E_2$ on cytokine response to exercise, additional research is imperative.

**Limitations**

There are several limitations to the current study. One it appears that the sample size was too small. Interleukin-6 data was underpowered, as the $\beta = 0.66$, rather the more acceptable 0.80. In order to reach statistical significance additional subjects are required. Furthermore, post-menopausal, amenorrheic, and oligomenorrheic women have different $E_2$ hormonal profiles compared to eumenorrheic and oral contraceptive users, potentially altering their immune response following exercise. Therefore, the results of this study are only relevant to eumenorrheic and oral contraceptive users. Lastly, immune response is modulated by the mode of exercise – eccentric exercise imposes a greater response compared to concentric (Bruunsgaard et al., 1997; Nieman et al., 1998). Running has an eccentric component; hence, theses results may be only applicable to runners.

**Future Directions**

Future studies should repeat the experiment with a greater number of subjects to reach a statistical power of 0.80. Additionally, only one cytokine was analyzed in the current study. In order to have a more comprehensive understanding of the relationship between $E_2$ and immune response, additional cytokines, both pro- and anti-inflammatory, should be studied following exercise at different $E_2$ levels. Lastly, other sex hormones are known to fluctuate across the menstrual, specifically progesterone (Williams & Larsen, 2003). Some have suggested a relationship exists between progesterone and cytokines at rest (Angstwurm et al., 1997; Morshita et al., 1999). Future studies should further explore this potential relationship with respect to exercise.
APPENDICES

A  Phone Screening Script
B  Informed Consent
C  Medical Screening Forms
D  Physical Screening Forms
E  Data Collection Forms
F  Assay Information
APPENDIX A

Phone Screening Script
Phone Script

1. Hello “name.” My name is Ashley Kallman from the Applied Physiology Laboratory at University of North Carolina at Chapel Hill. I am conducting a research study looking at the influence of different phases of the menstrual cycle on immune response to prolonged treadmill running. Would you be interested in participating?
   a. If yes, then ask: “I need to ask you a few questions regarding your health and training status. Do you mind if I ask you these questions?”
      i. If no, proceed to question 2
      ii. If yes, thank the individual for their time and explain: “Certain criteria must be met in order to participate in the study. I am unable to determine if you meet the criteria; therefore, I am unable to enroll you in the study. Thank you for your time, have a nice day.”
   b. If no, then “thank you for your time have a nice day.”
2. Are you between the ages of 18 and 30?
   a. If yes, proceed to 3
   b. If no, thank the individual and explain: “In order to participate in the study you must be between the ages of 18 and 30. Since you do not fall into the range I cannot enroll you in the study. Thank you for your time, have a nice day.”
3. Are you currently using oral contraceptive?
   a. If yes, thank the individual and explain: “In order to participate in the study you cannot be using an oral contraceptive. Since you are using an oral contraceptive I cannot enroll you in the study. Thank you for your time, have a nice day.”
   b. If no, proceed to question 4
4. Do you have a regular menstrual cycle? Meaning, does your cycle last approximately 25-35 days?
   a. If yes, proceed to 5
   b. If no, thank the individual and explain: “In order to participate in the study you must have a normal menstrual cycle duration between 25-35 days. Since
you do not have a normal menstrual cycle duration I cannot enroll you in the study. Thank you for your time, have a nice day.”

5. How long does your cycle typically last? Meaning, how many days from the start of menses to the next start of menses?

6. How long has your cycle been regular?
   a. If more than 6 months, proceed to question 7
   b. If less than 6 months, thank the individual and explain: “In order to participate in the study you must have a regular cycle for at least 6 months. Since your cycle has not been regular for at least 6 months I cannot enroll you in the study. Thank you for your time, have a nice day.”

7. Are you knowingly pregnant or trying to become pregnant?
   a. If yes, thank the individual for their time and explain: “In order to participate in the study you cannot be pregnant or trying to become pregnant. Since you are or trying to become pregnant I cannot enroll you in the study. Thank you for your time, have a nice day.”
   b. If no, proceed to question 8

8. Have you participated in a half-marathon race or a race of an equivalent distance within the last year?
   a. If yes, proceed to question 8
   b. If no, thank the individual and explain: “In order to participate in the study you must have completed a half marathon race or a race of equivalent distance in order to participate within the last year. Since you have not completed such a race I cannot enroll you in the study. Thank you for your time, have a nice day.”

9. Approximately when did the race take place?

10. What was your official finishing time?
    a. If time is less than 2 hours 30 minutes proceed to question 11
    b. If time is greater than 2 hours 30 minutes thank the individual and explain: “In order to participate in the study you must have completed a half marathon or a race of equivalent distance within 2 hours 30 minutes. Since you did not
complete within the time frame I cannot enroll you in the study. Thank you for your time, have a nice day.”

11. Have you sustained any recent injury for example, patellar tendonitis, illiotibial band syndrome, etc. within the last year?
   a. If yes, thank the individual and explain: “In order to participate in the study you must have no history of injury within the last year. Since you have sustained an injury I am unable to enroll you in the study. Thank you for your time, have a nice day.”
   b. If no, proceed to question 12

12. Are you currently engaged in consistent aerobic training?
   a. If yes, proceed to question 13
   b. If no, thank the individual and explain: “In order to participate in the study you must be currently involved in aerobic training. Since you are not I cannot enroll you in the study. Thank you for your time, have a nice day.”

13. How many days a week are you currently training?
   a. If more than 3 days proceed to question 14
   b. If less than 3 days thank the individual and explain: “In order to participate you must aerobically training a minimum of 3 days per week. Since you are not I cannot enroll in you the study. Thank you for your time, have a nice day.”

14. What is the average duration of your training sessions?
   a. If the sessions are at least 45 minutes they are included.
   b. If less than 45 minutes per session thank the individual and explain: “In order to participate in the study your training sessions must be at least 45 minutes in duration. Since they are not I cannot enroll you in the study. Thank you for your time, have a nice day.”

If the subject meets all the inclusion criteria then the orientation/familiarization session will be scheduled.
APPENDIX B

Informed Consents
Adult Subjects Informed Consent

Study #: 10-2109

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects
Biomedical Form

IRB Study #10-2109
Consent Form Version Date: 12/23/10

Title of Study: Influence of Estrogen on Cytokine Response to prolonged Treadmill Running

Principal Investigator: Ashley Kallman
UNC-Chapel Hill Department: Exercise and Sport Science
UNC-Chapel Hill Phone number: 919-923-9846
Email Address: kallman1@email.unc.edu
Faculty Advisor: Dr. Anthony C. Hackney

Study Contact telephone number: 919-923-9846
Study Contact email: kallman1@email.unc.edu

What are some general things you should know about research studies?
You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason.

Research studies are designed to obtain new knowledge that may help other people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?
Recent work has shown a negative relationship between concentration of estrogens and the inflammatory response, specifically cytokines, at rest. Females in the midluteal phase of the menstrual cycle, high estrogen concentration, exhibited significantly lower circulating cytokines compared to females in the midfollicular phase, low estrogen concentration. These demonstrated fluctuations at rest begs the question - Is there an altered cytokine response during exercise at different points within the menstrual cycle as estrogen changes?
To date, few exercise studies on human female subjects with respect to estrogen concentration and cytokines exist. The studies that do exist present divergent results. The studies performed have limitations; primarily small sample sizes, potential inaccurate menstrual phase determination, or dosage of exercise was not enough to provoke a response.

The purpose of this research study is to learn about the influence estrogen has on the cytokine response to prolonged treadmill running. The results of this study will add to the limited body of knowledge available on the influence of estrogen on cytokine expression in response to exercise within women, potentially providing insight as to how training regimens might be altered for optimal performance and minimal risk.

The main aim of the study is to determine if there is a significant difference in cytokine response at rest, immediately post exercise, 24-hours post exercise, and 72 hours post exercise between two phases of the menstrual cycle, midluteal and midfollicular. On separate days, you will perform an exercise test on a treadmill to determine your maximal aerobic capacity (VO₂max), two 90-minute running bouts at approximately 70% VO₂max, and have blood drawn before, immediately post, 24-hours post, and 72-hours post the 90-minute running bout. Blood samples will be assessed for estrogen and cytokine concentration and creating kinase levels.

You are being asked to be in the study because you are a healthy, highly trained woman between the ages of 18 and 30 with a normal menstrual cycle for at least 6 months. You have not used oral contraceptives for at least six months prior. You have completed at least one half-marathon in less than 2 hours 30 minutes within the last year. Have no major injuries within the last six months that limit ability to engage in exercise or if have sustained an injury are completely recovered and cleared by a physician to partake in exercise. Your current VO₂max is at least 45 ml/kg/min. Your current minimum training volume is 3-5 days a week, 45-120 minutes per session of aerobic activity.

**Are there any reasons you should not be in this study?**
You should not be in this study if you are knowingly pregnant or become pregnant during the study, if you have an irregular or absent menstrual cycle, are currently taking or have taken within the six months prior oral contraceptives, you have sustained an injury within the last six months that has limited your ability to exercise, use substances known to alter immune response (e.g. NSAIDS) the week before each 90-minute exercise session, or you become ill with immune responding conditions (i.e., colds, respiratory infections...etc.).

**How many people will take part in this study?**
If you decide to be in this study, you will be one of approximately 20 people in this research study.

**How long will your part in this study last?**
You will be enrolled in the study for approximately 8 weeks. Within the 8 weeks, 7 visits are made to the Applied Physiology Laboratory at the University of North Carolina at Chapel Hill.

- Visit 1: Orientation Session, duration is approximately 90 minutes
- Visit 2 (approximately 1-6 weeks after visit 1): Prolonged treadmill running bout with a before and immediately after exercise blood draws performed by a certified phlebotomist (NCPT 56147), duration is approximately 2 hours
- Visit 3: 24-hours after prolonged running bout blood draw, duration is approximately 30 minutes
- Visit 4: 72-hours after prolonged running bout blood draw, duration is approximately 30 minutes

Visits 5-7 will be a repeat of visits 2-4 approximately 2-6 weeks after visit 4. Blood specimens will be stored for 3 years following the completion of the study.

What will happen if you take part in the study?
Orientation/Familiarization Session, duration approximately 90 minutes (visit 1):

- The study protocol, schedule, inherent benefits, and potential risks will be explained to you, followed by signing the informed consent.
- You will go through a physical screening that includes completing the Department of Exercise & Sport Science (EXSS) medical history questionnaire and undergoing a physical examination and 12-lead electrocardiogram in the Applied Physiology Laboratory. The physical examination includes auscultation of blood pressure, review of heart sounds, and pulmonary assessment. The physical examination and 12-lead electrocardiogram will be conducted and assessed by Dr. Anthony C. Hackney.
- Height and mass will be obtained and you will be fitted for a heart rate monitor and then asked to rest lying down for 10 minutes. After obtaining your resting heart rate, you will be fitted for a mouthpiece that will be used to collect expired air.
- You will then perform a modified Astrand Protocol to volitional fatigue to determine VO2max. The protocol consists of 3-minute stages with progressive speed at 0% inclined to near max, then grade will be introduced to maximal exertion (e.g. 3 minutes: 6.0, 0%, 3 minutes: 7.5, 0%, 3 minutes: 9.0, 0%, 3 minutes, 9.0, 2.5%). Heart rate (HR), rating of perceived exertion (RPE), and expired air will be monitored throughout the test. VO2max attained will be used to determine running speed for the specific menstrual phase prolonged running bout for each individual subject.

Menstrual Phase Determination:

You will need to inform the principal investigator at the start of menses (the first menses following visit 1 to the lab), which will be denoted as day one. Scheduling of the prolonged treadmill running bout will correspond to a specific menstrual phase.

Prolonged treadmill running bout 1, duration approximately 2 hours (Visit 2 and 5):

The time lapse between visit 1 and visit 2 is not determined by investigators, rather it is determined based upon your menstrual cycle. The start of your menstrual cycle (e.g. menses, day 1) and the approximate length of your menstrual cycle (day 1 of menses to the start of the next menses) will determine when you are scheduled for the prolonged running bouts so as to ensure you are in the appropriate menstrual cycle phase. The approximate time between visit 1 and visit 2 can be 1-6 weeks. Between visit 1 and 24-hours before visit 2 you can partake in normal daily
activities and exercise training with no restrictions. Twenty-four hours prior to the prolonged running bout you will be asked to refrain from exercise, drink plenty of fluids, and eat a diet rich in carbohydrates.

- Upon arrival, you will be asked to urinate into a sterile specimen container. The urine will be assessed for hydration status. If the urine analysis comes back as dehydrated you will not participate in the prolonged running bout, you will be encouraged to consume plenty of fluids, and you will be rescheduled.

- If the urine analysis comes back normal you will be weighed, fitted with a HR monitor, and asked to rest lying down for 10 minutes. After, a resting HR will be recorded and a 1-teaspoon blood sample will be drawn from your arm by a certified phlebotomist (NCPT 56147), placed into a K3-EDTA blood collection tube and immediately put on ice. The blood sample will be used to confirm menstrual cycle status and resting IL-6, IL-1β, estrogen, and creatine kinase levels.

- You will then be transferred to the treadmill and fitted for a mouthpiece. You will be asked to sit quietly for 4 minutes as your expired air is collected to determine resting VO2.

- You will then have 10 minutes to warm-up: 5 minutes will be dedicated to easy walking on the treadmill followed by 5 minutes of stretching appropriate muscles used in the upcoming prolonged running bout (e.g. calf stretch, hamstring stretch, quadriceps stretch, and hip flexor stretch). During the walking, you will practice going on and off the mouthpiece as you are moving.

- Following warm-up, you will run on a treadmill at a 0% incline and a speed to elicit 70% VO2max. At 6 minutes, 26 minutes, 56, and 86 minutes you will be asked to return to the mouthpiece and expired air will be recorded for four minutes. This is to ensure appropriate intensity and make adjustments in running speed if necessary. Heart rate and RPE will be recorded during the last 10 seconds of minutes 9, 29, 59, and 89 of the running bout.

- Throughout the running bout you will have a fan to keep you cool, can drink water at your convenience, and listen to music.

- At the completion of exercise, another 1-teaspoon blood sample will be drawn following the same procedure, placed in K3-EDTA blood collection tube, and immediately placed on ice. This blood sample will be analyzed for IL-6 and IL-1β concentrations and creatine kinase activity. After blood collection you will cool down. The cool down will consist of walking on the treadmill at an easy pace for 5 minutes, stretching the muscles used during the prolonged running bout (e.g. calf stretch, hamstring stretch, quadriceps stretch, and hip flexor stretch), and resting quietly in a chair.

- Once your heart rate has returned to 100 bpm you are free to leave the laboratory.

Follow-up Blood Draws, duration approximately 30 minutes (visit 3, 4, 6, and 7):

At 24 and 72 hours after the running bout you will report to the laboratory for additional blood draws. You will be asked to rest lying down for 10 minutes. Blood samples will be obtained following the same blood draw procedures as explained above. These blood samples will be analyzed for IL-6 and IL-1β concentrations and creatine kinase activity. During the 72 hours of recovery from the exercise you are asked to refrain from performing any physical activity other than that of daily routine living.
Prolonged treadmill running bout 2, duration approximately 2 hours (visit 5):

You will be asked to repeat the aforementioned protocol (prolonged treadmill running bout with 24 and 72 hour follow-up blood draws – visits 5, 6, and 7) during two different phases of your menstrual cycle. The time frame between the prolonged running bouts is approximately 2 to 6 weeks. Between visit 4 and 24-hours before visit 5 you can partake in normal daily activities and exercise training with no restrictions.

Blood Analysis:

The blood samples will be separated by centrifuging and frozen until later analysis. The blood plasma will be analyzed for estrogen levels and immune markers. At the completion of the study the specimens will be stored for 3 years. This is further discussed in another consent form you will be asked to sign.

What are the possible benefits from being in this study?

Research is designed to benefit society by gaining new knowledge. You will not receive any direct benefit from participating in the study.

What are the possible risks or discomforts involved with being in this study?

The potential risks to you from participating in this study may be related to exercise or the blood draw process.

Potential risks associated with exercise are outlined by American College of Sports Medicine as: sudden cardiac death, musculoskeletal injury, and falling.

- The risk of sudden cardiac death is low in healthy individuals; however, to minimize risk a health history questionnaire and physical examination will occur prior to testing.
- To minimize risk of musculoskeletal injury a proper warm-up will be completed prior to all testing.
- Given the prolonged nature of the exercise bout dehydration is a potential risk. To minimize this risk your hydration status will be determined before testing begins ensuring you are in normal hydrated state; if dehydrated the testing session will be cancelled and rescheduled. You will be asked to consume plenty of fluids 24 hours before testing and encouraged to drink water throughout and after the running bout.
- The universal sign for stopping an exercise session will be explained to you prior to all testing sessions. Research technicians will closely monitor your status during the exercise sessions for signs and symptoms of fatigue or a cardiac event to reduce risk of injury or falling.

Furthermore, the potential risk of exercise for you will be minimal because you have performed similar exercise intensities and durations within previous training programs.

Risks associated with blood draws include infection, bruising of the area around the needle insertion, and dizziness/fainting.

- To minimize infection, cleaning of the puncture area and sterile equipment will be used.
- Proper needle gauge and firm pressure applied to the puncture following the blood draw will help minimize risk of bruising.
Following the blood draw, to minimize the risk of syncope you will be asked to move from a supine position to sitting and eventually standing slowly. Research technicians will monitor complexion and skin temperature for adverse signs. A certified phlebotomist will perform all blood draws. First aid procedures and universal precautions will be followed during blood draws and handling of blood samples. In addition, there may be uncommon or previously unknown risks that might occur. You should report any problems to the researchers.

**What are the risks to a pregnancy or to a nursing child?**
If you are a woman and you are planning to get pregnant, you should not be in the study.

**What if we learn about new findings or information during the study?**
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

**How will your privacy be protected?**
Following phone screening, an identification number will be assigned to you for future identification. A hard copy of records will be stored in a locked cabinet in the Applied Physiology Laboratory. Electronic records will be maintained on a secured, password-protected computer. All identifiable hard-copy files will be shredded and disposed of using UNC-CH mechanisms and procedures. Blood samples will be stored in a secured ultra-freezer behind a access code protected door within a laboratory involving only electric ID card access. These specimens will be encoded and labeled so that no personal identifying information will be revealed. The identification number will consist of a unique number along with phase and the sample time (e.g. 00913, 009 is the subject ID, 1 is indicative of menstrual phase, 3 is time sample). Study data and specimens will only contain the identification number. These numbers will be indiscernible unless access to the master list which will be locked in a file cabinet in the Applied Physiology Laboratory. Only the principal investigator and faculty advisor will have access to the records.

No subjects will be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies (for example, the FDA) for purposes such as quality control or safety.

**What will happen if you are injured by this research?**
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not
give up any of your legal rights.

**What if you want to stop before your part in the study is complete?**
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped.

**Will you receive anything for being in this study?**
You will not receive anything for taking part in this study.

**Will it cost you anything to be in this study?**
If you enroll in this study, the only cost to you will be transportation to the research site.

**What if you are a UNC student?**
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

**What if you are a UNC employee?**
Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

**What if you have questions about this study?**
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

**What if you have questions about your rights as a research subject?**
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

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Title of Study: Influence of Estrogen on Cytokine Response to Prolonged Treadmill Running
Principal Investigator: Ashley Kallman (kallman1@email.unc.edu, (919) 923-9846

Subject's Agreement:
I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

Signature of Research Subject __________________________ Date __________

Printed Name of Research Subject __________________________

Signature of Research Team Member Obtaining Consent __________________________ Date __________

Printed Name of Research Team Member Obtaining Consent __________________________
Stored Specimens Informed Consent

University of North Carolina at Chapel Hill
Consent for Storing Biological Specimens With Identifying Information

IRB Study #: 10-2109
Consent Form Version Date: 12/23/10

Title of Study: Influence of Estrogen on Cytokine Response to Prolonged Treadmill Running

Principal Investigator: Ashley Kallman
UNC-Chapel Hill Department: Exercise and Sport Science
UNC-Chapel Hill Phone number: (919) 923-9846
Email Address: kallman1@email.unc.edu
Faculty Advisor: Dr. Anthony C. Hackney
Email Address: ach@email.unc.edu
Study Contact telephone number: (919) 923-9846
Study Contact email: kallman1@email.unc.edu

What are some general things you should know about research?
Research is designed to gain scientific information that may help other people in the future. You may not receive any direct benefit from participating. There also may be risks.

You may refuse to take part in research. If you are a patient with an illness, you do not have to be in research in order to receive treatment.

Details are discussed below. It is important that you understand this information so that you can make an informed choice. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this specimen repository or “biobank?”
Research with blood, tissue or body fluids (specimens) can help researchers understand how the human body works. Research can also answer other questions by using specimens. Researchers may develop new tests to find diseases, or new ways to treat diseases. In the future, research may help to develop new products, such as drugs. Specimens are commonly used for genetic research. Sometimes researchers collect and store many specimens together and use them for different kinds of research, or share them with other scientists; this is called a specimen repository or “biobank.”

The purpose of this particular repository or biobank is to determine the influence of estrogen on the immune response to prolonged running. Within this study, analyses are limited to two pro-inflammatory cytokines as a result of the study being self-funded and resources limited. If more resources become available in the future, further analyses on other immune markers will be conducted to develop a better understanding of the influence of estrogen on immune response. Blood will be collected and separated by centrifuging and frozen until later analysis. The plasma will be analyzed for estrogen concentration and immune markers.
How will the specimens be collected?
Blood samples will be collected by a certified phlebotomist (NCPT 561471) via venipuncture method, placed into a 5ml K3-EDTA blood collection tube and immediately put on ice. The blood samples will be separated by centrifuging, plasma will be frozen at -80°C until later analysis.

What will happen to the specimens?
Blood samples will be stored in a secured ultra-freezer behind an access code protected door within a laboratory involving only electric ID card access. These specimens will be encoded and labeled in a fashion that no personal identifying information will be revealed. Only the principal investigator and faculty advisor will have access to the samples. The specimens will be destroyed within 3 years of the completion of the study.

What are the possible benefits to you?
Benefits to you are unlikely. Studies that use specimens from this repository may provide additional information that will be helpful in understanding the effect of estrogen on the immune response to prolonged running.

What are the possible risks or discomforts involved with the use of your specimens?
In the process of collecting the sample, risks associated with blood draws include infection, hematoma or bruising of the area around the needle insertion, and dizziness/fainting. A certified phlebotomist will perform all blood draws. First aid procedures and universal precautions will be followed during blood draws and handling of blood samples. To minimize infection, cleaning of the puncture area and sterile equipment will be used. Proper needle gauge and firm pressure applied to the puncture following the blood draw will help minimize risk of bruising. Following the blood draw, to minimize the risk of syncope you will be asked to move from a supine position to sitting and eventually standing slowly. Research technicians will monitor complexion and skin temperature for adverse signs. Research technicians are first aid, CPR, and AED certified.

There is a risk of breach of confidentiality. If this research involves genetics, there is also a potential risk for some of your relatives and other members of your ethnic group, since they share some of your genetic makeup.

Will there be any cost to you for storage of the specimens?
There will be no cost to you for the storage and use of the specimens for research purposes.

Will you receive anything for the use of your specimens?
You will not receive anything for taking part in this research.

Who owns the specimens?
Any blood, body fluids, or tissue specimens obtained for this purpose become the exclusive property of the University of North Carolina at Chapel Hill. This organization may retain, preserve or dispose of these specimens and may use these specimens for research that may result in commercial applications. There are no plans to compensate you for any future commercial use of these specimens.
How will your privacy be protected?
Following phone screening, an identification number will be assigned to you for future identification. A hard copy of records will be stored in a locked cabinet in the Applied Physiology Laboratory. Electronic records will be maintained on a secured, password-protected computer. The identification number will consist of a unique number for each individual subject, along with phase and the sample time (e.g. 00913, 009 is the subject ID, 1 is indicative of menstrual phase, 3 is time sample). Study data and specimens will only contain the identification number. These numbers will be indiscernible unless access to the master list which will be locked in a file cabinet in the Applied Physiology Laboratory. Only the principal investigator and their faculty advisor will have access to the records. All identifiable hard-copy files will be shredded and disposed of using UNC-CH mechanisms and procedures. Blood samples will be stored in a secured ultra-freezer within a laboratory involving only electric ID card access. These specimens will be encoded and labeled in a fashion that no personal identifying information will be revealed.

You will not be identified in any report or publication about research using your specimens. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

Will researchers seek approval from you to do future studies involving the specimens?
By signing this consent form, you are giving your permission for researchers to use your specimens as described above. Current and future research is overseen by a committee called the Institutional Review Board (IRB). The role of the IRB is to protect the rights and welfare of research participants. In some cases, the IRB may require that you be re-contacted and asked for your consent to use your specimens in a specific research study. You have the right, at that future time, not to participate in any research study for which your consent is sought. Refusal to participate will not affect your medical care or result in loss of benefits to which you are entitled.

Will you receive results from research involving your specimens?
Most research with your specimens is not expected to yield new information that would be meaningful to share with you personally. There are no plans to re-contact you or other subjects with information about research results.

Can you withdraw the specimens from the research repository?
If you decide that you no longer wish for the specimens to be stored, you should contact the researchers on the front page of this form. It is best to make your request in writing.

Any analysis in progress at the time of your request or already performed prior to your request being received by the researcher will continue to be used as part of the research study. Once the researchers have been notified, your remaining specimens would be destroyed. If you do not make such a request, the specimens may be stored forever. The researchers may choose to destroy the specimens at any time.
What will happen if you are injured by this research?
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from having your specimen collected. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

What if you have questions about this research?
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by e-mail to IRB_subjects@unc.edu.

Subject’s Agreement:
I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate. I agree to my specimen(s) being stored with the identifying code(s).

Signature of Research Subject ___________________________ Date ________________

Printed Name of Research Subject ___________________________

Signature of Research Team Member Obtaining Consent ___________________________ Date ________________

Printed Name of Research Team Member Obtaining Consent ___________________________
APPENDIX C

Medical Screening Form
Department of Exercise and Sport Science
Medical History

Subject: ______________________  ID: __________  Telephone: __________

Address: ______________________

Occupation: ________________  Age: __________

**Patient History**  
1. How would you describe your general health at present?  
   Excellent ______  Good ______  Fair ______  Poor ______

2. Do you have any health problems at the present time?  

3. If yes, please describe: ______________________  

4. Have you ever been told you have heart trouble?  

5. If yes, please describe: ______________________  

6. Do you ever get pain in your chest?  

7. Do you ever feel light-headed or have you ever fainted?  

8. If yes, please describe: ______________________  

9. Have you ever been told that your blood pressure has been elevated?  

10. If yes, please describe: ______________________  

11. Have you ever had difficulty breathing either at rest or with exertion?  

12. If yes, please describe: ______________________  

13. Are you now, or have you been in the past 5 years, under a doctor’s care for any reason?  

14. If yes for what reason? ______________________  

15. Have you been in the hospital in the past 5 years?  

16. If yes, for what reason? ______________________  

17. Have you ever experienced an epileptic seizure or been informed that you have epilepsy?  

18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year?  

19. If yes, name the disease: ______________________  

20. Have you ever been treated for or told you might have diabetes?  

21. Have you ever been treated for or told you might or low blood sugar?  

22. Do you have any known allergies to drugs?  

23. If so, what? ______________________
24. Have you ever been “knocked-out” or experienced a concussion? 
25. If yes, have you been “knocked-out” more than once? 
26. Have you ever experienced heat stroke or heat exhaustion? 
27. If yes, when? 

28. Have you ever had any additional illnesses or operations? (Other than childhood diseases) 
29. If yes, please indicate specific illness or operations: 

30. Are you now taking any pills or medications? 
31. If yes, please list: 

32. Have you had any recent (within 1 year) difficulties with your:  
a. Feet 
b. Legs 
c. Back 

Family History 
33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?  
a. Sudden death 
b. Cardiac disease 
c. Marfan’s syndrome 

Mental History 
34. Have you ever experienced depression? 
35. If yes, did you seek the advice of a doctor? 
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? 
37. If yes, please list condition and if you are currently taking any medication. 

Bone and Joint History 
34. Have you ever been treated for Osgood-Schlatter’s disease? 
35. Have you ever had any injury to your neck involving nerves or vertebrae? 
36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? 
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? 
38. Have you ever experienced any injury to your arms, elbows, or wrists?
39. If yes, indicate location and type of injury: _____________________________

40. Do you experience pain in your back? ________ ________
41. Have you ever had an injury to your back? ________ ________
42. If yes, did you seek the advice of a doctor? ________ ________
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? ________ ________
44. Do you think you have a trick knee? ________ ________
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint surgery that presently limits your physical capacity? ________ ________
46. If yes, indicate where: _____________________________

47. Have you ever had a bone graft or spinal fusion? ________ ________

Activity History
48. During your early childhood (to age 12) would you say you were:
   Very active _____ Quite active_____ Moderately active____ Seldom active____
49. During your adolescent years (age 13-18) would you say you were:
   Very active _____ Quite active_____ Moderately active____ Seldom active____
50. Did you participate in:
   a. Intramural school sports? ________ ________
   b. Community sponsored sports? ________ ________
   c. Varsity school sports? ________ ________
   d. Active family recreation? ________ ________
51. Since leaving high school, how active have you been?
   Very active _____ Quite active_____ Active_____ Inactive____
52. Do you participate in any vigorous activity at present? ________ ________
53. If yes, please list:
   Activity ________ Frequency ________ Duration ________ Intensity ________

54. How would you describe your present state of fitness?
   Excellent _____ Good _____ Fair _____ Poor _____
55. Please list the type(s) of work you have been doing for the previous ten years:
   Year _____ Work _____ Indoor/Outdoor _____ Location (city/state) _____

56. Whom shall we notify in case of emergency?
   Name: _____________________________
   Phone: (Home) _____________________________ (Work) _____________________________
   Address: _____________________________

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57. Name and address of personal physician: __________________________

________________________

All of the above questions have been answered completely and truthfully to the best of my knowledge.

Signature: __________________________ Date: ____________________
APPENDIX D

Physical Screening Form
Examination status: Approved  Disapproved

Department of Exercise and Sport Science
Physical Examination Screening

Name: _____________________ Age: _____ Gender: _______

Please respond to each of the following in writing.

Pulse rate and regularity: ___________ ECG Interpretation: ___________________________

Blood Pressure:
Supine: _______ Sitting: _______ Standing (Left side): _______
Squat: _______ Standing (Right side): _______

Marfan Syndrome evaluation: (Δ BP, Physical Char.) ________________________________

Palpation of Pulses: Carotid: _____ Radial: _____ Pedal: _____

Auscultation of the Lungs:
Back: Lower: _____ Middle: _____ Upper: _____
Front: Middle: _____ Upper: _____

Auscultation of Heart Sounds (Supine, Standing, Squatting)
Non-Specific HS: ______/_____/_____
Murmur: _____ Gallop: _____ Click: _____ Rub: _____ Click w/ Murmur: _____

Bruits: Carotid: _____ Abdominal: _____

Edema: Abdominal: _____ Calf: _____ Pedal: _____

Tenderness: Abdominal: _____ Other: _____

Xanthoma or xanthelasma: _____

Medical/Family History:
High Blood Pressure: _____ Diabetes: _____ CHD/CAD: _____

Last examination w/ physician: __________________
Medications (prescription/ counter): ____________________________

Examiner: ___________________________ Date: __________________
APPENDIX E

Data Collection Forms
Familiarization/Orientation Session

Subject ID: ___________    Date: ___________    Time: ___________

Height: ___________    Weight: ___________    Resting HR: ___________

Each stage is 4 minutes long. Please record average HR for the last 10 second of each minute within each stage. During the last 10 seconds of the stage record both HR and RPE. Regardless of if the stage is complete or not, get a final HR and RPE and hit STOP on the timer at the end of the test.

**Treadmill Stage 1:**
- Speed: ___________    Minute 1: HR: ___________
- Grade: ___________    Minute 2: HR: ___________
- Minute 3: HR: ___________
- Minute 4: HR: ___________
- RPE: ___________

**Treadmill Stage 2:**
- Speed: ___________    Minute 5: HR: ___________
- Grade: ___________    Minute 6: HR: ___________
- Minute 7: HR: ___________
- Minute 8: HR: ___________
- RPE: ___________

**Treadmill Stage 3:**
- Speed: ___________    Minute 9: HR: ___________
- Grade: ___________    Minute 10: HR: ___________
- Minute 11: HR: ___________
- Minute 12: HR: ___________
- RPE: ___________

**Treadmill Stage 4:**
- Speed: ___________    Minute 13: HR: ___________
- Grade: ___________    Minute 14: HR: ___________
- Minute 15: HR: ___________
- Minute 16: HR: ___________
- RPE: ___________

**Treadmill Stage 5:**
- Speed: ___________    Minute 17: HR: ___________
- Grade: ___________    Minute 18: HR: ___________
- Minute 19: HR: ___________
- Minute 20: HR: ___________
- RPE: ___________

The subject will give a 1 minute warning when the end of the test is nearing. I will count down that last minute. I will tell the subject to grab the handrails and walk the treadmill to a stop. **Once they grab the handrails, hit “STOP” on the treadmill.** When the treadmill stops, the subject will straddle the belt, I’ll remove the headgear, and then hit “START” on the treadmill so the subject can cool down. Subject will walk on the treadmill for a few minutes, then be transferred to the table, where they will lie down until HR is below 100.
### Prolonged Session 1

<table>
<thead>
<tr>
<th>ID:</th>
<th>Date:</th>
<th>Time:</th>
<th>Session:</th>
<th>Phase:</th>
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</table>

<table>
<thead>
<tr>
<th>Urine SG:</th>
<th>Weight:</th>
<th>BF%:</th>
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</table>

**SKF Site**

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Average of 2</th>
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<tbody>
<tr>
<td>Triceps</td>
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<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>Supraillium</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>Thigh</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
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</tbody>
</table>

Sum of SKF: mm

Calculated Exercise Intensity: ml/kg/min

Subsequent Speed: mph

Supine Resting HR: bpm

### Exercise Session (HR and RPE Recording)

<table>
<thead>
<tr>
<th>Minutes 6-10: VO_2 measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 minutes</td>
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<td>Notes:</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Minutes 26-30: VO_2 measurement</th>
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</thead>
<tbody>
<tr>
<td>30 minutes</td>
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<tr>
<td>Notes:</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Minutes 56-60: VO_2 Measurement</th>
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<tr>
<td>60 Minutes</td>
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<tr>
<td>Notes:</td>
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<table>
<thead>
<tr>
<th>Minutes 86-90: VO_2 Measurement</th>
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</thead>
<tbody>
<tr>
<td>90 Minutes</td>
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<td>Notes:</td>
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Plasma Volume Shift data

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<tr>
<th></th>
<th>Resting Hematocrit (Hct) and Hemoglobin (Hb) values</th>
<th>Immediate Post Hct and Hb values</th>
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</thead>
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<tr>
<td></td>
<td>Hct</td>
<td>Hb</td>
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<td>Pre 1</td>
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<tr>
<td>Pre 2</td>
<td>____</td>
<td>____</td>
</tr>
<tr>
<td>Pre 3</td>
<td>____</td>
<td>____</td>
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<td>Average</td>
<td>____</td>
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Prolonged Session 2

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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Calculated Ex. Intensity: _____ ml/kg/min  Subsequent Speed: _____ mph  Supine RHR: _____ bpm

**Exercise Session (HR and RPE Recording)**

<table>
<thead>
<tr>
<th>Minutes 6-10: VO₂ measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 minutes</td>
</tr>
<tr>
<td>HR _____</td>
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<tr>
<td>RPE _____</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Minutes 26-30: VO₂ measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
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<tr>
<td>HR _____</td>
</tr>
<tr>
<td>RPE _____</td>
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</tbody>
</table>

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<tr>
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<tr>
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<tr>
<td>HR _____</td>
</tr>
<tr>
<td>RPE _____</td>
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<tbody>
<tr>
<td>90 Minutes</td>
</tr>
<tr>
<td>HR _____</td>
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<tr>
<td>RPE _____</td>
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**Plasma Volume Shift data**

<table>
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<tr>
<th>Resting Hct and Hb values</th>
<th>Immediate Post Hct and Hb values</th>
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</thead>
<tbody>
<tr>
<td>Hct</td>
<td>Hb</td>
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<td>Pre 1</td>
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<tr>
<td>Pre 2</td>
<td></td>
</tr>
<tr>
<td>Pre 3</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX F

Assay Information
Estrogen Assay (Copyrighted by Abnova, Walnut, CA)

REAGENT PREPARATION
1. All reagents should be brought to room temperature (18-25°C) before use.
2. Samples with expected Estradiol concentrations over 1000 ng/ml may be quantitated by dilution with diluent available from Abnova, Inc.

ASSAY PROCEDURE
1. Secure the desired number of coated wells in the holder.
2. Dispense 25 µl of standards, specimens and controls into appropriate wells.
3. Dispense 100 µl of Estradiol-HRP Conjugate Reagent into each well.
4. Dispense 50 µl of rabbit anti-Estradiol(E2) reagent to each well.
5. Thoroughly mix for 30 seconds. It is very important to mix them completely.
6. Incubate at room temperature (18-25°C) for 90 minutes.
7. Rinse and flick the microwells 5 times with distilled or deionized water. (Please do not use tap water.)
8. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
9. Incubate at room temperature (18-25°C) for 20 minutes.
10. Stop the reaction by adding 100 µl of Stop Solution to each well.
11. Gently mix 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
12. Read absorbance at 450 nm with a microtiter well reader within 15 minutes.
Manual Procedure
1. Prepare CK Working Reagent according to instructions.
2. Zero spectrophotometer at 340 nm with distilled water.
3. For each sample and control, add 1.0 mL Working Reagent to cuvet or test tube and warm to 37°C for 4 minutes.
4. Add 0.05 mL (50 μL) sample to its respective tube and mix gently.
5. Read and record absorbance at 2 minutes. Continue incubating at 37°C and record absorbance again at 3 and 4 minutes. Rate should be constant.
6. Determine the average absorbance per minute (ΔA/min), multiply by factor 3376 for results in U/L.

NOTE: If cuvet is not temperature controlled, incubate samples at 37°C between readings.
Interleukin-6 Assay (Copyrighted by Biolegend, Sand Diego, CA)

Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. Prepare 500 μL of the 500 pg/mL top standard by diluting 12.5 μL of the standard stock solution in 487.5 μL Assay Buffer A. Perform six two-fold serial dilutions of the 500 pg/mL top standard in separate tubes. Thus, the human IL-6 standard concentrations in the tubes are 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL and 7.8 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL).

3. Wash plate 4 times with at least 300 μl 1X Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.

4. Add 50 μL of Assay Buffer A to each well that will contain either standards or samples.

5. Add 50 μL/well of standards or samples to the appropriate wells.

6. Seal the plate with a Plate Sealer included in the kit and incubate at room temperature for 2 hours with shaking.

7. Dump the plate contents in a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3.

8. Add 100 μL of Human IL-6 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
9. Dump the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3.

10. Add 100 μL of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.

11. Dump the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 3. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

12. Add 100 μL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells with higher concentrations of human IL-6 should turn a blue color. It is not necessary to seal the plate during this step.

13. Stop the reaction by adding 100 μL of Stop Solution to each well. The blue color should change to yellow color.

14. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.
Interleukin-1β Assay (Copyrighted by Biolegend, San Diego, CA)

Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. Prepare 500 µL of the 125 pg/mL top standard by diluting 3.13 µL of the standard stock solution in 496.9 µL Assay Buffer D. Perform six two-fold serial dilutions of the 125 pg/mL top standard in separate tubes. Thus, the human IL-1β standard concentrations in the tubes are 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL, 3.9 pg/mL, and 2 pg/mL, respectively. Assay Buffer D serves as the zero standard (0 pg/mL).

3. Wash plate 4 times with at least 300 µL 1X Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.

4. For measuring samples of cell culture supernatant:
   a) Add 50 µL of Assay Buffer D to each well that will contain either standard dilutions or samples.
   b) Add 50 µL/well of standard dilutions or samples to the appropriate wells.

For measuring serum or plasma samples:
   a) Add 50 µL/well of Matrix E to each well that will contain the standard dilutions and 50 µL/well of Assay Buffer D to each well that contains samples.
   b) Add 50 µL/well of the prepared standard dilutions to the standard wells and 50 µL/well of serum or plasma samples to the sample wells.
5. Seal the plate with a Plate Sealer provided in the kit and then incubate at room temperature for 2 hours with shaking at 200 rpm on a plate shaker.

6. Discard the plate contents in a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3.

7. Add 100 µL of the Human IL-1β Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 3.

9. Add 100 µL of Avidin-HRP D solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.

10. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 3. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

11. Add 100 µL of Substrate Solution F to each well and incubate for 20 minutes in the dark. Wells with higher concentrations of human IL-1β should turn a blue color. It is not necessary to seal the plate during this step.

12. Stop the reaction by adding 100 µL of Stop Solution to each well. The blue color should change to yellow color.

13. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.
REFERENCES


interleukin-6 alpha 1 acid glycoprotein, and C-reactive protein. Journal of Laboratory and Clinical Medicine, 130 (1): 69-75.


