UNIVERSITY OF CALGARY

# Pregnancy and Fever: Further on Endogenous Glucocorticoids

by

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## *ABSTRACT*

Fever, an important component of the host's defence response to immune challenge, is absent or attenuated in rats near the term of pregnancy. The present experiments were carried out to determine the role of endogenous glucocorticoids in mediating the altered response to exogenous pyrogen (i.e., *E coli* LPS). For the experiments, Metyrapone —a glucocorticoid synthesis inhibitor— was administered to near-term pregnant rats prior to an $EC_{100}$ dose of *E coli* LPS. Administration of LPS following vehicle resulted in a decrease in core temperature (i.e., hypothermia). Prior administration of Metyrapone, however, which abolished the corticosterone response and altered the pyrogenic/cryogenic cytokine response to LPS, eliminated hypothermia and restored the febrile response. Our results provide evidence that endogenous glucocorticoids play a role in mediating the altered febrile response to immune stimuli observed in rats near the term of pregnancy.

## *ACKNOWLEDGEMENTS*

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## *DEDICATION*

This thesis is dedicated to a wonderful, selfless, and caring person. A person who gave when there was nothing left to give, and who prevailed over every obstacle in her path, while ensuring that I would have the same opportunity in life. This is dedicated to my loving mother, Janice Ann-Marie Alexander.

## *TABLE OF CONTENTS*

## *LIST OF TABLES*

## *LIST OF FIGURES AND ILLUSTRATIONS*

## *LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE*

| | |
|---|---|
| AA | Arachidonic acid |
| APR | Acute phase response |
| BBB | Blood-brain barrier |
| CNS | Central nervous system |
| csf | Cerebrospinal fluid |
| CORT | Corticosterone |
| COX | Cyclooxygenase |
| HPA | Hypothalamic-pituitary-adrenal |
| I.C.V. | Intracerebroventricular |
| IL-1β | Interleukin-1 beta |
| IL-1r I | Interleukin-1 receptor (type I) |
| IL-1r II | Interleukin-1 receptor (type II) |
| IL-1ra | Interleukin-1 receptor antagonist |
| IL-6 | Interleukin-6 |
| I.P. | Intraperitoneal |
| I.V. | Intravenous |
| LPS | Lipopolysaccharide |
| mRNA | Messenger ribonucleic acid |
| MET | Metyrapone |
| NFκB | Nuclear factor-kappa B |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NTS | Nucleus of the tractus solitarius |
| OVLT | Organum vasculosum lamina terminalis |
| PVN | Paraventricular nuclei |
| $PLA_2$ | Phospholipase $A_2$ |
| PEG | Polyethylene glycol |
| PGE | E series prostaglandins |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGE_2$ | Prostaglandin $E_2$ |
| PGES | Prostaglandin E synthase |
| $PLA_2$ | Phospholipase $A_2$ |
| POA | Preoptic anterior (hypothalamus) |
| POLY I:C | Polyinosinic:polycytidylic acid |
| TLR3 | Toll-like receptor 3 |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumour necrosis factor-alpha |
| TNFsR | Tumour necrosis factor soluble receptor |

# *Chapter One:*

## *INTRODUCTION*

Pregnancy is a period associated with a number of reversible physical changes to accommodate the demands of a developing fetus. Fever during pregnancy poses short and long-term physiological challenges to the well-being of both the mother and fetus.

History has documented the extent to which 'balance and well-being' of the body have been in the thoughts of great thinkers. In 400 B.C. Hippocrates, the "Father of Medicine", said to his students, "Let thy food be thy medicine and thy medicine be thy food". Through his words, Hippocrates might have paved the way for Leonardo da Vinci's initial pondering on metabolism in the 1500's, and Antoine Lavoisier's ideas in 1770. By the 19$^{th}$ century, physiologists were contemplating the biological bases of eating and drinking. For example, Claude Bernard brought to light his concept of processes that defend physiological states against disturbances from outside the body. In his words, ''stability of environment implies an organism so perfect that it can

continually compensate for and counterbalance external variations." This viewpoint later compelled Walter B. Cannon to publish *The Wisdom of the Body* in 1932, which shifted perceptions from a rigid, non-fluctuating internal body environment as emphasised by Bernard, towards the more realistic notion of a ''dynamic steady-state.'' Cannon created the term: 'homeostasis', to define his developing perspective (32). To him, this term described "a fairly constant or steady state, maintained in many aspects of the bodily economy even when they are beset by conditions tending to disturb them (63)." As such, homeostasis is now known today as the ability of the body to maintain a consistent 'interior milieu' in the face of the bodily stressors which tend to bring the organism out of physiological balance. The homeostatic system is understood as an open system that employs interdependent regulatory subsystems to counteract deviation from a dynamic equilibrium. Bernard referred to this trait as the essential condition of a free and independent life (19; 63). This freedom makes it possible for an organism to adapt and survive in varying environments. At times when environmental extremes cause an extended challenge to the typical regulatory parameters used by the organism, a prolonged shift in the dynamic steady state can also occur. Mrosovsky studied this phenomenon and defined the concept as rheostasis (129). In complex organisms, the regulation of internal core temperature is not only one of the key aspects of adaptive homeostasis and rheostasis; it is also the central influence on the events of a febrile episode during pregnancy.

## 1.1 Thermoregulation

The regulation of core temperature varies among organisms. There are those whose internal temperature is dependent on or dictated by the external environment (i.e., poikilotherms), and those who maintain a stable internal body temperature regardless of external influence (i.e., homeotherms). Homeothermy is defined as "the pattern of temperature regulation in a tachymetabolic species in which the cyclic variation in core temperature, either nychthemerally or seasonally, is maintained within arbitrarily defined limits despite much larger variations in ambient temperature" (190). In homeotherms, thermoregulation is a part of the manifested activities to the over-shadowing concept of homeostasis put forth by Cannon.

In mammals, deep-body temperature measurements have been noted to stay close to 37.0°C (68). In spite of this consistency, rhythmic alterations in physiology and behaviour occur in mammals with a frequency of about one cycle/day (72). These systematic fluctuations have been defined by Halberg and Barnum as 'circadian' (72). Circadian systems can be altered or shifted by environmental stimuli and may be important to daily homeostatic adjustments, or longer-term rheostatic changes. For example, zeitgebers (external time cues to internal processes) such as sunlight provide indicators to biochemical variations in the body (8).

Circadian augmenting controls are endogenously manifested, with the main controlling area located in the suprachiasmatic nucleus, a set of cells situated within the hypothalamus (121). Although circadian systems are apparent in daily changes (i.e., hormone levels, sleep patterns), the core temperature of a healthy homeotherm remains

very constant. Numerous theories have been derived to explain this strict control of temperature. For instance, Satinoff has posed a 'multiple thermostat' theory, as there is evidence showing the likelihood that the two main controlling elements of thermoregulation are innervated independently (162). Given that homeotherms are thought to have evolved from poikilotherms, it is probable that some efficient thermoregulatory traits were retained, and thus possible that there is more than one central thermostat for the organism. To Satinoff, parallel thermoregulatory systems would operate dependently of each other in a hierarchical mode whereby the activity of lower structures would be facilitated and inhibited by those above (162).

Chief regulation of overall core temperature controlling systems has been speculated to originate within the hypothalamus (73; 150). The preoptic anterior regions of the hypothalamus (POA) act as the prime comparator to internal temperature in relation to the external environment. This brain region is responsible for determining the organism's optimal basal core temperature, and directs any subsequent modifications needed. The POA hypothalamus holds a dense cluster of specialized neurons that are sensitive to temperature input (73; 132). These thermo-sensitive neurons can accept central brain input to achieve a thermal steady-state – a set-point. Set-point is defined as "the value of a regulated variable which a healthy organism tends to stabilize by the process of regulation" (190). Hammel's set-point theory describes a "virtual set" or "reference" temperature that determines both the type and the magnitude of response (73). When there are deviations in the organism's actual temperature from the given set-point, thermo-sensitive neurons can additionally accept input via the afferent division of the peripheral nervous system through thermo-sensors embedded in the skin, along the

spinal cord, and from other deep-body thermoreceptor sites (73; 132). Summation of the hypothalamic set-point with afferent input generates an error signal and stimulates thermoregulatory effector pathways to evoke actions that realign core temperature with the set-point. The system can be broken down into five major components (67): 1) the peripheral and deep-body thermoreceptors, which provide afferent temperature input to the central nervous system (CNS); 2) the set-point, which is the central reference value that the organism uses to achieve a steady state; 3) the neurons of the CNS, which act as the integrator for the set-point and afferent inputs; 4) the efferent controlling elements, which effect the alteration of body temperature; and 5) the organism's core temperature, which is the controlled system (Figure 1-1).

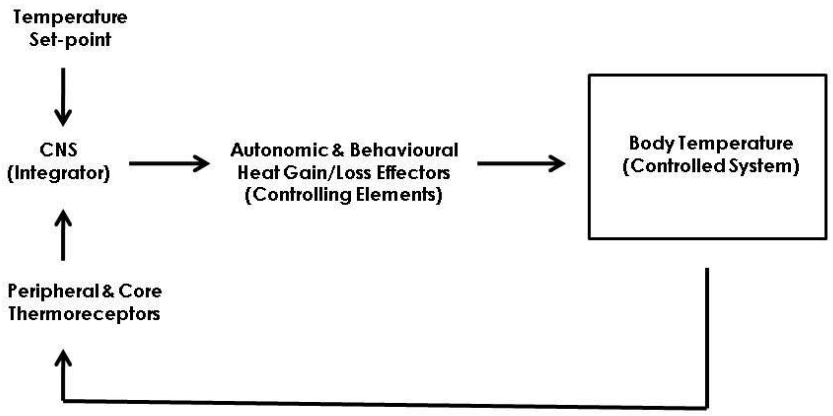


**Figure 1-1: Thermoregulatory control system in homeotherms as a feedback loop. When a difference is detected between the summation of input from thermoreceptors compared with the CNS set-point, the appropriate effector mechanisms are activated to bring core body temperature back to accordance with the set-point. Adapted from Gordon (67).**

The most noticeable processes stem from two separate controlling elements within the efferent peripheral nervous system: the autonomic nervous system and the somatomotor nervous system. The primary action that the body uses to control temperature is through autonomic vasoaction of the vasculature. At times when heat gain is needed, vasoconstriction reduces blood flow to the periphery, conserving heat. Additional heat can be produced by shivering, which is the continuous rapid contraction of skeletal muscles at a rate of 10 to 20 oscillations per second (25; 42). Further, non-shivering thermogenesis which involves the use of brown adipose tissue deposits is capable of oxidizing lipids to produce heat, and is used by small, infantile, or cold adapted mammals (25; 42; 175). An increase in the rate of metabolic activities of all living cells can also create additional heat and is influenced by the '$Q_{10}$ effect'. A normal $Q_{10}$ of 3 indicates that a 10.0°C rise in temperature will result in a three-fold increase in biochemical reactions, and thus can amplify core temperature quickly. Conversely, when heat loss is required, peripheral vasodilation increases blood flow available to be shed into the external environment via a combination of conduction, convection, radiation, and evaporation from the skin. Aside from autonomic processes, the somatomotor system can stimulate the physical relocation of the homeotherm to a warmer/cooler ambient temperature or arouse the organism to engage external mechanisms to achieve the same result.

The body can be categorized into five states of thermoregulation, according to Gordon (66). A state of normothermia is present when the body's core temperature is equal to the set-point, and is the default condition that would define the 'normal' physiology of a healthy being. Hypothermia and hyperthermia are the two states which

stray below and above normothermia. There are two routes to the state of hypothermia, one derived from bodily submission to external factors, and one stemming from internally-orchestrated means. A forced hypothermia occurs whenever an organism is subjected to a relatively cold environment, such that core temperature cannot be maintained and falls below set-point. A regulated hypothermia occurs anytime set-point is reset below core temperature by neurological means. The same two routes of onset are true of the excessive heat realized during a state of hyperthermia. Forced hyperthermia occurs when core temperature is elevated by non-neurological means (e.g., exercise, hot environment) above set-point; whereas a regulated hyperthermia is experienced when set-point is reset to a higher value than core temperature by the hypothalamus. As stated, normothermia is the physiological status quo; however, many challenges that the homeotherm will go through in life can act to alter this balance. Fever presents one such challenge.

### 1.2 Fever

Fever is a period of regulated increase in core temperature (66). This means that there has been a neurological elevation in set-point beyond the organism's basal core temperature. The elevation of set-point has been hypothesized to occur in response to an increase in circulating pyrogenic substances interacting with the hypothalamic POA region (42). Endogenous pyrogens are thought to affect the response characteristics of thermo-sensitive neurons by lowering the firing threshold for cold-sensitive neurons, but raising the threshold for warm sensitive neurons (73). This action would inhibit warm-

sensitive neurons, limiting efferent signals to autonomic heat-loss effector mechanisms such as vasodilation of the peripheral vessels; whereas the excitation of cold-sensitive neurons would further stimulate heat-gain effectors such as shivering and non-shivering thermogenesis (42; 73). Augmentation of hypothalamic activity denotes a shift in set-point which will be defended as long as the pyrogenic influence is present. Once the influence is abated the set-point reverts to its primary position. Hypothalamic thermo-sensitive neurons which were augmented are reversed to their original state, and heat gain/loss mechanisms which were employed or inhibited are restored to their initial state.

The onset of fever is numerous and diverse in form with variations in the magnitude and duration of the responses. In most species, dependant on the intensity of the causative factors that induce the fever, the subsequent response has been noted to occur in discreet phases (154; 186). These phases are denoted by distinct increases or decreases in core temperature over a period of time establishing a separate febrile plateau from the previous. In the monophasic fever, there is a single burst of thermoregulatory effector neuron activity, and a single rise in core temperature (151). Polyphasic responses represent two or more phases. They are noted to occur when the severity of the febrile induction is increased (151). In rats challenged by lipopolysaccharide (LPS), these phases have remarkably precise timing (186), which remains the same for different preparations of LPS and different rat strains (149). The administration of a small, near-threshold dose of LPS (e.g., 1 $\mu g \cdot kg^{-1}$ in a rat) in the subject's thermo-neutral ambient temperature zone, brings about a monophasic (*Phase I*) fever peaking between 1 to 1.5 hours post-injection (151). If the administered dose to the rat increases to 3 $\mu g \cdot kg^{-1}$, the febrile response is composed of two core temperature rises, with the second peak (*Phase*

*II*) occurring between 2 to 2.5 hours post-injection (151; 154). Doses increasing from 5 $\mu g \cdot kg^{-1}$ to lethal results in at least a triphasic response, with *Phase III* occurring 5 to 6 hours post-injection (151; 154).

Febrile initiation is also diverse in its origin (156). For instance, in an acute response immunological processes that have been triggered to enable host defence mechanisms subsequently evoke the hypothalamic augmentations that lead to a febrile response. However, this is not merely by chance. It has been said that elevating the core temperature speeds up some important immunological reactions, whereas a temperature increase hinders the proliferation of some pathogens with strict temperature preferences (93). In this manner, evolution has retained the febrile mechanism as part of the immune response as it plays a role in regaining normal physiology and the restoration of homeostasis.

### *1.2.1 Febrile Mechanism*

Part of the immunological response to a foreign substance is the activation of the Acute Phase Response (APR), which is the upregulation of numerous reactants (i.e., coagulation factors, complement component system) (23). The hallmark of the APR is fever, which stems from a highly regulated process of pyrogen and cryogen and /or antipyretic synthesis and release (42). Exposure of the organism to an exogenous pyrogen (e.g., *Escherichia coli* lipopolysaccharide; polyinosinic:polycytidylic acid) directs cells of the immune system to synthesize and secrete specific cytokines as endogenous pyrogens. Evidence shows that the cytokines interleukin-1β (IL-1β),

interleukin-6 (IL-6), and perhaps tumour necrosis factor alpha (TNF-α) could be the most important pyrogenic molecules to the augmentation of CNS set-point (42; 46). During an APR, these cytokines are released in an increased amount to circulate in the bloodstream. Speculation has occurred on how the cytokines then transduce their signalling to CNS apparatus. More than 100 years ago, Welch forecasted that the cytokines travel to the vessels of the brain to engage the hypothalamus (203), a postulation which is still accepted today. There are four major mechanisms by which periphery pyrogenic molecules may engage the CNS: 1) by binding to endothelial receptors of periventricular structures to elicit central signalling (21); 2) through the organum vasculosum lamina terminalis (OVLT), or other circumventricular organs (21); 3) by stimulation of sensory nerves, primarily the vagus (22); 4) or perhaps by active transport into the brain tissues.

The final common pathway of each speculated route of peripheral signalling is the enhanced expression of neural prostaglandin $E_2$ ($PGE_2$) (42; 58). $PGE_2$ is thought to be the key neural factor to augmenting the firing of hypothalamic thermo-sensitive neurons which effects an increase in core temperature (Figure 1-2). The following sections will delve deeper into this mechanism and its key mediators.

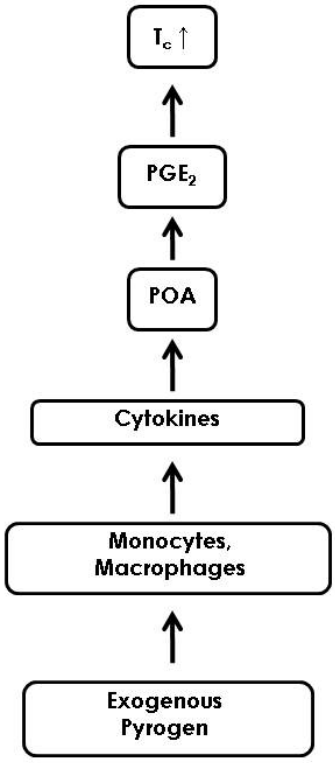


**Figure 1-2: Simplified schematic depicting the classical concept of the exogenous pyrogen induced febrile mechanism adapted from Blatteis (22). An exogenous pyrogen stimulates immune cells to up-regulate pro-inflammatory cytokine secretion. Cytokines interact with the POA hypothalamus, evoking increased central $PGE_2$ which influences autonomic heat gain/retention effectors to activate thus raising core temperature.**

*1.2.1.1 Phase I*

Current evidence is now suggesting that pyrogenic cytokines may not be present during the first phase of fever (22). Studies completed in guinea pigs, mice, and rats show that core temperature rises within 10 minutes of endotoxin administration; however the cytokine TNF-alpha is not detected within the plasma until 30 minutes or more, post-

administration (166). In the polyphasic fever, a specific time delay sequence is observed between phases. The mechanism involved in *Phase I* is a parallel upward shift of the threshold for activation of the different thermoregulatory effectors such as cold thermogenesis, peripheral vasomotor tone, and respiratory evaporative heat loss (151). Thus, this phase denotes a shift in thermoregulatory set-point. *Phase I* is characterised by the upregulation of the functional couple COX-2 and mPGES-1 in the peripheral LPS-processing organs and the hypothalamus (82). Studies have shown marked pyrogenic activity of $PGE_2$ when administered intravenously as a complex with albumin (152), and the expression of $PGE_2$-synthesizing enzymes in the liver and lungs but not in the brain (82). The proposed mediator of *Phase I* has thus been said to be peripheral $PGE_2$ originating in sources such as the lungs or the liver, rather than its central expression. The macrophages found in the liver are known as Kupffer Cells, after Karl Wilhelm von Kupffer who observed them first in 1876, and are the largest group of macrophages produced in the body – a major source for cytokines (47). The Vagus nerve has also been deemed integral to the mechanics of *Phase I*. In a recent study, Niijima showed that injecting IL-1β into hepatic portal vein increases activity of the Vagus nerve and induces enhanced c-fos production in the NTS (135). Vagotomies performed independently by Sehic *et al.* (165) and Simons *et al.* (171) were shown to inhibit the febrile responses to LPS in rats and guinea pigs.

The Complement system is a biochemical cascade of the immune system that destroys pathogens in the host and may also play an important role in the development of *Phase I*. There is recent evidence that complement-depleted guinea pigs cannot develop fevers, possibly due to a lack of components C3a and C5a (22). Furthermore, Kupffer

cells in the liver express receptors for C components (22). During *in-vitro* tests where complement components were added to Kupffer cells, the cells expressed $PGE_2$ synthesis within only minutes (74; 75; 144). Hepatic vagal fibres express the $EP_3$ receptor, which $PGE_2$ readily binds to (51). Peripherally produced $PGE_2$ could then bind to the $EP_3$ receptor on local vagal afferents, innervating the vagus nerve, and consequently stimulating ventrolateral medulla and NTS noradrenergic A1 and A2 projections into the POA hypothalamus (22). Activation of the A1 and A2 cell group fibres increases norepinephrine release into the POA, leading to the direct inhibition of warm-sensitive neurons, or the synthesis of central $PGE_2$. It has been documented that in both the peripheral and the central nervous systems, the stimulation of noradrenergic neurons induces the postsynaptic release of $PGE_2$ (18). Furthermore, norepinephrine microinjected into the POA of conscious guinea pigs also evokes a core temperature rise (145). Linthorst *et al.*, Sehic *et al.*, and Wieczorek *et al.*, propose that initial febrile phase is mediated and sustained by the consequent activation of α1 adrenergic receptors without a major intervention of $PGE_2$ (109; 165; 204). Further support for this position can be seen in work by Jansky and Vybiral who revealed that in rabbits, peripheral administration of LPS induces instant vasoconstriction and the attenuation of panting, whereas cold thermogenesis is not influenced (85). This means that the early febrile response is solely due to inhibition of heat loss mechanisms, and not due to heat production (figure 1-3).

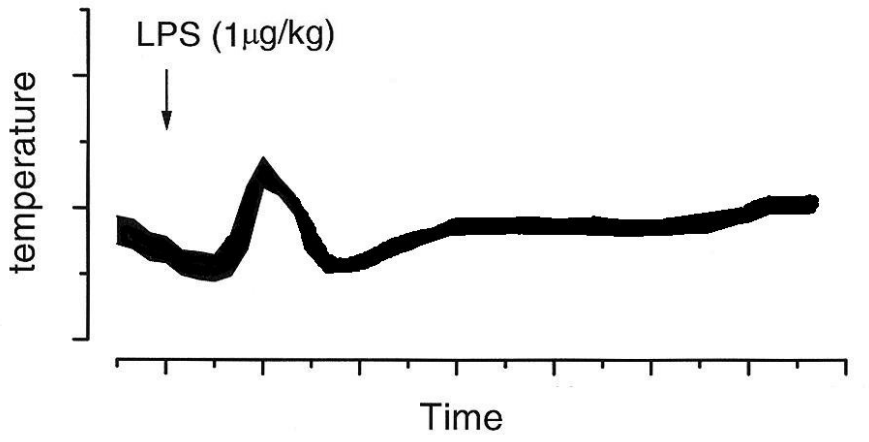


**Figure 1-3: Generalized sketch of monophasic febrile response. A near-threshold dose of LPS elicits one rapid increase in core temperate which later returns to baseline.**

1.2.1.2 *Phases II & III*

Unlike the parallel shift of thermoregulatory set-point characterized by *Phase I*, febrile *Phase II* and *Phase III* involves a threshold dissociation (151). That is, in these latter phases the threshold for heat loss effectors remains elevated, as typically occurs in *Phase I*; however, threshold for heat gain effectors decreases by several degrees (198) creating a wide dead band zone between upper and lower thresholds. Core temperature regulation within this zone thus shifts from homeothermic 'defended' regulation, to the passive heat transfer between body and the environment, characteristic of poikilothermy (151) leaving behavioural thermoregulation as the only means of regulation (151). As a result, these latter phases are denoted by distinct decreases or plateaus in core

temperature, followed by further elevations in temperature as additional pyrogenic factors again begin to exert their influence on the hypothalamus (151) (figure 1-4).

The elevation in core temperature of the polyphasic fever has been said to be mediated by bursts of underlying $PGE_2$ synthesis, and its arrival to the POA hypothalamus (151; 182). *Phase II* and *Phase III* have shown distinct patterns in the transcription of $PGE_2$-synthesizing enzymes in response to LPS. The most remarkable event of *Phase II* is a forceful transcriptional upregulation of all of the necessary components for $PGE_2$ synthesis ($sPLA_2$, COX-2, and mPGES-1) in the periphery, and in the brain (83; 151). The level of $PGE_2$ in the bodily fluids is not only dependant on its synthesis, but also on the rate of its degradation (143). Downregulation of proteins such as carbonyl reductase (CR) and 15-hydroxy-PG-dehydrogenase (15-PGDH), which are involved in the catabolism of $PGE_2$ in peripheral organs can support an increase in prostanoid levels (83; 151). This boosts the blood-to-brain gradient of $PGE_2$ available to interact with peripheral neural afferents of the POA hypothalamus.

During *Phase III*, further upregulation of $sPLA_2$ and mPGES-1 is noted to be expressed in the liver and hypothalamus (83; 151). In addition, the downregulation of $PGE_2$-catabolizing enzymes continues to occur, along with a strong attenuation of the expression of the prostaglandin transporter (PGT) and multiorganic anion transporter (MOAT), proteins involved in the carrier-mediated cellular uptake of $PGE_2$ (83; 151). Thus, it is hypothesized that by these latter phases, a ramped synthesis of both central and peripheral $PGE_2$ synthesis, along with a strong inhibition of $PGE_2$ catabolism drives the elevation in core temperature (83; 151).

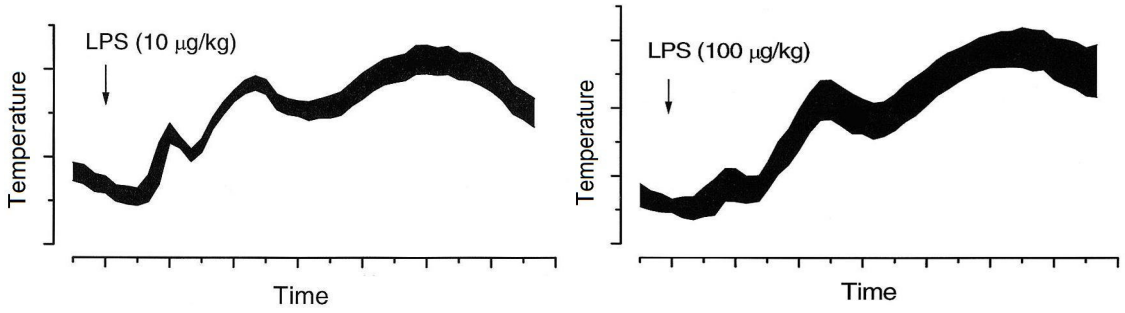


**Figure 1-4: Generalized sketch of moderately-high and high dose polyphasic febrile responses in rats. Left sketch depicts the triphasic temperature response to a moderately-high dose of LPS. Right sketch shows the response to a dose ten times greater, in which the appearance of *Phase I* of the response has been diminished. Adapted from Romanovsky *et al*. (153).**

A pyrogen is defined as a substance that causes the elevation of body temperature, or in other words, a stimulant capable of invoking an elevation in thermal set-point (42). Pyrogens have been separated into two classes dependant on whether they originate outside (exogenous) or inside (endogenous) the body.

1.2.1.3 Exogenous Pyrogen

Exogenous pyrogens represent an array of substances external to a host that are capable of inducing a febrile response in the organism (81). This may include sources such as bacteria, viruses, fungi, and chemical substances. One of the most widely known and researched biological substances that trigger a febrile response in humans and other organisms is lipopolysaccharide (LPS). LPS is a large cell-wall component of gram-negative bacteria, and is classified as an endotoxin given that its toxic element is a

structural entity of the bacteria and is only released when the microorganism is lysed. There are three covalently-bonded domains to the structure of LPS: (139) a proximal hydrophobic domain known as Lipid A which anchors the endotoxin to the outer monolayer cell wall of the bacteria and gives the structure it's toxicity (139); a non-repeating, oligosaccharide core section; and a distal O-antigen polysaccharide tail section which determines the antigenic properties of the endotoxin (42; 139). The implementation of LPS in medical science has been an important step to the study of fever and immunological responses in mammals. LPS mimics infection as it engages similar physiological pathways that an actual pathogenic bacterial infection would cause, but in a controlled manner without colonization or unforeseen sepsis.

Pathogen-associated molecular patterns are non-host molecular structures which are recognized, and activate innate immune responses to defend the host. The introduction of exogenous pyrogens into an organism can trigger a myriad of physiological consequences and reactions, such as the initiation of the APR. In the case of LPS, it causes similar immunological actions to occur, but also engages many signalling pathways such as the nuclear factor-kappa B, MAPK/ERK, and STAT5 pathways once the cell wall is lysed and the toxic lipid A portion begins to circulate. A lipid transfer protein in the host plasma known as LPS Binding Protein delivers lipid A to the Cluster of Differentiation molecules (CD14) on the surface of a circulating macrophage (36). The Lipid A/CD14 complex and one further binding protein, MD-2, then bind to the Toll-Like Receptor 4 (TLR4), also present on macrophage outer membranes (36). The formation of this heterodimer is an integral step in sensitizing TLR4 to engage with cytosolic adapter proteins such as MyD88 which is thought to

activate protein kinase (e.g., IRAK-1, IRAK-4, MAPK/ERK) signalling pathways resulting in the translocation of nuclear factor-kappa B into the cell nucleus, and the subsequent upregulation of gene transcription and synthesis of mediators such as pyrogenic cytokines (Figure 1-3).

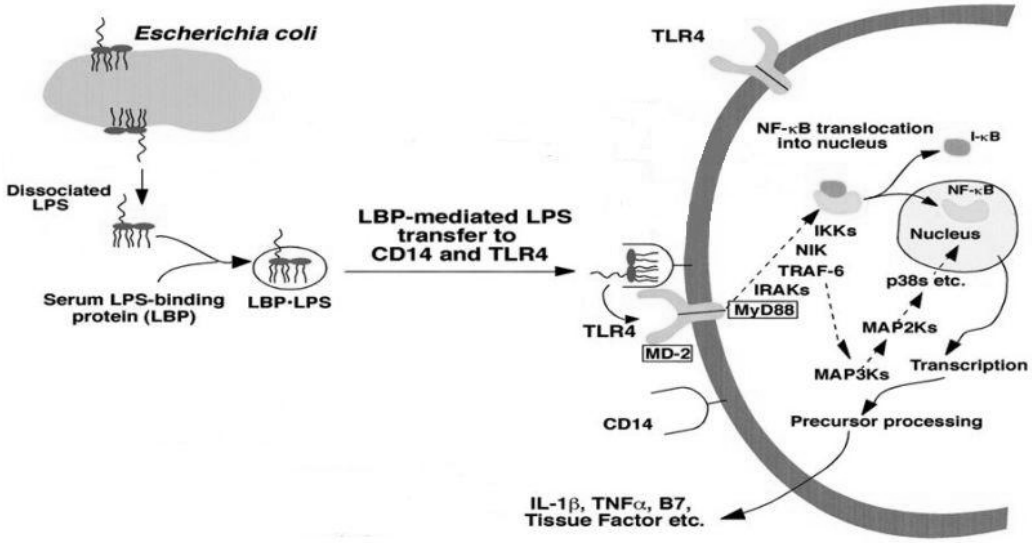


**Figure 1-5: LPS stimulated MAPK/NFκB signalling pathway. LPS (Lipid A) forms a complex with CD14 and MD-2 to bind to TLR4 activating intercellular protein kinases which bring about the translocation of NFκB into the cell nucleus, and the upregulation of cytokine synthesis. Adapted from Raetz (147).**

Although LPS was the agent utilized to induce a febrile response in this report, there is evidence that other substances can engage similar pathways to upregulating pro-inflammatory cytokine production *in vivo*. For example, unlike bacterial infection, little is known about sickness responses to viral infection. Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic double stranded RNA viral mimic that has been used

successfully in immunology and fever studies to reproduce the effects of viral infection in a body (62). Poly I:C administration stimulates antiviral activities of the innate immune system without the unmanageable replication attributes of a live viral entity. Similar to LPS, Poly I:C activates the APR and can also induce pyrogenic cytokine synthesis (i.e., IL-1β, IL-6, and TNF-α), leading to a febrile response. Viral double stranded RNA such as Poly I:C can induce cytokine synthesis by passing through the lipid bylayer of host cells and binding to the Toll-Like Receptor 3 (TLR3) on endosomal membranes (34). This initiates the TRIF/IRF3/NFκB cascade which cumulates in interferon α and β, and pyrogenic cytokine secretion from the cell (34).

The onset of fever and its maintenance is a highly regulated event involving various mediators. As evident in both the bacterial and viral routes described, exogenous pyrogens do not directly manipulate the final pathways to a febrile response, but provide the initiating elements to engage this machinery. The role of interacting with the neural centers and influencing an adjustment in CNS set-point is the responsibility of endogenous pyrogen.

#### 1.2.1.4 Endogenous Pyrogen

In 1948, Beeson discovered that granulocytes from peritoneal exudates which were not contaminated by exogenous pyrogen, could themselves produce a pyrogenic substance (14). Evidence from subsequent investigators confirmed that the material that Beeson discovered was not an altered form of endotoxin but rather a separate substance, and hence was termed ‘leucocyte pyrogen’ or ‘endogenous pyrogen’. Endogenous

pyrogens are defined as heat labile proteins and lipids that have been synthesized within an organism's body, and cause fever (81). The production of these proteins can occur in response to injury, trauma, or stress; but it is also often due to stimulation by exogenous pyrogen (94). Of the multiple endogenous substances that have been acknowledged as pyrogenic, cytokines are thought to be the key intermediary between exogenous pyrogen stimulation and CNS pathways which elicit an eventual febrile response. As discussed previously, cytokines are primarily secreted by activated mononuclear phagocytes. Of those produced, parenteral administration of IL-1β, IL-6, and TNF-α in numerous animal models have all independently proved to evoke febrile responses similar to responses initiated by exogenous pyrogen (i.e., LPS, Poly I:C) (94).

IL-1 increases adhesion factors on the surface of endothelial cells to influence leukocyte migration (78). IL-1 exists as two biochemically separate proteins, interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β). Although both are thought to share the same cellular receptor, IL-1β is deemed to be the only type of IL-1 secreted as an endogenous pyrogen because IL-1α is proposed to be cell-associated (78; 94). Long *et al.* have reported that after using LPS to evoke a febrile response, an IL-1β antiserum reduced the fever by 57% (110). IL-6 is a myokine that can be increased in production by muscle contractions, or secreted by osteoblasts in response to a requirement of further osteoclast formation. It is also produced by T cells and macrophages in response to immune system activation, and binds to its cell-surface receptor to stimulate inflammation. TNF-α is a 185 amino acid glycoprotein that regulates immune cells and induces cell death by binding to its TNF-R1 and TNF-R2 receptors as an anti-tumoural factor (189). Endogenous IL-6, interferon- α, and interferon-γ have all shown pyrogenic

roles in fever production, as has TNF-α. Interferon-γ enhances the production of IL-1β; whereas TNF-α can cause the further induction of plasma IL-1β and IL-6 (31).

Cytokines are relatively large molecules. For instance, IL-1β has a molecular mass of 17.5 kDa (45) and cannot diffuse across the blood-brain barrier (BBB). Given this apparent complication, multiple routes have been postulated as to how endogenous pyrogen evokes further downstream CNS signalling (Figure 1-4). Not all theories regarding this stage of febrile signal transmission view the BBB as an obstacle. The current primary hypothesis claims that cytokines do not penetrate the brain at all, but bind to their cell receptors along the BBB to generate the expression of central pyrogenic mediators within the preoptic nucleus (118; 163). In this respect, the BBB is no longer seen as an obstacle, but as an active signal transducer. Two distinct cell types have been identified as possible mediators of this path: brain endothelial cells, and perivascular microglial cells (151). The brain capillaries are formed from non-fenestrated, highly differentiated endothelial cells that are connected together by tight junctions. Perivascular cells are bone marrow derived, vascular-associated macrophages that comprise most of the brain's macrophage population. The predominant signal transducing agent of the two is currently unclear. Data from Matsumura and Kobayashi indicate that endothelial cells could be the central entity to the mechanism after an $_{I.P.}$ administration of LPS or IL-1β (118; 151); whereas Elmquist *et al.*, and Schiltz and Sawchenko claim that perivascular cells display a greater sensitivity to synthesize the majority of brain $PGE_2$ after $_{I.V.}$ injection of the same pyrogens (151; 163). What is clear to proponents on both sides of the debate is that both cell types have been shown to rapidly induce $PGE_2$-synthesizing enzymes within the brain after peripheral

administration of LPS, IL-1β, or TNF-α (151). Furthermore, both sides agree that of the various routes speculated on, pyrogenic signal transmission from humoral factors, into CNS mediators, is best explained by way of active signal transduction by perivascular and endothelial cells.

Circumventricular structures such as the OVLT have also been deemed of particular importance to this stage of the mechanism. The OVLT contains capillaries which are fenestrated, thus lacks a molecule-filtering BBB (79), and lies in close proximity and exactly mid-line to the POA region of the hypothalamus. Therefore, it has been thought that the OVLT provides a direct link for subsequent neural tissue signalling by the blood-borne pyrogen (76). However, over the past two decades, multiple experiments using electrolytic lesioning of the OVLT have drawn inconclusive results (either exaggerated, attenuated, or non-effect on the febrile response) (155). Furthermore, most positive findings supporting the importance of the OVLT, do indicate that the *lamina terminalis* (anteroventral periventricular nucleus, ventromedial preoptic area, and medial preoptic nucleus) is integral to fever signalling, but fail to distinguish which of the four signalling mechanisms are primarily activated in the process (151).

An alternate theory suggests that cytokines are actively transported across the BBB by cytokine specific protein carriers. However, the physiological significance of this mechanism has been difficult to prove although the list of cytokines utilizing carrier transport to cross the BBB has grown in recent years (11). Instead, it has recently been put forth that perhaps the cytokines can diffuse across the basal lamina into the brain parenchyma (122).

Once the cytokines have successfully crossed, or interacted with the BBB (Figure 1-4), the next stage of signalling can proceed. One CNS mediator that has been of focus to the transmission of febrile signalling is the E series prostaglandins (PGE).

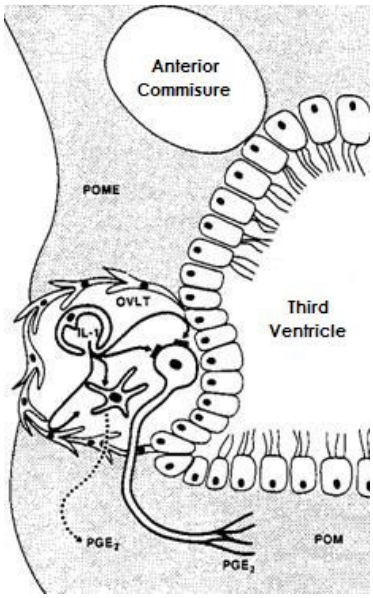


**Figure 1-6: Postulated mechanisms of endogenous pyrogen signal transmission on the OVLT. Endogenous pyrogen (e.g., IL-1β) interacts with endothelial cells and neuron projections to stimulate $PGE_2$ formation in the medial preoptic nucleus. Adapted from Coceani (37).**

1.2.1.5 Prostaglandin

The humoral transmission of pyrogenic signalling by way of endogenous pyrogen has long been deemed an important, if not necessary step in febrigenesis; however, it has not been identified as the final stage in the process. Milton & Wendlandt conducted numerous studies on the actions of prostaglandins on the CNS mediation of fever. Their

initial observations in 1970 showed that microgram amounts of $PGE_1$ injected into the cat's lateral cerebral ventricle resulted in a typical endotoxin-induced febrile response (123). The drug 4-acetamidophenol, which they found to consistently prevent systemic endotoxin-induced fevers, did not obstruct fevers caused by $_{I.V.}$ $PGE_1$ micro-injection (123). This gave credence to the possibility that the prostaglandins can be a key element in the final neural pathway of fever generation. Since then, evidence has shown that of the types of prostaglandins synthesized in the body, the E series prostaglandins are the principle CNS mediators of the febrile response, with emphasis on $PGE_2$. In experiments on fevered cats, radioimmunoassay of csf (cerebrospinal fluid) samples showed that essentially all of the activity was due to $PGE_2$ (56). Further, there is recent evidence that the OVLT is extremely sensitive to $PGE_2$ during fever (184).

Prostaglandins are a form of lipid compound originating from fatty acids. CNS mediated fever production is primarily focused on the formation of prostaglandin from arachidonic acid (AA) derivatives. The first step in the production of prostaglandin is the freeing of AA from phospholipids. Phospholipases, including phospholipase C/diacylglycerase and phospholipase $A_2$ ($PLA_2$), are enzymes that deacylates fatty acids from a phospholipid. $PLA_2$ facilitates most of this action in normal brain cells, removing the fatty acid chain and creating a lipid molecule with an open binding site, in this case, AA. In the second step, freed AA is acted upon by the enzyme cyclooxygenase (COX), which converts AA into prostaglandin $H_2$ and prostaglandin $G_2$ (173). The COX enzyme is expressed in two isoforms (172): COX-1 which is constitutively expressed; and COX-2, the NFκB, ERK/MAPK, STAT5 inducible form thought to play a role in inflammation and fever. Prostaglandin $H_2$ and $G_2$ are then rapidly acted upon by various prostaglandin

synthases to form the final products: prostaglandin $D_2$, prostaglandin $F_2$, and prostaglandin $E_2$ (173). Of the active synthases, cPGES, mPGES-1, and mPGES-2 can all facilitate the formation of $PGE_2$ (71), with mPGES-1 being the main enzyme that parallels COX-2 expression in experiments that employed IL-1β (71) (Figure 1-5).

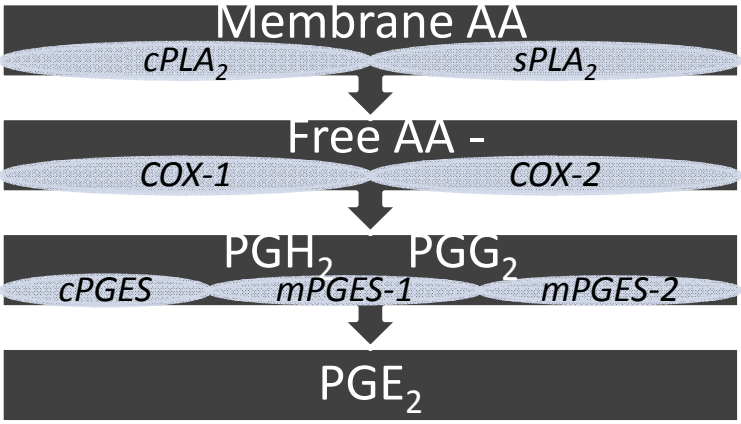


**Figure 1-7: Prostaglandin $E_2$ synthesis pathway. Membrane bound AA is freed by phospholipases cPLA2 and sPLA2. AA is then converted by COX-1 and COX-2 into the intermediaries $PGH_2$ and $PGG_2$. $PGE_2$ synthases cPGES, mPGES-1, and mPGES-2 rapidly convert the intermediary prostaglandins into $PGE_2$.**

The brain parenchyma is thought to have few prostaglandin catabolising agents (151). Peripheral $PGE_2$ arriving into the brain, along with centrally released $PGE_2$, would be free to quickly accumulate in the hypothalamus and potently act on the thermo-sensitive neurons (151). There are at least four receptor variants that $PGE_2$ can bind to in the mammalian body: the $EP_1$, $EP_2$, $EP_3$, and $EP_4$ subtypes (151; 173). Evidence has suggested that the binding of $PGE_2$ to the $EP_3$ receptor on GABAergic neurons expressed in the POA can be an integral step to the febrile response (106; 195). Activation of this

receptor hyperpolarizes the neural cell and is said to decrease intracellular second-messenger cyclic adenosine monophosphate (cAMP) (105) and cyclic guanosine monophosphate (cGMP) (181) levels, creating an inhibitory effect that widens the gap between resting membrane and threshold potentials. Furthermore, a differential effect could be occurring between the $EP_3$, and $EP_4$ types (105). Experiments by Oka *et al.*, using $_{I.C.V.}$ injections of the EP3 agonist ONO-AE-248 and the EP4 agonist ONO-AE1–329 into the brains of rats, have resulted in a 1.5°C fever and a 0.5ºC hypothermia respectively (137). The distribution of binding sites within the brain is ubiquitous, perhaps suggesting their importance to diverse CNS functions. Studies in monkey and rat brains have identified high density $PGE_2$ binding sites in locales near the OVLT and anterior hypothalamus including the anterior wall of the third ventricle, medial preoptic area, paraventricular nuclei (PVN), and the nucleus of the tractus solitaries (NTS). This suggests the importance of these locations to the febrile mechanism (119; 201).

### 1.3 Antipyresis

Historically, man has searched for means of reducing the body's core temperature for therapeutic benefit. Hippocrates used willow bark which contains glucoside salicin for this purpose (70). von Gerhardt first prepared acetylsalicylic acid in 1853 — a drug which is still commonly used world-wide to reduce core temperature and alleviate inflammation. Fever is a complexity of regulated responses. In addition to the upregulation and release of endogenous pyrogenic substances, the body also produces agents known as antipyretics that work to limit the intensity of the temperature increase

(99). In contrast to cryogens, which cause a cooling of the body due to their presence, regardless of the existence or absence of fever (66; 81), antipyretics are agents that nullify the actions of circulating fever-induced pyretic substances (81). Antipyretics do not affect basal core temperature in afebrile subjects, but will counteract the effects of pyrogens under febrile conditions, creating an empirical upper limit (112). In this manner, plasma concentration of antipyretics can directly influence the severity of the febrile response. Early 'fever sensitization' work by Pittman and Cooper into the inability of newborn lambs to mount an initial febrile response to endotoxin or prostaglandin micro-injection lead to Kasting's conclusion that fever in the ewe and newborn lamb can be suppressed close to term and shortly after delivery by some endogenously produced substance (88). Various hormones such as neuropeptides (e.g., arginine vasopressin), cytokines (e.g., interleukin-10, interleukin-1 receptor antagonist), nitric oxide, and corticosteroids (e.g., melanocortins, corticosteroids, ACTH) have all been reported to show pyrogen inhibiting properties. Significant elements to this list are arginine vasopressin (88), nitric oxide (69), interleukin-1 receptor antagonist (7), and the glucocorticoids (38), as they have all shown to have altered characteristics in their antipyretic actions during pregnancy and thus may be important to understanding the febrile process during gestation.

### *1.3.1 Arginine Vasopressin*

Arginine vasopressin (AVP) is produced in the supraoptic and paraventricular nuclei of the hypothalamus and stored within the posterior pituitary (214). It is released mostly in response to a diminishing free water content within the body (159). Although evidence from Cooper *et al.* indicates that peripheral concentrations may not play a role in antipyresis (43), there are reports of an antipyretic influence when it is localized within the brain. For instance, there are many AVP, $V_1$-type receptor sites in the brain. Of these sites, only the ventral septal area has been consistently identified with AVP-mediated antipyresis. Micro-injections of AVP into this area following endotoxin or $PGE_2$ results in significant decreases in core temperature response in the sheep (88), rat (161), rabbit (133), and guinea pig (210). AVP blockade by antagonism has also proved to induce antipyresis. Cooper *et al.* outlined their use of $d(CH_2)_5Tyr(Me)AVP$, a vasopressin $V_1$ receptor antagonist which was injected into ventral septal area 15 minutes before the administration of human purified IL-1 into a lateral cerebral ventricle (44). The result was a dose-dependent enhancement of the induced fever, an effect which was not noted to occur in the afebrile rat (44). AVP antibody perfusions into the ventral septal area, which would neutralize vasopressin activity, also greatly enhances fevers (114).

Haemorrhage, causes a massive systemic release of AVP to aid in fluid retention, but is also released in the brain and csf. Kasting *et al.* rapidly removed 20% of circulating blood and noted an attenuation in endotoxin-induced fever (90). Furthermore, major neural sources of AVP are said to reside in the paraventricular nucleus and bed

nucleus of the stria terminalis (211), where electrical stimulation of this area has been shown to reduce the hyperthermic severity of $_{I.V.}$ $PGE_1$ administration (133).

### *1.3.2 Interleukin-1 receptor antagonist*

Cytokines, such as IL-1β are deemed to be important systemic transmission elements of the febrile response. Apart from IL-1α, and IL-1β, another 40% structurally homologous protein is also secreted from blood monocytes and macrophages. First observed in the urine of fevered patients (108) and human volunteers injected with endotoxin (48), IL-1ra is a molecule with the effect of competing for the same binding sites on the IL-1 receptor as IL-1α, and IL-1β; and unlike these two forms, it does not render any apparent biological response. Occupation of the IL-1 receptor by IL-1ra diminishes the pyrogenic influence of circulating IL-1β, making it a potent antipyretic factor. *In vitro* evidence has shown that this protein has the ability to inhibit the biological activities of IL-1, including $PGE_2$ formation (7). *In vivo*, mice deficient of IL-1ra displayed an increased lethality to LPS administration; whereas over-expression of IL-1ra in these mice showed increased LPS survival from controls (78), a result which was also replicated in similar studies with rabbits (136) and rats (3).

### *1.3.3 Nitric Oxide*

Nitric Oxide (NO) is a molecular gas formed by three nitric oxide synthase (NOS) isoforms based on the cell types they were first derived from: neuronal NOS, endothelial

NOS, and inducible NOS (178). Most organ systems depend on NO as a paracrine messenger molecule, stimulating cGMP formation (20; 159), and notably enabling endothelium-derived relaxing factor to facilitate action as a potent vasodilator (159). Generally, neuronal and endothelial NOS are constitutively active. In contrast, the transcriptional attributes of inducible NOS can be dramatically enhanced, particularly by inflammatory cytokine expression leading to large quantities of NO (159). Furthermore, NOS containing neurons have been identified in the hypothalamic POA (20), leading to research that now provides evidence that neural NO could influence the neural aspects of thermoregulation, particularly as an antipyretic. For example, both the central (183) and peripheral (69) administration of NO donors evokes hypothermia. Whereas, hyperthermic responses in body temperature are noted in LPS injected (183), restrained (117), and exercising (103) rats after central administration of a NOS inhibitor.

### *1.3.4 Glucocorticoids*

Glucocorticoids are a class of endogenous steroid hormone with multiple physiological effects on cardiovascular, metabolic, immunological, and homeostatic functions (13). The principal active glucocorticoid produced endogenously is corticosterone in rodents, and cortisol in humans (5). Glucocorticoids impart their actions in most cells by binding to the cytosolic glucocorticoid receptor, translocating the activated complex to the cellular nucleus, bringing about the transactivation or transrepression of various genes (13).

Glucocorticoid release is the end product of negative feedback control with a neuroendocrine system involving the hypothalamus, pituitary, and adrenal glands, called the hypothalamic-pituitary-adrenal (HPA) axis. The processes in this system begin in the hypothalamus where corticotropin-releasing hormone (CRH), a factor produced by hypophysiotropic neurons of the paraventricular nucleus, is transported down through the hypothalamic-hypophyseal portal system to the anterior pituitary. CRH then acts on cells in the anterior pituitary called corticotropes which secrete stored adrenocorticotropic hormone (ACTH) into the circulation. ACTH travels via the bloodstream to the middle and inner layers of the adrenal cortex, the zonae fasciculata and zona reticularis, respectively, where it interacts with a specific G protein-coupled membrane bound receptor (41). This interaction activates cyclic AMP and stimulates steroidogenesis and the release of glucocorticoids into the circulation. Synthesized glucocorticoids exert their biological actions while circulating, but also provide hypothalamic feedback to dampen further ACTH production. Jacobson and Sapolsky indicate specific corticosteroid receptor sites in the hippocampus which are proposed to play important roles in the negative feedback inhibition exerted by glucocorticoids (84). Further, binding to the corticosteroid receptor in the pituitary could inhibit the expression of pro-opiomelanocortin in corticotropes.

Glucocorticoid synthesis by the activated HPA axis during an acute phase response has been commonly viewed as being engaged by the same mediating factors that produce fever. Cytokines such as TNF-α and IL-1β, are said to be potent ACTH secretagogues in the rat and mouse, acting via transcriptional stimulation of CRH producing neurons in the hypothalamic paraventricular nucleus (192). IL-6

administration brings about HPA activation, but at a slower rate when compared to the other cytokines (98). Lesions applied to the PVN in the rat greatly reduced this apparent cytokine-influenced activation of the HPA (98). Recent research by Quan *et al.* examined IL-1β mRNA expression in the brain and found that systemic LPS might induce central cytokine production, further activating the HPA axis (146). AVP has been thought to be significant in activation of the HPA axis. Yasin *et al.* evaluated the possibility of IL-1β and IL-6 directly activating AVP and oxytocin neurosecretory systems. They found that IL-1β produced a dose-dependent increase in the release of AVP and oxytocin, which further increased plasma ACTH (209). There are reports that neural injections of $PGE_2$ in the rat have led to increased circulation of ACTH and corticosterone (148); whereas COX inhibitors used to reduce $PGE_2$ production have been shown to reduce plasma ACTH secretion after TNF-α and IL-1β administration (91; 170). It is speculated that central and/or peripheral opioid receptors play a role in systemic IL-1β stimulation of the HPA axis. In rats, pretreatment with naloxone methiodide decreases IL-1β-induced fos-immunoreactivity of CRH neurons in the medial parvocellular paraventricular nucleus (29).

Glucocorticoids have been shown to suppress the febrile response to bacterial endotoxin which can be observed by the elevated LPS-induced fevers in rats after adrenalectomy (39), or glucocorticoid receptor blockade (120). Further, pretreatment with glucocorticoid type I or II receptor antagonists, have shown to elevate LPS-induced $PGE_2$ production within the brain (202). Glucocorticoid receptor blockade has also been successful in reversing the activation of the HPA axis due to psychological stress (35).

In terms of their antipyretic effects, glucocorticoids have shown to possess these attributes through various interactions initiated via binding to its cellular receptor, nuclear translocation, and binding to DNA components. Glucocorticoids engage an immunosuppressive mechanism which inhibits genes coding for inflammatory cytokine production (i.e., IL-1β, IL-6, and TNF-α), as well as COX-2 and $PLA_2$ expression (1; 38). Cellular transcription factor activity can also be reduced by glucocorticoid-receptor complex activation. The receptor complex interacts with transcription factors such as NF-κB, preventing its typical nuclear transactivation (1). Additionally, glucocorticoids induce the synthesis of lipocortin-1, a protein that suppresses eicosanoid synthesis (176). Lipocortin-1 readily binds to plasma membrane surface receptors and calcium, antagonizing the interaction of $PLA_2$ with phospholipids such as AA, perhaps by modulating the signalling pathways involved (176). Glucocorticoids also upregulate the synthesis of IL-1ra (1; 26), a cytokine which reduces the biological effects of IL-1β. Furthermore, glucocorticoids also increase IL-1r type II synthesis. The IL-1r type II varies from its type I counterpart in that it has no known signal transduction capability (26). This receptor variant has a greater affinity for IL-1β than IL-1α, or IL-1ra, and thus preferentially binds with IL-1β. This consequence prevents IL-1β from binding with the type I variant, capable of intracellular signal transmission (26).

## 1.4 Thermoregulation in Pregnancy

Pregnancy is a unique period in which a number of reversible physical changes occur within the maternal body to accommodate the demands of initiating, and sustaining the development of the fetus. During this time dramatic changes take place in the reproductive system, however other body systems are also altered to aid in fetal development. For instance, greater energy demands are met with a 30% increase in blood volume, and a 20% boost in respiratory activity to address elevated $O_2$ expenditure, and $CO_2$ removal. Also, increased metabolic demands by the fetus drive the mother to augment her nutritional requirements. In addition to these changes, pregnancy is a period in which variations occur in thermoregulatory mechanisms. Baseline decreases in maternal core temperature have been documented to occur as gestation proceeds in rats (92), sheep (102), rabbits (131), and humans (17). Although the initiating factor for this temperature decrease is currently unknown, it has been shown by Eliason and Fewell to be of a 'regulated' origin and thus normal physiology, as core temperature follows a decreased set-point (52). Fewell clearly showed this trend in rats monitored throughout gestation, finding that baseline core temperature is decreased from day 15, onward to parturition (57).

The reason for the maternal temperature decrease is unclear. Part of this thermoregulatory shift may involve the augmentation of hormonal expression associated with pregnancy. The secretion of the two main pregnancy hormones, estrogen, by way of the placenta, and progesterone, via the fetal adrenal cortex, increases steadily throughout gestation. However, near term, progesterone synthesis is reduced (140), while estrogen

levels surge (167). Progesterone is known to have thermogenic properties and its presence will consequently raise body temperature by 0.3 – 0.5°C (134). Thus, a reduction of progesterone can drop the maternal core temperature by this factor. Conversely, elevated estrogen can reduce core temperature (187). Furthermore, blood corticosterone, angiotensin II, luteinizing hormone, and prolactin levels all rise in near-term rats (10; 50; 87; 126).

A pregnancy-induced alteration of central prostaglandin receptor expression, or the antagonism of $PGE_2$ with its receptors may also factor in to decrease maternal core temperature. The binding of $PGE_2$ to the EP3 receptor on GABAergic neurons expressed in the POA is said to be an important step to engaging autonomic effectors that raise body temperature. A decrease in the expression of EP3 during pregnancy may consequently work to reduce hypothalamic set-point. However, Mouihate *et al.* recently reported that EP3 expression in the rat diencephalon does not change throughout gestation and lactation, nor does it change in males (127). On the other hand, Oka *et al.* has provided evidence that I.C.V. injections of the EP4 agonist ONO-AE1–329 into the rat brain, have brought about hypothermic responses (137). Thus it is possible that an increase in the expression of EP4 receptor might modulate the gestational decrease in core temperature to some degree.

## 1.5 Fever in Pregnancy

In 1978, Kasting, Veale, and Cooper reported that near-term, sheep fail to develop a fever in response to endotoxin and leukocyte pyrogen (89). At that time, there was no published information on the response of ewes to the effects of endotoxin near term. The group showed that while all non-pregnant control animals responded with fever following each $_{I.V.}$ injection of both the endotoxin *Salmonella abortus equi* (SAEP), and a granulocyte-derived endogenous pyrogen (EP), eight of the nine ewes tested failed to develop a fever to SAEP at days 1 and 2 prepartum, and three of seven of those ewes remained afebrile to EP challenge within 2 days of parturition (89). Since then, it has been shown in various laboratories that the febrile response to pyrogenic stimuli is altered late in gestation. Martin *et al.* demonstrated this effect further in the rat, showing that twenty-four hours before parturition, they also do not develop fevers to endotoxin (116).

Males and non pregnant females react to pyrogenic influence with elevated and sustained increases in their core temperatures as described in the preceding sections. In near-term guinea pigs (212), sheep (89), and rats (116), the febrile responsiveness to a physical stressor such as exogenous pyrogen (e.g., LPS) or endogenous pyrogen (e.g., IL-1ß) is attenuated. In many cases, attenuation of this response is first preceded by a hypothermic period (Figure 1-6). The exact mode of this attenuation is currently unknown, although some reports indicate that this response may be dissociated from the common LPS modes of febrigenesis (128). During pregnancy numerous maternal physiological elements can be altered by the developmental requirements of the fetus, and the attenuation in febrile responsiveness may be an important end result.

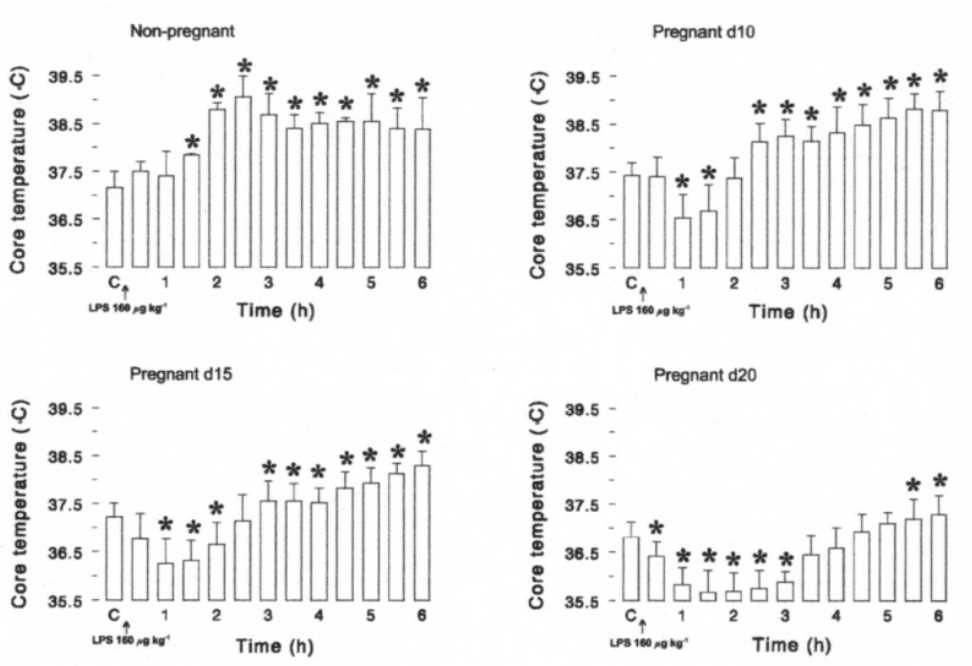


**Figure 1-8. Core temperatures measured before (C) and after (arrow) intraperitoneal administration of *E. coli* LPS (160 µg kg$^{-1}$) in non-pregnant and pregnant rats on days 10, 15, and 20 of gestation. From Fofie and Fewell (60).**

## 1.6 Pregnancy Induced Antipyresis

### *1.6.1 Arginine Vasopressin*

The role of AVP as a distinct mediator of the attenuated febrile response observed during late gestation is uncertain. Hypothalamic levels of AVP have been said to increase during late gestation. Landgraf *et al.* showed increases in AVP presence in both the ventral and mediolateral septal areas of the rat brain (104). Caldwell indicated significant increases in AVP expression in the supraoptic nucleus and PVN (30). In

contrast, systemic circulation of AVP has been noted to remain unchanged near term (174).

### *1.6.2 Interleukin-1 receptor antagonist*

IL-1ra may be an essential component to antipyresis in near-term females. The direct antagonistic actions of IL-1ra on the biological activities of IL-1β, one of the principle factors to febrigenesis, can have significant attenuating effects on the febrile response (6). Fofie and Fewell have indicated that levels of IL-1ra increase significantly on day 15 of gestation in pregnant rats as compared to circulating levels of the cytokine on days 10 and 20 in non-pregnant models (60). Although elevated on day 15 of pregnancy, concentrations of IL-1ra returned to baseline by day 20 (60). Thus, IL-1ra can play multiple gestation-dependant roles to near-term antipyresis in rats, as this cytokine could be essential to mechanisms influencing core temperature on day 15 of gestation; whereas the dominant modulating action may shift to another mechanism (e.g., increased glucocorticoid synthesis) by day 20. However, during LPS challenge late in pregnancy, it has also been documented that IL-1ra expression in rats is elevated; whereas IL-1β levels remain at baseline (61). This evidence makes a strong case for the IL-1ra as a mediator to the attenuated febrile response during late gestation.

### *1.6.3 Nitric Oxide*

Along with the ability for NO production to be enhanced by inflammatory cytokines under normal physiology, pregnancy seems to further extend the antipyretic effects of NO in near-term females. It is known that LPS-challenged near-terms have lower hypothalamic COX-2 and csf $PGE_2$ levels (80; 113; 127). NO has been shown to inhibit COX-2 mediated $PGE_2$ activity (124). Recent data have been published showing that neurons containing NO synthase have been identified in the POA hypothalamus (20), and that these neurons increase their production of NOS during the latter stages of pregnancy (205).

### *1.6.4 Opioids*

The CNS contains an analgesic system that suppresses pain by blocking the release of substance P via presynaptic inhibition (138). This activity inhibits ascending spinal cord transmission to higher brain levels for pain perception. The system depends on the opiate receptors, which facilitate the binding of endogenous ligands such as endorphins, enkephalins, and dynorphin. Further evidence has come to light supporting the significance of antipyresis by glucocorticoids, through the responsiveness of the HPA axis. Reports indicate that late in gestation there is a role for endogenous opioids in the attenuation of the HPA axis (27). As stated previously, the responsiveness of the HPA axis to psychogenic stressors is strongly attenuated in late pregnancy (27; 86). This attenuation has been said to be secondary to a central opioid mechanism that emerges

during pregnancy, with the NTS playing a vital role (27; 49). NTS neurons express proenkephalin-A mRNA, and the NTS architecture contains μ-opioid receptors which would support an autoinhibitory role of enkephalin on the NTS.

### *1.6.5 Glucocorticoids*

During the late stages of gestation in the rat, glucocorticoid concentrations and its production increase to significant levels (197). However, this increase in production does not follow the typical pathway of the HPA axis as glucocorticoid production is ramped markedly relative to concentrations of plasma CRH and ACTH. In a study by Atkinson and Waddell, plasma corticosterone and ACTH detection showed divergent paths as the common slope of this association increased considerably after mid-gestation, indicative of higher corticosterone levels for a given concentration of ACTH (10). Consequently, it has been postulated that an increase in adrenal gland sensitivity is likely occurring during this period. As previously stated, ACTH interacts with a specific membrane receptor in the adrenal cortex, thus perhaps a pregnancy-induced increase in receptor presence is providing this increased ACTH sensitivity. In addition to the increased responsiveness of the adrenal gland, an elevation of pregnancy hormones may also supply a catalyst action to glucocorticoid creation. The hormone estradiol significantly increases near parturition (167). Its presence has been noted to potentiate the effects of ACTH on corticosterone synthesis within the adrenal cortex (40). Furthermore, observing the glucocorticoid biosynthesis pathway, progesterone is an early intermediate that is vital to corticosterone

synthesis. Progesterone levels continually increase throughout the course of pregnancy and thus could provide the necessary precursors for increased corticosterone production.

In terms of their effects on fever during pregnancy, glucocorticoids are viewed to have very distinct antipyretic characteristics (188). Glucocorticoids have an immunosuppressive mechanism (111) that inhibits a host of genes coding for cytokine production, including two important pyrogens, IL-1β and IL-6. Further, as an anti-inflammatory effect, glucocorticoids induce the synthesis of lipocortin-1 (111; 176), which can readily bind to cell walls and calcium, preventing the interaction of phospholipase A2 with AA. Moreover, both isoforms of the COX enzyme are also suppressed (213). All of this translates into a reduction in PGE, which has already been noted as a key component to the neurological portion of the febrile signal transduction process. Glucocorticoids synthesis suppresses the febrile response to endotoxin in many ways. Elevated LPS-induced fevers occur in rats after adrenalectomy (ADX) or glucocorticoid receptor blockade (39; 120). Pretreatment with glucocorticoid type I or II receptor antagonists also elevate LPS-induced $PGE_2$ production within the brain (202). The binding of the glucocorticoid to its soluble receptor initiates a translocation into the nucleus and the subsequent downregulation of the transcription of pyrogenic cytokines such as IL-1, IL-6, and TNF-α, and the upregulation of antagonistic substances including lipocortin-1, and IL-1ra (1).

Utilizing the information from the current body of knowledge on fever and its mechanisms, in conjunction with the recently discovered attributes of the glucocorticoids as a very potent antipyretic, the stage has been set for a very intriguing study which may unravel a key element to the near-term febrile response to bacterial endotoxin.

## 1.7 Specific Aims & Hypothesis

The purpose of this study was to explore the role of glucocorticoids on the known attenuated febrile response to exogenous pyrogen in near-term pregnant female rats. Fever is the distinguishing characteristic of the acute phase response under normal physiology. It was speculated that elevated basal concentrations of glucocorticoids, typical during the latter stages of pregnancy, are a key modulator of the attenuated febrile response. Thus, the inhibition of endogenous glucocorticoid production would result in a restoration of the febrile response similar to what is seen in the non-pregnant female.

Metyrapone (MET) or 2-methyl-1, 2-di-3-pyridyl-1-propanone is a glucocorticoid inhibiting drug. The main study would specifically test the hypotheses that, (i) the administration of MET would cause the complete inhibition of corticosterone synthesis in near-term female rats; (ii) glucocorticoid inhibition by MET administration in near-term female rats would not produce a significant alteration in their baseline core temperature; (iii) the pretreatment of near-term female rats with MET would facilitate significant increases in core temperature during the resultant febrile response elicited by exposure to a high-dose of exogenous pyrogen; and (iv) MET pretreatment in near-term female rats will cause an alteration in the plasma concentrations of specific pyrogenic/antipyretic cytokines following a high-dose challenge with an exogenous pyrogen.

*Chapter Two:*

# *MATERIALS AND METHODS*

## 2.1 Housing

Experiments were carried out on non-pregnant (260.1 ± 16.4 g), and time-mated pregnant female Sprague-Dawley rats (319.0 ± 22.2 g) (Charles River Laboratories). Upon arrival to the laboratory, each animal was weighed and transferred to a plexiglass cage where it was individually housed. The cage was lined with Aspen-Chip Laboratory Bedding (Northeastern Products) and placed inside an environmental chamber where temperature was maintained at 25.0 ± 1.0°C and humidity kept at 40%. The chamber was set on a 12H:12H light/dark cycle with lights on at 7 AM. Food (Lab Diet 5001) and water (standard tap water) was available to each animal *ad libitum*. All procedures were carried out in accordance with ethical codes provided by the Canadian Council on

Animal Care, with the approval of the Animal Care Committee of the University of Calgary.

**2.2 Surgical Procedure**

Each animal was anesthetized, and implanted with a battery-operated telemetry device for abdominal temperature measurements (TA-F40; Data Sciences International), and a catheter made from silicon tubing (Dow Corning Silastic; Helix medical) for intraperitoneal injections.

For anesthesia, each animal was placed within a cylindrical chamber (Kent Scientific Corporation) containing 2% halothane in oxygen. Following anesthesia induction, the animal was switched over to a breathing circuit atop the surgical platform and maintained in a surgical plane of anesthesia with 1.5 – 2% halothane in oxygen and adjusted relative to the noted signs of depth of anesthesia as the procedure progressed. The animal's skin was then shaved and cleaned with betadine and 70% ethanol solutions prior to the first incision. An initial incision was made into the cutaneous layer, over the mid to lower abdomen, followed by blunt dissection to lift the skin from the subcutaneous layers. The catheter was then tunneled from the incision, to an area between the inter-scapular region, where a second small incision was made, and the catheter externalized. The animal was then placed in the supine position, and a laparotomy was preformed along the linea alba of the mid/lower abdomen. The internal end of the catheter was then secured to the muscular wall of the abdomen with a suture leaving about 2-3 cm of the end of the catheter pushed into the abdomen and allowed to

settle freely. Next, an antibiotic ointment (Polysporin) was applied to the telemetry device to aid in insertion, and the device was pushed inside the abdomen to float freely. The abdominal wall was then closed using non-absorbable, multi-filament surgical silk (Ethicon, Johnson & Johnson) sutures. A topical spray (Gentocin) was applied to the sutured wound. Use of the same suturing technique closed the cutaneous layer and a spray bandage (Opsite) was applied to this area. Next, the animal was turned over into the prone position to secure the catheter. A small 3 millimetre clipping of silicone tubing of same diameter as the catheter was stretched over the externalized portion of the catheter to act as a bead to which the suture thread could be anchored. The catheter was then secured to the skin on the upper scapula area of the animal with a suture at the bead. A small volume of saline was then injected into the catheter to ensure it has not accidentally become occluded along its length during the procedures. After this, the open end of the exteriorized catheter was closed off with a knot in the catheter about 5 cm from the tip. The anesthetic was turned off, and the animal was allowed to breathe oxygen until adequate signs of recovery were noted. The subject was then placed into a clean cage, returned to the chamber, and laid on a piece of paper towel to recover.

## 2.3 Experimental Protocol

### *2.3.1 Preliminary Experiments*

#### 2.3.1.1 Metyrapone Solubility Tests

Metopirone (aka. Metyrapone (MET), Sigma-Aldrich, St Louis, MO) is a glucocorticoid inhibiting drug (164). Given that MET is sparingly soluble in water and physiological solutions, a series of tests were carried out to determine a method to increase its solubility in physiological solutions. For the task, both a polyethylene glycol solution (average molecular weight 400, Sigma-Aldrich, St Louis, MO), and (2-Hydroxypropyl)-β-cyclodextrin solution (45% w/v in water, Sigma-Aldrich, St Louis, MO) were deemed to be possible effective solvents according to the literature details. The ability for each solvent to disperse and dissolve MET at 35, 75, 100, and 200 mg/mL was tested. All testing with the cyclodextrin solution was completed in its full concentration; however, PEG effectiveness was tested in various ratios of dilution ranging from 100% down to 5% PEG, with the remaining volume composed of saline.

On the day of experimentation, the required dry weight of MET to be tested was weighed using a scale and transferred to a 5 mL beaker. Next, depending on which solvent was being tested, either 0.33 mL of saline, cyclodextrin solution or PEG was added to the beaker containing the MET. The beaker containing the solute and solvent was then placed on a hot plate set to approximately 37.0 ºC to replicate core temperature, and a sterile magnetic stirring stick was dropped into the beaker to rapidly circulate the

substances to aid in dissolution. For each trial, the time at which the solvent was added to MET was recorded and qualitative observations of the homogeneity, fluidity, clarity, and overall assessment of the potential for the solution to be injected without compromising its concentration was observed (Tables 2-1 to 2-6).

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| Saline | 50 | 100 | 1.0 | Suspension of MET bubbles within saline | No |
| Saline | 100 | 100 | 1.0 | Suspension of MET bubbles within saline | No |

**Table 2-1: Qualitative assessment of the ability of 1 mL of 0.9% sterile saline to incorporate 50 and 100 $mg \cdot mL^{-1}$ Metyrapone into solution.**

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| Cyclodextrin | 35 | 100 | 0.33 | No suspensions; clear, fluid liquid | Yes |
| Cyclodextrin | 75 | 100 | 0.33 | No suspensions; clear, fluid liquid; slight yellow tint | Yes |
| Cyclodextrin | 100 | 100 | 0.33 | No suspensions; clear liquid; viscous; deeper yellow tint | Yes |
| Cyclodextrin | 200 | 100 | 0.33 | Suspensions; oil/water appearance; viscous; deep yellow tint | No |

**Table 2-2: Qualitative assessment of 0.33 mL, Cyclodextrin solution's ability to incorporate 35, 75, 100, and 200 $mg \cdot mL^{-1}$ Metyrapone into solution. Shaded area denotes the highest concentration of MET solution deemed adequate for use.**

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| PEG | 35 | 5 | 0.33 | Oily appearance; film residue | No |
| PEG | 35 | 10 | 0.33 | Slight yellow tint; clear liquid | Yes |
| PEG | 35 | 15 | 0.33 | Slight yellow tint, clear liquid | Yes |
| PEG | 35 | 40 | 0.33 | Very clear liquid | Yes |

**Table 2-3: Qualitative assessment of 5, 10, 15, and 40%, 0.33 mL Polyethylene Glycol's ability to incorporate 35 $mg \cdot mL^{-1}$ Metyrapone into solution. Shaded area denotes lowest possible percentage of solvent for viable use.**

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| PEG | 75 | 10 | 0.33 | Cloudy appearance; suspensions | No |
| PEG | 75 | 15 | 0.33 | Slight yellow tint; clear liquid | Yes |
| PEG | 75 | 40 | 0.33 | Very clear liquid | Yes |

**Table 2-4: Qualitative assessment of 10, 15, and 40%, 0.33 mL Polyethylene Glycol's ability to incorporate 75 $mg \cdot mL^{-1}$ Metyrapone into solution. Shaded area denotes lowest possible percentage of solvent for viable use.**

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| PEG | 100 | 15 | 0.33 | Cloudy; oil-water appearance | No |
| PEG | 100 | 20 | 0.33 | Slight yellow tint; clear liquid | Yes |
| PEG | 100 | 40 | 0.33 | Slight yellow tint, clear liquid | Yes |
| PEG | 100 | 100 | 0.33 | Clear liquid; solids not dissolved | No |

**Table 2-5: Qualitative assessment of 15, 20, 40, and 100%, 0.33 mL Polyethylene Glycol's ability to incorporate 100 $mg \cdot mL^{-1}$ Metyrapone into solution. Shaded area denotes lowest possible percentage of solvent for viable use.**

| *Solvent* | *MET Concentration ($mg \cdot mL^{-1}$)* | *Percent Solvent (%)* | *Volume (mL)* | *Result* | **Effective?** |
|---|---|---|---|---|---|
| PEG | 200 | 20 | 0.33 | Cloudy; suspensions | No |
| PEG | 200 | 40 | 0.33 | Yellow tint; clear liquid | Yes |

**Table 2-6: Qualitative assessment of 20, and 40%, 0.33 mL Polyethylene Glycol's ability to incorporate 200 $mg \cdot mL^{-1}$ Metyrapone into solution. Shaded area denotes lowest possible percentage of solvent for viable use.**

2.3.1.2 Corticosterone Inhibition via Metyrapone in Non-Pregnant Rats

The ability of metyrapone to inhibit endogenous glucocorticoids was then assessed. Experiments from our laboratory have determined that 160 µg/kg LPS from Escherichia Coli (Sigma-Aldrich, St Louis, MO) corresponds to the $EC_{100}$ dose, or the lowest dose producing a maximal core temperature response (60). Tests were conducted in non-pregnant female rats pre-treated with metyrapone and then challenged with an $EC_{100}$ dose of LPS to stimulate a maximal febrile response. The level of corticosterone expressed following the treatment was then compared to the expression of the hormone in animals that were similarly challenged without metyrapone pretreatment.

Thirty-two non-pregnant rats were randomly assigned to one of four experimental treatment groups so that each subject received only one treatment consisting of two separate intra-peritoneal injections. The treatments were divided into the following injection combinations: *Vehicle and Vehicle*, *Vehicle and LPS*, *MET and Vehicle*, or *MET and LPS*. The doses of Metyrapone used in this study were 12.5, 25, 50, 100

mg/kg. The dose of LPS that was administered was160 µg/kg, while the vehicle used was a solution of 40% PEG and 60% sterile saline. 40% PEG was chosen as the vehicle as it was deemed to be the best solvent to dissolve MET into solution as determined by the results of preliminary testing (section 3.1.1). On a given experimental week, eight animals were sub-divided into the four experimental groups so that each group contained two animals. Surgery occurred three days following the eight animals' arrival into the environmental chamber (corresponds with day fifteen of gestation in pregnant models); the animals were given a five-day recovery period within the environmental chamber. All animals were studied once, eight days after their arrival into the lab (corresponding with day twenty of gestation in pregnant models). On that day, the experiment proceeded with inter-peritoneal substance injections, followed by plasma collection from one animal in each experimental group.

Sixty minutes following the second ($T_2$) injection of 12.5, 25, 50, or100 mg/kg (see section 2.4.3 *Conditions of Injection*), the trunk blood of one animal in each experimental group was collected by decapitation. There are few data published in the literature on the pharmacokinetics of metyrapone; however details from one reputable source identify the half-life of the drug as approximately 1.9 hours (185). Thus, 2 hours following the metyrapone injection ($T_1$) was determined to be a reasonable time-point to assess the drug's effect on plasma corticosterone levels.

### *2.3.2 Main Study*

One hundred and twenty-eight pregnant rats were randomly assigned to one of four experimental treatment groups so that each subject received only one treatment consisting of two separate intra-peritoneal injections. The treatments were divided into the following injection combinations: *Vehicle and Vehicle*, *Vehicle and LPS*, *MET and Vehicle*, or *MET and LPS*. The doses of Metyrapone and LPS used in this study were 50 mg/kg and160 µg/kg respectively, and the vehicle used was a solution of 40% PEG and 60% sterile saline. The Metyrapone dose represents the dose which effectively inhibited glucocorticoid synthesis in non-pregnant animals as determined by the results of preliminary testing (section 3.1.2). The dose of LPS used is in accordance with previous dose-response data gathered and published by our lab detailing it as the $EC_{100}$ dose, or the lowest possible dose that produces a maximal core temperature response in Sprague Dawley rats. Forty percent polyethylene glycol was chosen as the vehicle as it was the solvent deemed to adequately dissolve metyrapone into solution as determined by the results of preliminary testing (section 3.1.1). On a given experimental week, eight animals would be ordered and transported into the lab for experimentation. These eight animals were then sub-divided into the four experimental groups so that each group contained two animals. One animal from each experimental group would participate in the *Temperature Assessment* series of experiments, and one animal would participate in the *Plasma Assessment* series of experiments. All animals were studied once on day twenty of gestation, with twenty-one days being the typical gestational period for Sprague Dawley rats.

2.3.2.1 Temperature Assessment

Following the second ($T_2$) injection (see section 2.4.3 *Conditions of Injection*), the core temperature of one member of each experimental group was monitored for 24 hours. During this time period, core temperature was recorded by the implanted telemetry device/PC at each two minute time interval to observe core temperature fluctuations in each animal as varied febrile responses occurred due to administered substances.

2.3.2.2 Plasma Assessment

Following the second ($T_2$) injection (see section 2.4.3 *Conditions of Injection*), the trunk blood of one member of each experimental group was collected by rapid decapitation (see section 2.4.4 *Conditions of Plasma Analysis*). Plasma was collected from the subject at one of three time points (Min 120, 180, 300). The resultant plasma was later analyzed for the presence and concentration of corticosterone, TNF-α, IL-1β, and IL-6 which are antipyretics/pyrogens known as important mediators of the febrile mechanism. The collection of plasma at various points along the experimental timeline provided extensive glucocorticoid/cytokine data for further interpretation.

## 2.4 Experimental Procedures

### *2.4.1 Handling*

From the day of arrival, each animal was handled by the same investigator, every second day, for the course of the experimental period. On the day of handling, the animal's cage was removed from the chamber and placed on a counter. The cage lid was opened and the investigator's hand slowly inserted to give the animal time to become accustomed to the scent. Next, a blue drape with a medium-sized hole cut into the center was prepared to wrap the animal with. The hole was positioned in a manner that it fell over the scapula area where later an implanted catheter was exteriorized. The animal was then wrapped with moderate firmness within the drape and held in place for about twenty seconds to simulate the exact same technique which was employed when the injections occurred.

### *2.4.2 Conditions of Observation*

All animals were housed and observed from their respective plexiglass cage within the environmental chamber. Each animal's core temperature was monitored via the implanted biotelemetry device transmitting to a platform antenna (Physio Tel CTR 86 Receiver; Data Sciences International) which the cage was placed atop. Radio transmissions were sampled for 10 seconds every two minutes from each animal. The sample was then sent by the receiver to a PC located outside of the chamber and

averaged. The resultant core temperature was displayed to the monitor, and saved to the hard drive (Figure 2-1).

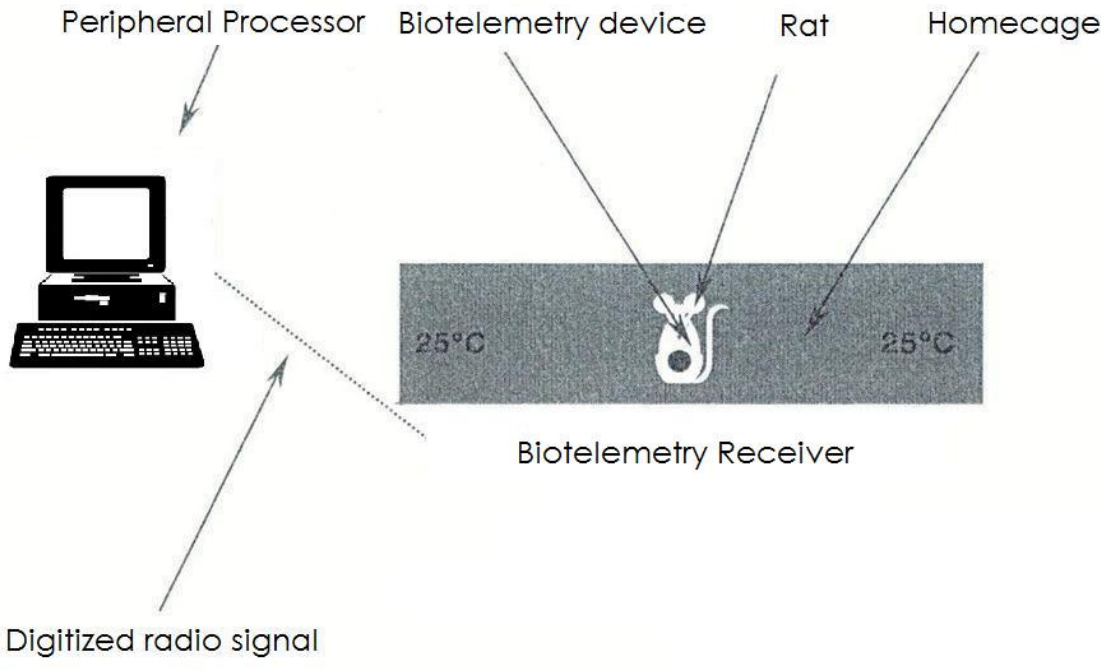


**Figure 2-1: Diagram showing the experimental set-up for core temperature transmission and recording from chronically instrumented rat inside its cage. Adapted from Fofie (59).**

### *2.4.3 Conditions of Injection*

All injections began at 11 AM to allow for circadian rhythm considerations. Weights were obtained on the day before experimentation by removing each animal temporarily from its cage and placing it atop a digital scale. On injection day, the chamber was left undisturbed without human intrusion to minimize undue stress to the testing group. Eight animals were paired into four separate groups (Group 1-4)

depending on the type of injections they were to receive (Table 2-7). The two animals constituting group 1 received two separate injections of 1 mL/kg 0.9% sterile saline as vehicle allotments. The two animals in group 2 received one vehicle injection, followed by an injection of LPS. The two group 3 subjects received an initial injection of Metyrapone, followed by a vehicle injection. In the final group, the two animals of group 4 received an injection of Metyrapone, followed by an injection of LPS.

| *Treatment Group* | | *T1 Injection* | *T2 Injection* |
|---|---|---|---|
| 1 | Animal 1<br>Animal 2 | Vehicle (40% PEG) | Vehicle (40% PEG) |
| 2 | Animal 3<br>Animal 4 | Vehicle (40% PEG) | LPS (160 µg/kg) |
| 3 | Animal 5<br>Animal 6 | MET (50mg/kg) | Vehicle (40% PEG) |
| 4 | Animal 7<br>Animal 8 | MET (50mg/kg) | LPS (160 µg/kg) |

**Table 2-7: Table showing randomly assigned treatment groups for eight subjects during the week of an experiment. Each group contained two animals that received the same injection regime.**

Beginning at 11AM, the first animal was removed from the environmental chamber and brought in its cage to a central table for the first ($T_1$) of two substance injections. The lid of the cage was removed and the animal was handled with a blue drape in the exact manner as all previous handling sessions, with the externalized catheter portion protruding from the hole in the centre of the drape. A prepared injection device was removed from a water-bath which was used to pre-heat the injectate to typical core temperature (37.0°C). The injection device was constructed using a 21 gauge, 1" blunt needle (Monoject) attached to a sterilized 3-way stopcock. Connected to the stopcock

were two 1 mL syringes. One syringe contained the prescribed injectate indicated for the particular treatment group and the other syringe contained 0.5 mL of sterile 0.9% saline. For delivery, the knotted end in the catheter was quickly snipped off and the blunt needle inserted into the open catheter. The injectate was slowly introduced, followed by the volume of saline from the second syringe to ensure the desired substance was flushed completely through the catheter, and into the peritoneal cavity. After completion of the injection, the animal was placed back in its cage and returned to the environmental chamber.

One hour later, the second injection ($T_2$) occurred. The injection process was repeated in the exact manner of the $T_1$ injection, however, now each animal received either vehicle or LPS (Table 2-7). After completion of the injection, the animal was again placed back in its cage, and returned to the environmental chamber (Figure 2-2 (A)).

### *2.4.4 Conditions of Plasma Analysis*

The trunk blood of half of the animals experimented on was collected at certain instances following the second ($T_2$) injection (Figure 2-2 (B)). At the appropriate time, each animal was removed from the environmental chamber and brought to a central table. The animal was removed from the cage, and the trunk blood was collected by rapid decapitation by way of guillotine, into a 15 mL heparinized plastic centrifuge tube set on ice. The blood sample within the centrifuge tube was then further cooled on ice for fifteen minutes, and subsequently spun on high speed in a centrifuge for fifteen minutes. The resultant supernatant was then extracted via pipette into a smaller specimen vial and

stored at – 72ºC until glucocorticoid/cytokine assays occurred on a later date. All plasma samples were shipped together on dry ice to our lab's collaborating partner Donna Farley, at the University of Iowa to be analyzed. Serum corticosterone was measured using a Coat-A-Count Rat Corticosterone solid-phase radioimmunoassay (RIA) kit from Siemens Medical Solutions Diagnostics (Los Angeles, CA). Sensitivity of the corticosterone RIA was 5.7 ng/mL and the intra- and inter-assay coefficients of variation averaged 8% and 10%, respectively. Serum IL-1β was measured using a quantitative sandwich enzyme immunoassay (ELISA) technique employing a polyclonal antibody specific for rat IL-1β (Quantikine Rat IL-1β/IL-1F2, R&D Systems, Minneapolis, MN). Sensitivity of the method was typically 15 pg/mL and the intra- and inter-assay coefficients of variation averaged 6.4% and 4.9%, respectively. Serum IL-6 was measured using a quantitative sandwich enzyme immunoassay (ELISA) technique employing a monoclonal antibody specific for rat IL-6 (Quantikine Rat IL-6, R&D Systems, Minneapolis, MN). Sensitivity of the method was typically 21 pg/mL and the intra- and inter-assay coefficients of variation averaged 6.7% and 7.6%, respectively. Serum TNF-α was measured using a quantitative sandwich enzyme immunoassay (ELISA) technique employing a monoclonal antibody specific for rat TNF-α (Quantikine Rat TNF-α/TNFSF1A, R&D Systems, Minneapolis, MN). Sensitivity of the method was typically 5 pg/mL and the intra- and inter-assay coefficients of variation averaged 3.1% and 9.4%, respectively.

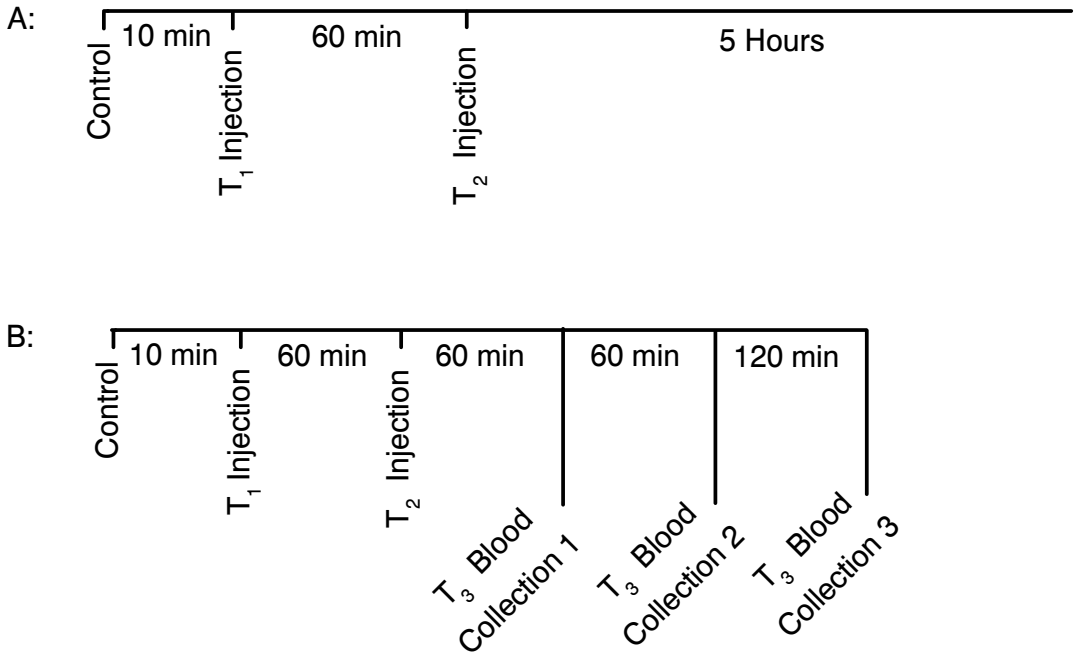


**Figure 2-2: Timeline for experimental protocols. Animals used in the Temperature Assessment followed timeline A. A control period was established, followed by two substance injections. Animals used in the Plasma Assessment followed timeline B with a control period, two substance injections, and trunk blood collection occurring at one of the three $T_3$ blood collection points.**

## 2.5 Statistical Analysis

Data were analyzed statistically using Sigma Stat (Version 3.5). Significance was deemed acceptable at a confidence level of 95% for all measures. Data are expressed as means ± standard deviation.

### *2.5.1 Main Study –Temperature Assessment*

Core temperature data in near-term pregnant females were statistically analyzed by a two-factor analysis of variance (ANOVA) with repeated measures over time. The factors used for the analysis were Treatment Group (*Vehicle and Vehicle*, *Vehicle and LPS*, *MET and Vehicle*, or *MET and LPS*) and Time. Significance was then evaluated and identified with a Student Newman-Keuls post-hoc analysis for multiple comparisons.

### *2.5.2 Main Study – Plasma Assessment*

Data obtained from the *Plasma Assessment* series of experiments was statistically analyzed by a two factor analysis of variance (ANOVA). The factors used for this analysis were Treatment Group (*Vehicle and Vehicle*, *Vehicle and LPS*, *MET and Vehicle*, or *MET and LPS*) and Time. Once again, any significance was evaluated with a Student Newman-Keuls post-hoc analysis for multiple comparisons.

# *Chapter Three:*

# *RESULTS*

## 3.1 Preliminary Experiments

### *3.1.1 Metyrapone Solubility Tests*

The ability of three different solvents (saline, cyclodextrin, and polyethylene glycol) to dissolve various concentrations of metyrapone was tested (refer to Tables 2-1 to 2-6). Based upon assessment of homogeneity, fluidity, clarity, and overall potential for the solution to be injected without compromising the concentration of metyrapone transferred from the body of syringe, through the needle, down the length of the catheter, and into the peritoneum, cyclodextrin and PEG but not saline were deemed to be appropriate solvents. Forty percent PEG in sixty percent saline was used as the solvent for metyrapone in the Main Study because of its known neutral physiological effects on

the body, and that it is already an inactive component of MET. Furthermore, Roozendaal *et al*. successfully dissolved metyrapone in forty percent PEG and sixty percent saline (158).

### *3.1.2 Corticosterone Inhibition via Metyrapone in Non-Pregnant Rats*

A dose-response assessment of MET's potential to inhibit endogenous glucocorticoids following LPS challenge was conducted prior to its use in the main study. Preliminary tests were performed on non-pregnant female rats using an LPS $EC_{100}$ dose in conjunction with various doses of MET to compare the resulting corticosterone levels with that of a Vehicle-LPS stimulated group. Basal concentration of corticosterone in the Vehicle-Vehicle group was 112 ± 33 $ng \cdot mL^{-1}$ (mean ± SD; n=4). Vehicle-LPS administration brought about a significant increase in plasma corticosterone to 639 ± 33 $ng \cdot mL^{-1}$ (mean ± SD; n=4; Figure 3.1). MET administration had a dramatic influence on the circulating levels of corticosterone following LPS, with each dose reducing the relative concentration of the hormone by at least half of that observed in the Vehicle-LPS group. 50 $mg \cdot kg^{-1}$ metyrapone appeared to be the lowest dose that resulted in maximal inhibition of corticosterone levels following LPS. Thus, 50 $mg \cdot kg^{-1}$was used for experimentation in the main study.

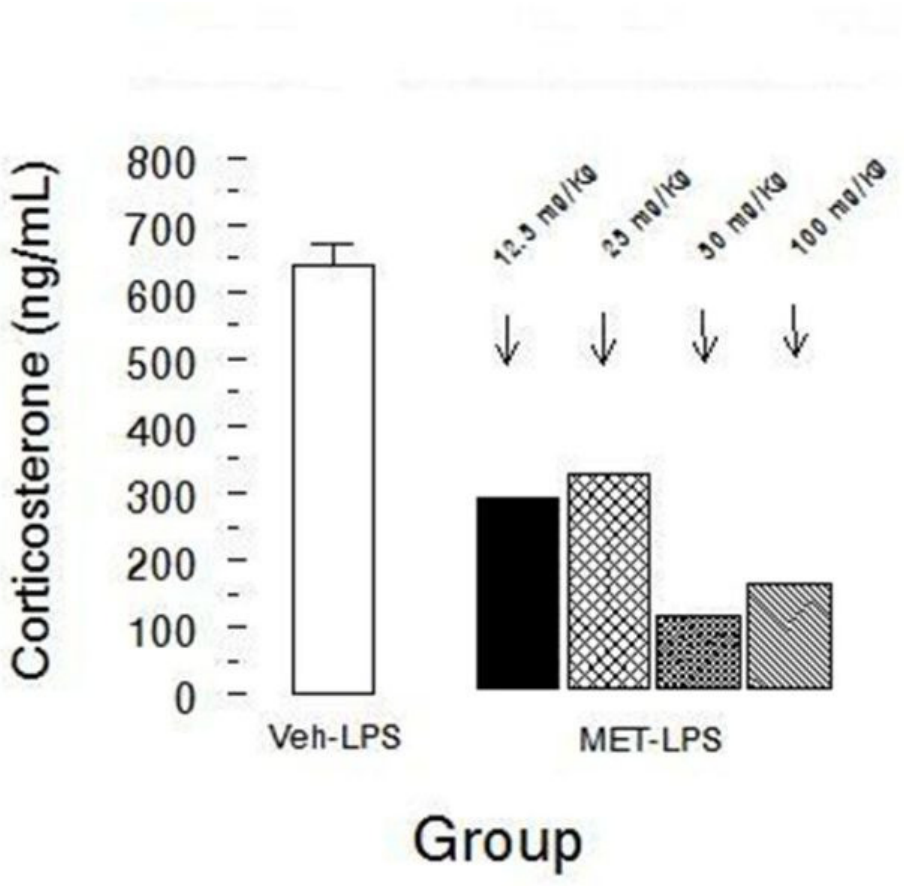


**Figure 3-1: Plasma corticosterone responses in non-pregnant female SD rats. Responses are shown for animals who received an intraperitoneal pretreatment with either Vehicle (40% PEG) or Metyrapone (12.5, 25, 50, 100 mg·kg$^{-1}$), 60 minutes prior to an *E. coli* LPS (160 µg·kg$^{-1}$) $_{I.P.}$ administration. Data for Veh-LPS are presented as means ± SD (n=4). Data for MET-LPS are n=1 per MET concentration.**

## 3.2 Main Study

### *3.2.1 Corticosterone Inhibition via Metyrapone in Near-Term Rats*

The ability of 50 $mg \cdot kg^{-1}$ MET to inhibit endogenous glucocorticoid synthesis in near-term rats following a high-dose LPS challenge was assessed. Figure 3-2 shows the expression of corticosterone in near-term pregnant rats at one, two, and four hours following the $_{I.P.}$ administration of either *Vehicle - Vehicle*, *Vehicle - LPS*, *MET - Vehicle*, or *MET - LPS*.

Basal measurements of corticosterone were 167 ± 19, 215 ± 35, and 402 ± 47 $ng \cdot mL^{-1}$ at one, two, and four hours, respectively, in rats treated with Vehicle-Vehicle. A significant increase in corticosterone expression is noted following Vehicle-LPS treatment at all time points. This elevation, is dramatically attenuated when pretreatment with MET occurs before LPS administration (Figure 3-2).

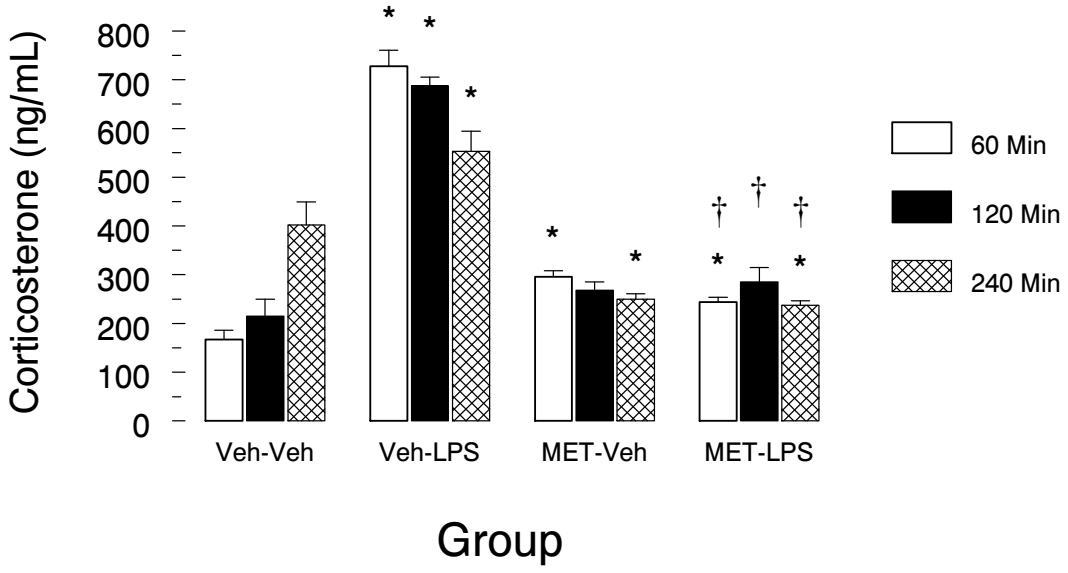


**Figure 3-2: Plasma corticosterone responses in female Sprague Dawley rats on day 20 of gestation. Animals received an intraperitoneal pretreatment injection with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to receiving an $_{I.P.}$ injection of either Vehicle or *E. coli* LPS (160 µg·kg$^{-1}$). The chart shows the four combinations of treatment groups and depicts corticosterone responses in each group at 60, 120, and 240 minutes following the second injection. Data are presented as means ± SD (n=8). * P <0.05 *versus* Veh-Veh at the same instant. † P <0.05 *versus* Veh-LPS at the same instant.**

### *3.2.2 Effect of Metyrapone Administration on Baseline Temperature*

To determine the effect of 50 $mg \cdot Kg^{-1}$ MET administration on baseline temperature in near-term pregnant rats, the change in core temperature from baseline (C), following the $_{I.P.}$ administration of either *Vehicle - Vehicle*, *Vehicle - LPS*, *MET - Vehicle*, or *MET - LPS* was assessed.

Figure 3-3 shows that there were no lasting, significant changes from control measurements of core temperature in animals who received either MET or vehicle followed by vehicle. Baseline control core temperature in the Veh-Veh group was 36.7 ± 0.1 (n=7); in the MET-Veh group, this property was 36.8 ± 0.1 (n=8). There were no significant differences in core temperature between these two groups at any time.

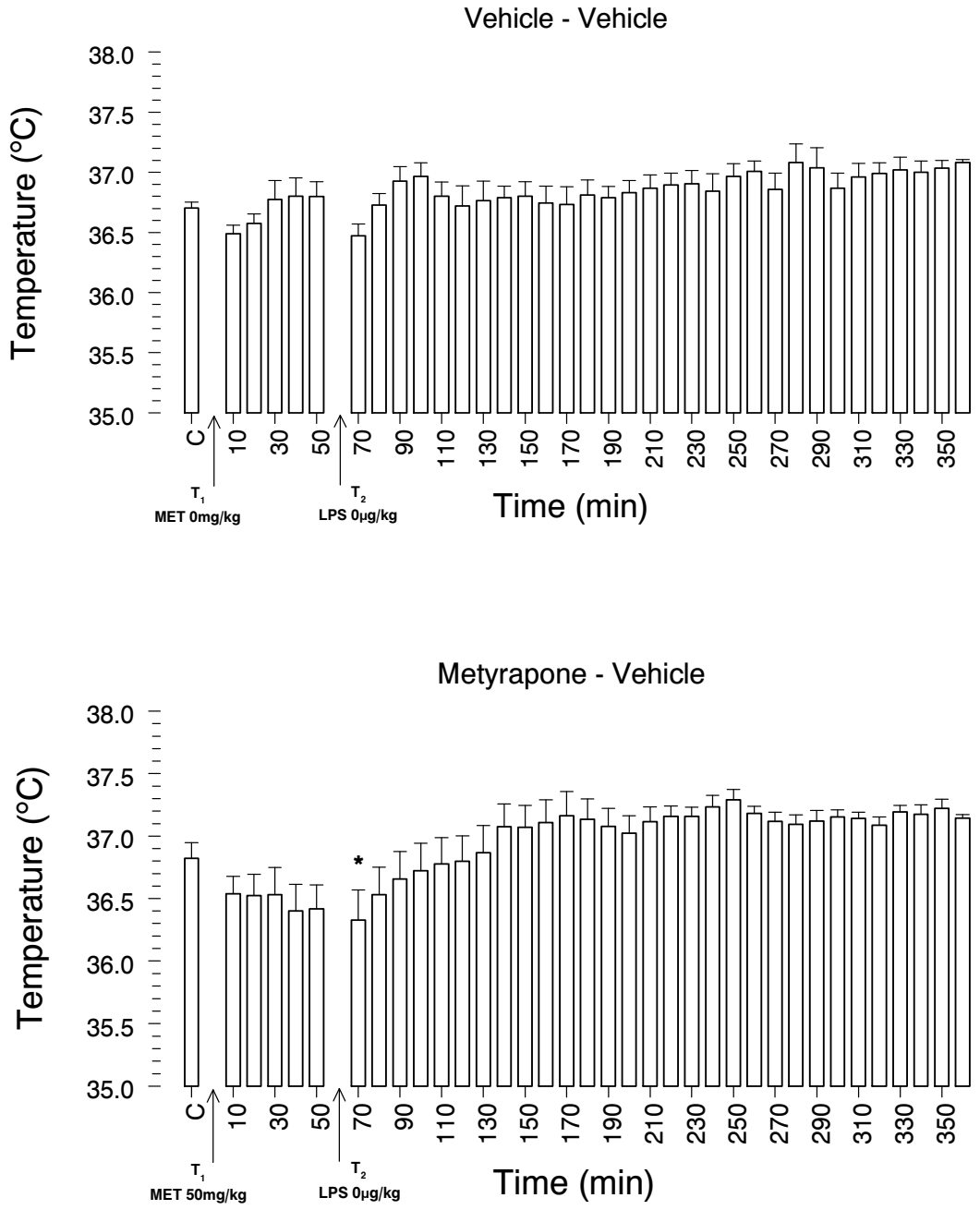


**Figure 3-3: Core temperature responses in pregnant female Sprague Dawley rats on day 20 of gestation. Responses are shown for animals who received an $_{I.P.}$ pretreatment with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to a Vehicle (40% PEG) $_{I.P.}$ administration. Data are presented as means ± SD (Vehicle-Vehicle, n=7; Metyrapone-Vehicle, n=8). * P <0.05 *versus* Control (C).**

### *3.2.3 Effect of Metyrapone Administration on the Febrile Response to LPS*

The effects of the pretreatment of near-term female rats with MET (50 $mg \cdot kg^{-1}$) on the febrile response elicited by exposure to a high-dose (160 $\mu g \cdot kg^{-1}$) of the exogenous pyrogen LPS was assessed. Figures 3-4, 3-5, and 3-6 show core temperature changes, from baseline (C), in near-term pregnant rats following an $_{I.P.}$ administration of either *Vehicle - Vehicle*, *Vehicle - LPS*, *MET - Vehicle*, or *MET - LPS*.

Core temperature responses of animals who received Vehicle followed by Vehicle or LPS were also observed. Control core temperature in the Veh-Veh and Veh-LPS groups were 36.7 ± 0.1 (n=7) and 36.9 ± 0.0 (n=7), respectively. Administration of LPS following Vehicle resulted in a significant decrease in core temperature with a latency, duration and magnitude of 90 minutes, 70 minutes, and -0.8°C, respectively. No significant changes in core temperature were observed in animals that received Vehicle following Vehicle (Figure 3-4).

Core temperature responses of animals who received MET followed by Vehicle or LPS are shown in figure 3-5. Control core temperature in the MET-Veh group was 36.8 ± 0.1 (n=8); and it was 36.8 ± 0.1 (n=5) in the MET-LPS group. Administration of LPS following MET resulted in a significant increase in core temperature with a latency, during and magnitude of 290 minutes, at least 70 minutes, and 1.1°C, respectively.

Figure 3-6 shows and allows comparisons of core temperature responses in animals pre-treated with either Vehicle or MET followed by LPS. It is evident that the hypothermic period noted in the Vehicle-LPS group has been abolished and additional elevations in core temperature are manifested by the administration of MET.

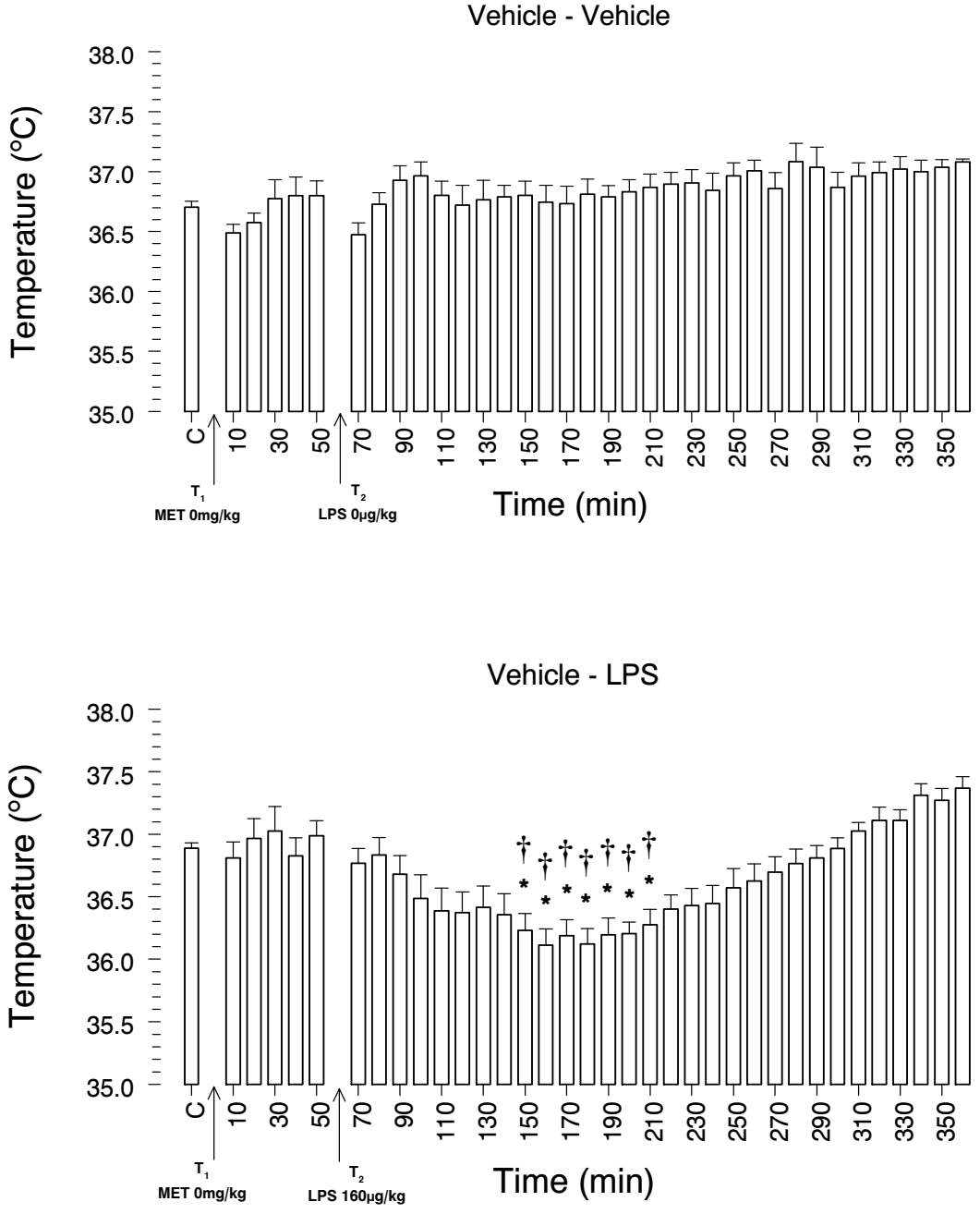


**Figure 3-4: Core temperature responses in pregnant female Sprague Dawley rats on day 20 of gestation. Responses are shown for animals who received an $_{I.P.}$ pretreatment with Vehicle (40% PEG), 60 minutes prior to either a Vehicle (40% PEG) or an *E. coli* LPS (160 µg·kg$^{-1}$) $_{I.P.}$ administration. Data are presented as means ± SD (Vehicle-Vehicle, n=7; Vehicle-LPS, n=7). * P <0.05 *versus* Control (C). † P <0.05 *versus* Vehicle-Vehicle at the same instant.**

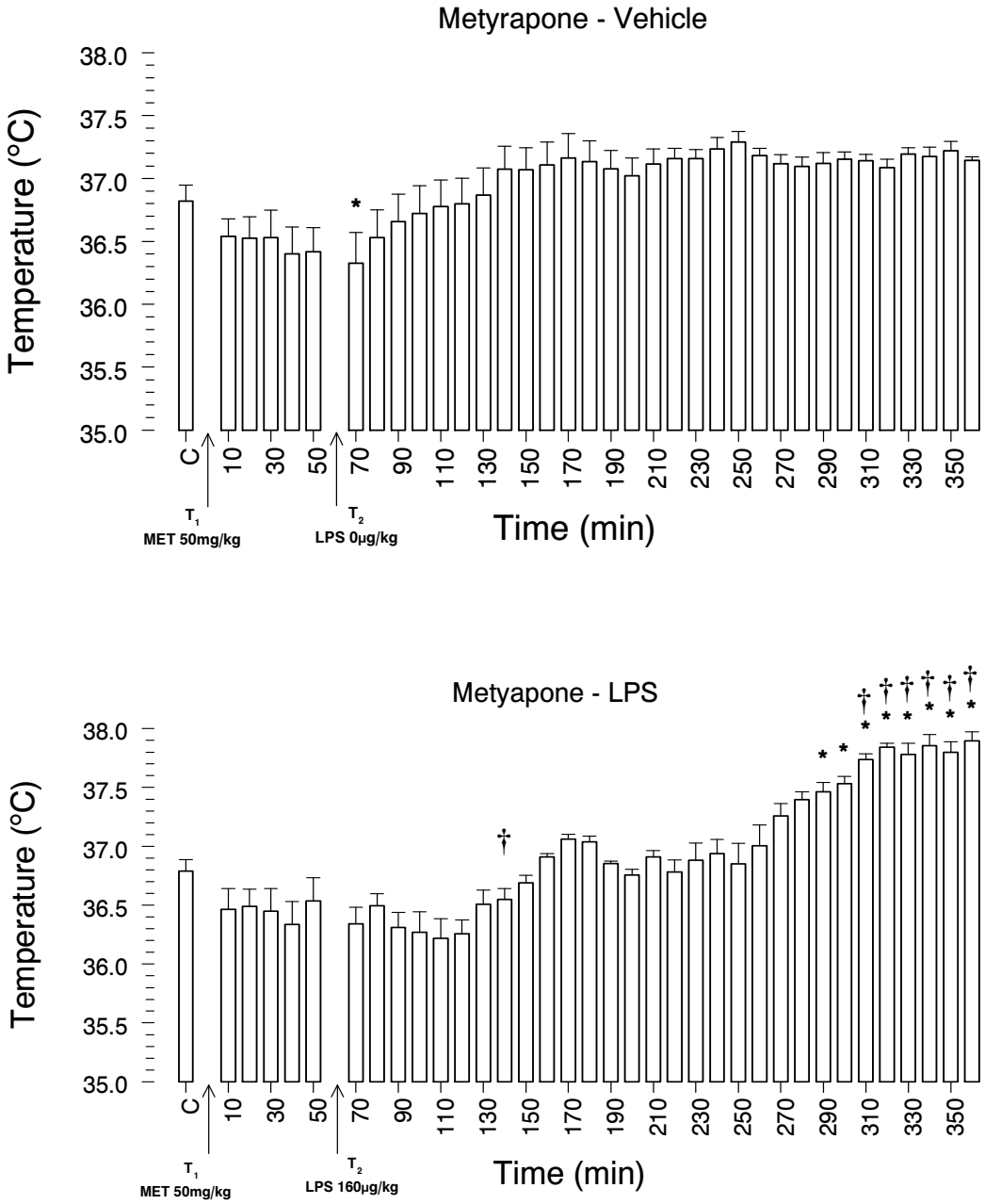


**Figure 3-5: Core temperature responses in pregnant female Sprague Dawley rats on day 20 of gestation. Responses are shown for animals who received an I.P. pretreatment with Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to either a Vehicle (40% PEG) or an *E. coli* LPS (160 µg·kg$^{-1}$) I.P. administration. Data are presented as means ± SD (Metyrapone-Vehicle, n=8; Metyrapone-LPS, n=5). * P <0.05 *versus* Control (C). † P <0.05 *versus* Metyrapone-Vehicle at the same instant.**

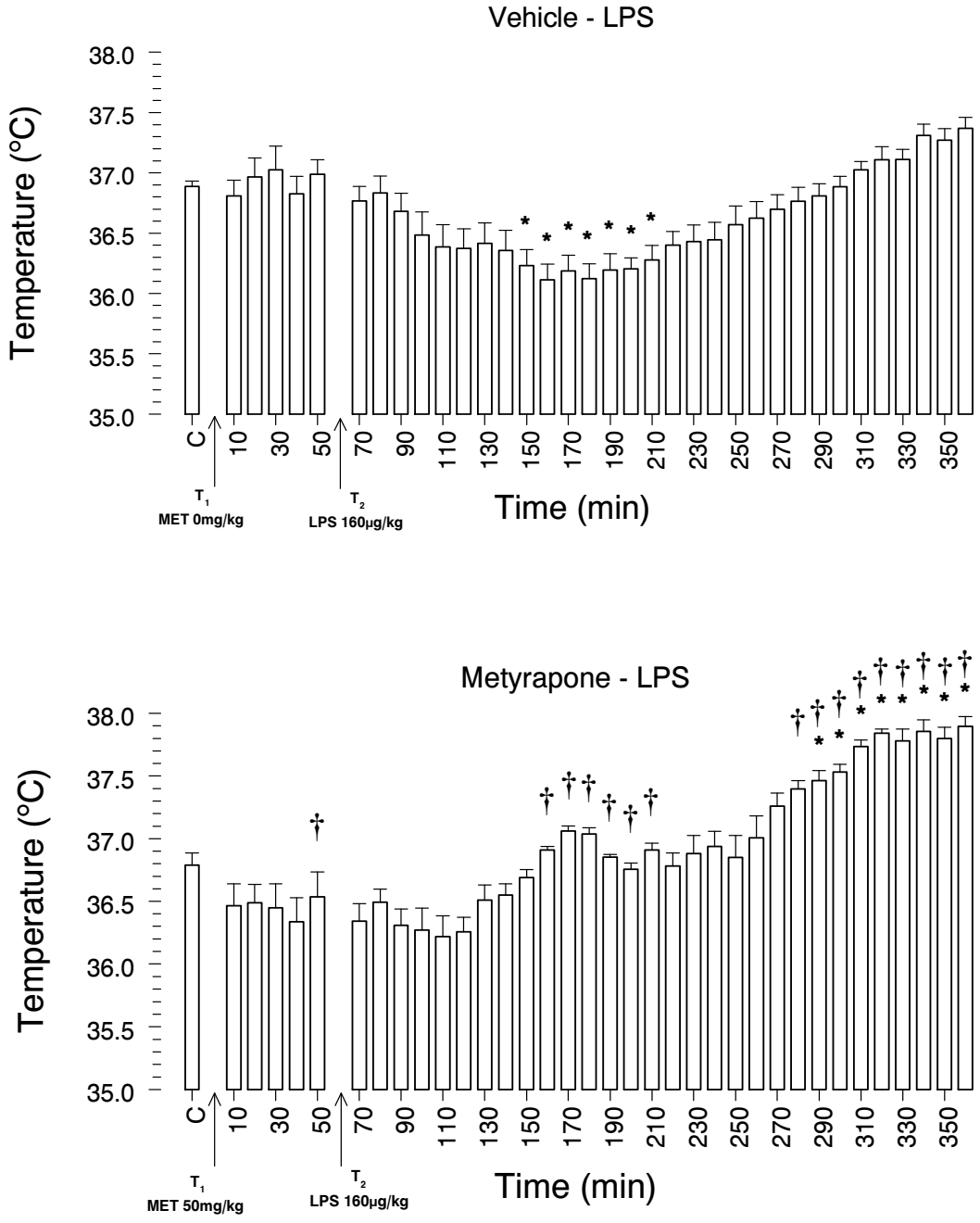


**Figure 3-6: Core temperature responses in pregnant female Sprague Dawley rats on day 20 of gestation. Responses are shown for animals who received an $_{I.P.}$ pretreatment with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior an *E. coli* LPS (160 µg·kg$^{-1}$) $_{I.P.}$ administration. Data are presented as means ± SD (Vehicle-LPS, n=7; Metyrapone-LPS, n=5). * P <0.05 *versus* Control (C). † P <0.05 *versus* Vehicle-LPS at the same instant.**

### *3.2.4 Effect of Metyrapone Administration on Plasma Cytokines*

The effects of the MET pretreatment (50 $mg \cdot Kg^{-1}$) in near-term rats prior to a challenge with LPS (160 $\mu g \cdot kg^{-1}$) was also examined. The changes in expression of plasma hormones at one, two, and four hours following the $_{I.P.}$ administration of either *Vehicle - Vehicle*, *Vehicle - LPS*, *MET - Vehicle*, or *MET – LPS* in near-term pregnant rats are shown in figures 3-7, 3-8, and 3-9.

Basal expression of IL-1β in the Vehicle-Vehicle group were 15 ± 0, 15 ± 0, and 58 ± 43 $pg \cdot mL^{-1}$ at one, two, and four hours, respectively. Regardless of the presence of metyrapone pretreatment, plasma IL-1β levels did not vary significantly following the administration of LPS. Both Vehicle-LPS and MET-LPS administrations elicited similar significant increases in IL-1 β expression (Figure 3-7).

Basal IL-6 levels were 35 ± 7, 10 ± 0, and 17 ± 7 $pg \cdot mL^{-1}$ at one, two, and four hours, respectively. The administration of Vehicle-LPS significantly elevated the presence of IL-6, whereas metyrapone pretreatment prior to LPS accentuated this expression (Figure 3-8).

Basal levels of TNF-α were 6 ± 0.9, 5 ± 0.2, and 6 ± 0.5 $pg \cdot mL^{-1}$ at one, two, and four hours, respectively. An overall significant increase in plasma TNF-α is noted in response to Vehicle-LPS; however, metyrapone pretreatment significantly diminished this LPS-induced response to near basal levels (Figure 3-9).

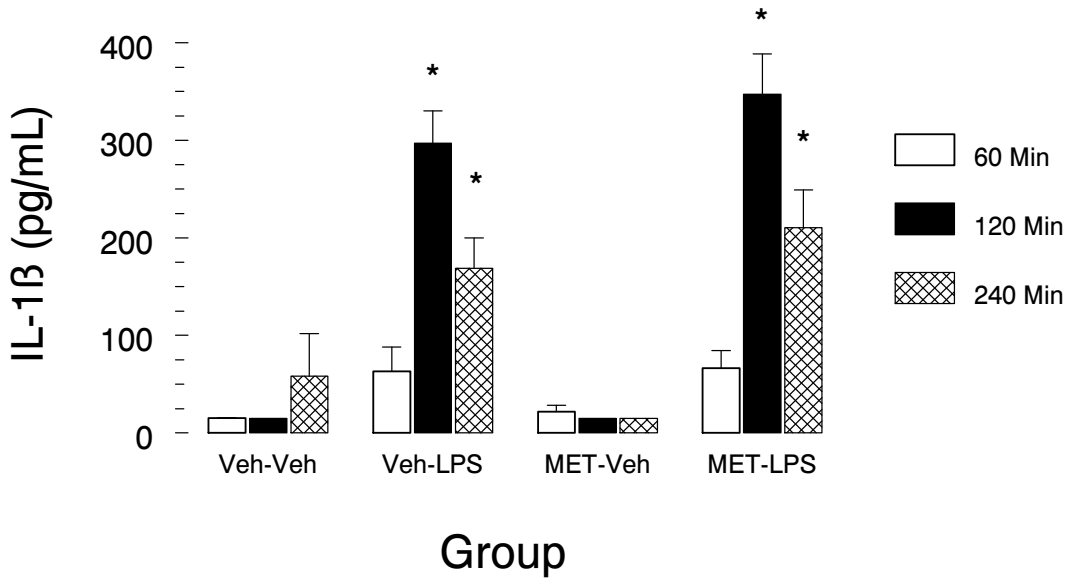


**Figure 3-7: Plasma IL-1β responses in female Sprague Dawley rats on day 20 of gestation. Animals received an $_{I.P.}$ pretreatment injection with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to receiving a following $_{I.P.}$ injection of either Vehicle or *E. coli* LPS (160 µg·kg$^{-1}$). The chart shows the four combinations of treatment groups and depicts Plasma IL-1β responses in each group at 60, 120, and 240 minutes following the second injection. Data are presented as means ± SD (n=8). * P <0.05 *versus* Veh-Veh at the same instant.**

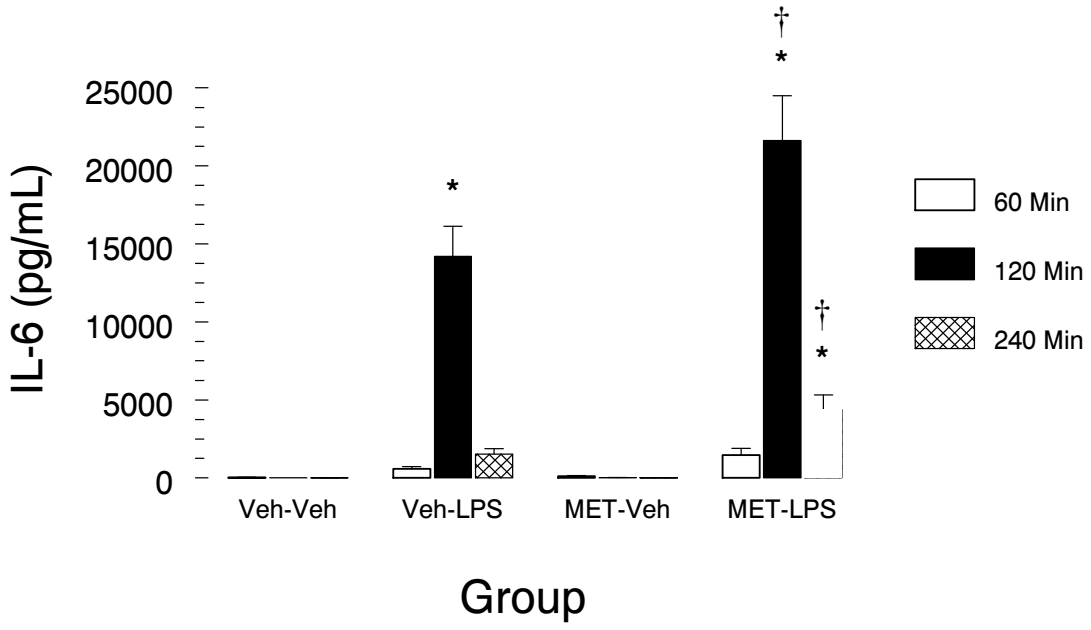


**Figure 3-8: Plasma IL-6 responses in female Sprague Dawley rats on day 20 of gestation. Animals received an I.P. pretreatment injection with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to receiving a following I.P. injection of either Vehicle or *E. coli* LPS (160 µg·kg$^{-1}$). The chart shows the four combinations of treatment groups and depicts IL-6 responses in each group at 60, 120, and 240 minutes following the second injection. Data are presented as means ± SD (n=8). * P <0.05 *versus* Veh-Veh at the same instant. † P <0.05 *versus* Veh-LPS at the same instant.**

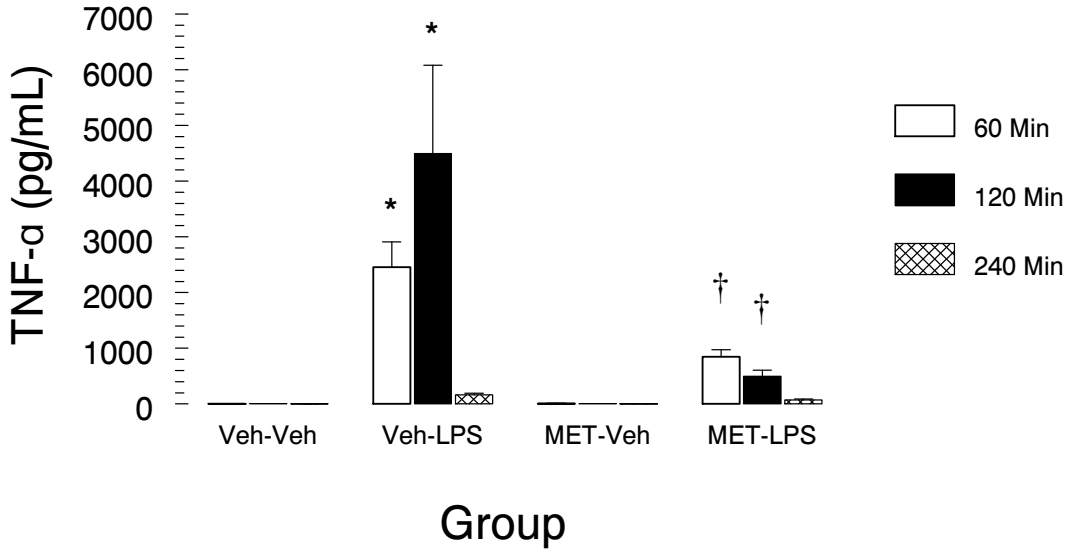


**Figure 3-9: Plasma TNF-α responses in female Sprague Dawley rats on day 20 of gestation. Animals received an I.P. pretreatment injection with either Vehicle (40% PEG) or Metyrapone (50 mg·kg$^{-1}$), 60 minutes prior to receiving a following I.P. injection of either Vehicle or *E. coli* LPS (160 µg·kg$^{-1}$). The chart shows the four combinations of treatment groups and depicts TNF-α responses in each group at 60, 120, and 240 minutes following the second injection. Data are presented as means ± SD (n=8). * P <0.05 *versus* Veh-Veh at the same instant. † P <0.05 *versus* Veh-LPS at the same instant.**

# *Chapter Four:*

# *DISCUSSION*

## 4.1 Summary and Conclusions

Pregnancy is a unique event in which a number of reversible physical changes occur within the maternal body. During the final days of gestation, there is an increasing impairment of the mechanisms that allow female mammals to develop a fever. In near-term pregnant rats, exposure to non-infectious (e.g., forced swimming) or high dose infectious-mimicking stimuli (e.g., LPS, IL-1β) result in a hypothermic response preceding an attenuated or absent febrile response. This phenomenon was first identified by Kasting *et al.,* who reported that the febrile response in the ewe to endotoxin or leukocyte pyrogen is attenuated in the period from two to five days pre-partum, to several hours post-partum (89). Martin *et al.* demonstrated this effect further by showing that rats do not develop fevers to endotoxin (116). The experiments outlined in this thesis

further clarify the intricacies of the febrile response and its mechanism of action in near-term pregnant female rats stimulated by a bacterial endotoxin. The results of these studies revealed the following findings: (i) the administration of MET (50 $mg \cdot kg^{-1}$) brought about the complete inhibition of corticosterone synthesis in near-term female rats; (ii) glucocorticoid inhibition by MET (50 $mg \cdot kg^{-1}$) administration in near-term female rats did not produce a significant alteration in their baseline core temperature; (iii) the pretreatment of near-term female rats with MET (50 $mg \cdot kg^{-1}$) facilitated significant increases in core temperature during the resultant febrile response elicited by exposure to a high-dose (160 $\mu g \cdot kg^{-1}$) of the exogenous pyrogen LPS; and (iv) MET (50 $mg \cdot kg^{-1}$) pretreatment in near-term female rats caused an alteration in the plasma concentrations of specific pyrogenic/antipyretic cytokines following a high-dose (160 $\mu g \cdot kg^{-1}$) challenge with the exogenous pyrogen LPS.

The initial finding shows that plasma corticosterone expression, which is markedly elevated in response to high-dose stimulation with LPS, is significantly attenuated to basal levels when pretreatment with metyrapone occurs before LPS administration. Similar to a physical challenge, psychogenic stressors can also activate the HPA axis, leading to an elevation in corticosterone. Studies by Roozendaal *et al*. and Wright *et al*. confirm our findings as they also used metyrapone to successfully reduce the elevation of plasma corticosterone induced by forced water-maze swimming in male rats with partial hippocampus lesions (158; 206). The inhibiting effect of metyrapone is dependent on the target action of the drug in the glucocorticoid biosynthesis pathway in the rat. The final step in the process is the hydroxylation at $C_{11}$ by cytochrome $P450C_{11}$ which forms corticosterone. Metyrapone is highly selective in this process as it inhibits

steroid 11β-hydroxylase and thus stalls the electron transport actions of P450$C_{11}$. The result is that the essential hydroxylation of $C_{11}$ cannot occur, thus halting the formation of corticosterone (Figure 4-1).

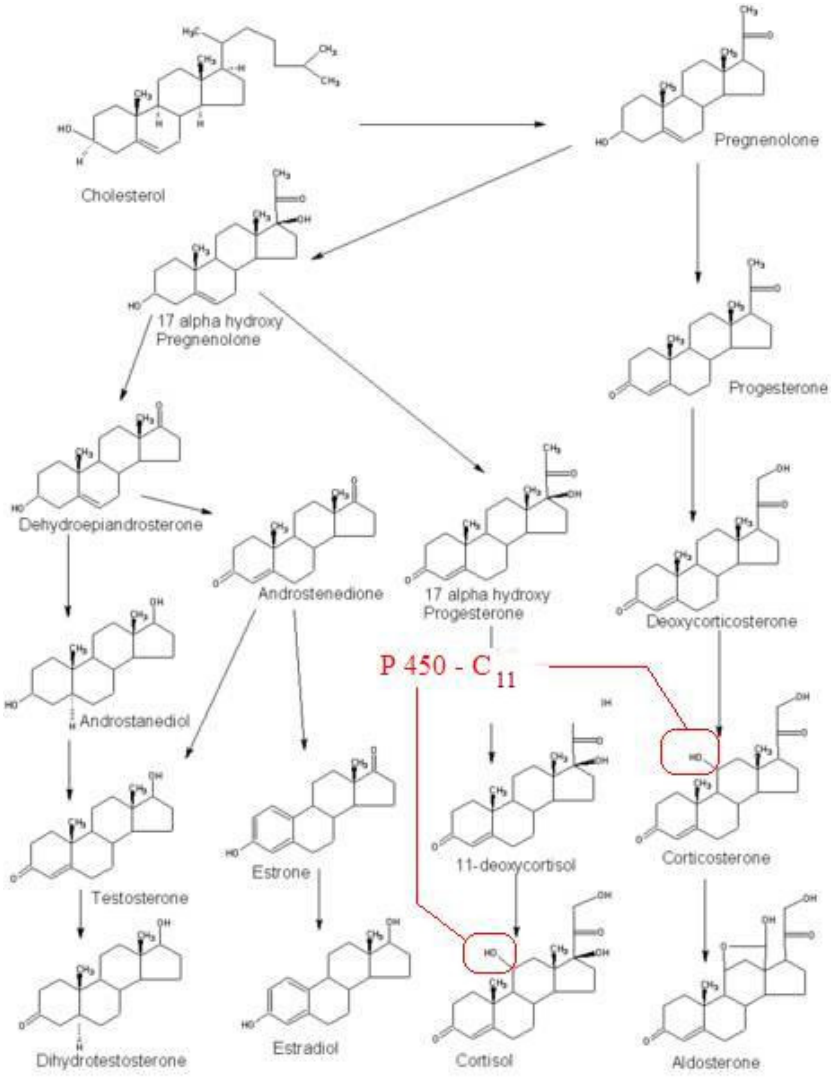


**Figure 4-1: Target of Metyrapone's action during steroidogenesis. The figure shows the location on which cytochrome P450$C_{11}$ acts to facilitate the final step in the biosynthesis of corticosterone and cortisol. Metyrapone selectively inhibits the actions of P450$C_{11}$ which results in an impediment of glucocorticoid synthesis. Adapted from Sigma Aldrich Ltd.**

The second finding indicated that administration of the glucocorticoid synthesis inhibitor had no ill effects on baseline temperature. This brought confidence in the selectivity and neutral effects of metyrapone on physiology. Metyrapone administration induced an insignificant depression in temperature that did not reappear at any other times following the injection.

The third set of experiments found that metyrapone administration prior to an $EC_{100}$ LPS challenge altered the febrile response from the expected response which occurred in the pregnant rats that were pre-treated with vehicle. To our knowledge, this is the first report of a febrile response in near-term rats that emulates the response that is typically noted in non-pregnant females stimulated by an $EC_{100}$ bacterial endotoxin challenge.

Fofie and Fewell used a similar $EC_{100}$ dose of LPS in near-term rats and reported a hypothermic (instead of a febrile) response with latency, duration, and magnitude of approximately 0.5 h, 2.5 h, and -1.2°C, respectively (60). Our study noted similar responses in non pre-treated rats and brought confidence to their use as a reference on which to base the effects of metyrapone. Corticosterone inhibition by metyrapone induced an immediate insignificant drop in core temperature followed by two significant increases, with core temperature still dramatically elevated from baseline five hours after LPS administration.

Our data show that in near-term rats pre-treated with the glucocorticoid synthesis inhibitor, the hypothermic period and attenuated fever noted to occur following high-dose LPS challenge has been abolished and replaced with a polyphasic febrile profile. These observations are consistent with reports of a multi-phase febrile response, evoked by

relatively high doses of pyrogen administered to non-pregnant animals (151; 154). *Phase II* of this response peaks within 2 to 2.5 hours, and *Phase III* peaks between 5 and 6 hours (153). This is in contrast to a monophasic response whereby a relatively weak pyrogenic stimulant brings about one significant core temperature rise that peaks between 1 to 1.5 hours later (151).

According to Romanovsky *et al*., in many experiments that involve an acute, stressful injection of pyrogen, *Phase I* is often absent from the febrile response (153). Our results clearly show a period of time from the LPS injection in which temperature does not waver far from baseline, followed by two distinct temperature rises peaking at approximately 1.8 hours and 5 hours, respectively. Thus, our results could portray an absence of febrile *Phase I*, with the hypothermic response replaced by the expression of *Phase II*, which is typically masked, and a distinct *Phase III*.

It has been thought that glucocorticoids play an integral role in the modulation of the febrile mechanism. For example, Coelho *et al.* conducted a study on male Wistar rats that were either adrenalectomized or treated with a synthetic glucocorticoid to determine if the febrile response, similar to the inflammatory response, could be modulated by endogenous glucocorticoids (39). The results showed an enhanced febrile response to LPS from the adrenalectomy, and an abolished febrile response following glucocorticoid treatment with dexamethasone (39). In a recent study, Moore and Fewell showed that pretreatment of the glucocorticoid receptor antagonist RU38486 in near-term rats prior to an LPS challenge, limited the core temperature drop within three hours of the LPS administration (125). However the study was complicated by the fact that RU38486 has been found to antagonise the progesterone receptor. Based on this study, it is difficult to

delineate the temperature responses mechanism because progesterone is a hormone with thermogenic properties (134). The present study takes these results to the next step by not only fully abolishing the hypothermic response in near-term rats, but also by restoring a significant febrile response associated with LPS stimulation through using a drug that is highly selective to glucocorticoid inhibition solely.

The fourth experimental finding in this thesis provides new details on the expression of IL-1β, IL-6, and TNF-α, and how their expression is modulated by the inhibition of plasma glucocorticoids. The analysis of IL-1β levels uncovered a trend of elevated expression from basal levels following both the LPS-only stimulation (vehicle-LPS), and after the Metyrapone-LPS treatment. IL-6 expression was also amplified in the near-term rat following stimulation with LPS, and this effect was enhanced by the attenuation of corticosterone from metyrapone pretreatment. A significant TNF-α expression, appearing faster than any of the other cytokines, was also noted to occur following LPS challenge; however, pretreatment with metyrapone dramatically reduced circulating TNF-α to near-basal levels following the LPS challenge. Based on these results, it is concluded that the elevated plasma corticosterone in near-term rats upon high-dose LPS challenge does not change the expression of IL-1β, but partially masks the full expression of IL-6. This, in turn, could help amplify TNF-α synthesis which could have already been rapidly influenced directly by corticosterone elevation.

Fofie *et al.* described how a differential pyrogenic and antipyretic/cryogenic plasma cytokine response may be in effect during a febrile response (61). They reported that following an $EC_{100}$ dose of LPS, non-pregnant rats expressed a statistically greater abundance of IL-1β, IL-6, IL-1ra and TNF-α as compared to that observed following the

administration of vehicle; whereas this mechanism is speculated to be altered in pregnant females (61). Additional data from Fofie *et al*. showed that LPS administration caused a statistically significant expression of the antipyretic/cryogenic cytokines (IL-1ra and TNF-α), but not of pyrogenic cytokines (IL-1β and IL-6) in pregnant rats on day 20 of gestation (61). In contrast, evidence has suggested that there is no change in plasma levels of any of the pro-inflammatory cytokines, anti-inflammatory cytokines, or even corticosterone following LPS administration near term (128). The present study opposes this position to some extent. For instance, reports indicate that IL-1β plasma levels do not rise significantly in response to LPS stimulation near term (61; 128). Mouihate *et al*. reported no change in IL-1β expression in response to LPS challenge during gestation (128). The reason for these findings might be that Mouihate *et al*. used a smaller LPS dose than used in the experiments in this thesis or the fact that the group reasoned that the attenuated febrile response is specific to the immediate days before parturition, but is unaltered on day fifteen or during lactation. Thus, Mouihate *et al*. made their hormone comparisons between day 15, day 22 and lactation periods.

The reasoning above may be contradicted by evidence from Fofie and Fewell who tracked the core temperature changes in non-pregnant rats, and rats on day 10, day 15, and day 20 of gestation in response to varying doses of LPS (60). The data showed obvious febrile profile alterations onward from day ten using smaller LPS doses than those used by Mouihate *et al*. Plasma corticosterone also increases progressively from day ten to reach a maximum by parturition (10). Furthermore, Fofie and Fewell have indicated that levels of IL-1ra increase significantly on day 15 of gestation in pregnant rats as compared to days 10 and 20 in non-pregnant models (60). Thus, multiple sources

of evidence suggest that there is an alteration in elements of the febrile mechanism prior to, and through day 15.

The present studies did not compare hormone levels between animals on different gestation days, but only those on day 20 of a 21 day gestation period and clearly showed a significant increase in IL-1β following LPS challenge near-term. Fofie *et al.* also state that IL-6 levels did not elevate significantly following LPS in their experiments on near-term rats (61). However, their data showed a slight cytokine increase from baseline after LPS administration, particularly two hours following treatment. This is consistent with our data, which showed a marked increase in IL-6 two hours following LPS. It needs to be noted that our injection occurred at noon whereas Fofie *et al.* began their study at 10 AM. Thus, the difference in expression between the two studies can be explained partly by the circadian variations that produce the basal cytokine level.

The increase in TNF-α following LPS administration observed in the present set of experiments correlates well with similar published observations. A recent report by Akarsu and Mamuk noted a dramatic increase in TNF-α following LPS administration in rats that resulted in hypothermic responses (2). In reaction to LPS administration, TNF-α double receptor knockout mice develop enhanced fevers (96). Tollner injected a high dose of LPS into male rats and observed the rapid induction of high concentrations of TNF-α in the plasma and peritoneal lavage fluid (193). Fofie *et al*. also reported significant increases in TNF-α after LPS stimulation in near-term rats (61).

**4.2 Mechanisms of the Attenuated Febrile Response Near Term of Pregnancy**

It has been proposed that glucocorticoids play a role in dampening the effects of exposure to exogenous and endogenous pyrogen, especially latter in pregnancy when it is elevated in the maternal circulation. Very little is known about the mechanics of this hypothermic response. There is, however, compelling evidence that its regulation has a hypothalamic origin, rather than being the result of a forced thermoregulatory response (194). Typically, glucocorticoid expression in the rat is influenced by the actions of the HPA axis; yet, marked changes occur in the HPA axis throughout the term of pregnancy and into lactation (10; 50). The glucocorticoid synthesis mediator ACTH does not elevate from mid-gestation, whereas there is a definite rise in corticosterone from this point forward (10). A steady core temperature decrease up to parturition is an overt trait of this response.

Maternal elevation of glucocorticoids have been studied in rats throughout the course of pregnancy (10). Evidence indicates that plasma corticosterone increases from 141± 27 $ng \cdot mL^{-1}$ progressively from day ten to reach a maximum 286 ± 28 $ng \cdot mL^{-1}$ by parturition (10). The magnitude of this gestation-dependant rise from non-pregnant levels of expression can be further elucidated by data from our laboratory, where the basal level of glucocorticoid in non-pregnant rats was about 40 $ng \cdot mL^{-1}$ (Alexander, unpublished observations, and Fofie, unpublished observations). The present study confirmed the pregnancy-related increase in basal glucocorticoid expression. Notably though, four hours following the second vehicle injection, in the vehicle-vehicle group, plasma corticosterone levels increased from values noted 60 minutes and even two hours

post $T_2$ injection. The vehicle injections (40% PEG) evoke no physiological stressors that would lead to an increased corticosterone response. Therefore, the corticosterone increase can be explained by circadian rhythm. This is further supported by data from Atkinson and Waddell, who studied the daily changes in plasma levels of corticosterone from diestrus through gestation and into lactation (10). They found that on the day before parturition, basal corticosterone levels at 8 AM were on the order of 100 $ng \cdot mL^{-1}$, while at 4 PM, the hormone level had risen to approximately 430 $ng \cdot mL^{-1}$, the highest peak at any time during their study. These data correlates well with our measurements, which also took place at 4 PM.

The significance of the ACTH-glucocorticoid dissociation can be understood through evidence that high levels of ACTH can compromise fetal survival (208); whereas the amplification of lipolysis by elevated glucocorticoids might be assisting maternal metabolism, which is predominantly catabolic during the latter stage of gestation to meet increased fetal demand (97). A rise in ACTH receptor expression in the adrenal cortex may explain an increased corticosterone release, despite a relatively low level of ACTH. Alternately, it has been shown in humans and indirectly in rats, that the placenta also produces bioactive ACTH (200), of which not all is detected by immunoreactivity. Therefore, it is likely that the placenta might be an additional influence on basal glucocorticoid production that is worthy of further study.

Prolonged increases in glucocorticoid levels have been linked to adverse effects on the maternal body (e.g., electrolyte abnormalities and hypertension) (164) and on the offspring (e.g., detrimental programming effects increasing the chances of disease during adulthood) (28). The increase in plasma glucocorticoid may be important late in

gestation for preparing the mammary glands for lactation and for mobilizing maternal energy stores (97). However, because the HPA axis has been altered in its control over glucocorticoid synthesis at this time, it is doubtful that this event would occur without an over-shadowing system to limit the degree of hormone expression. During this stage an opioid mechanism has been shown to arise (27). Autoinhibition of the NTS by an elevated expression of proenkephalin-A and a significant increase in expression of the μ-opioid receptor along the NTS can blunt NTS POMC neurons and anterior pituitary corticotropes during gestation, reducing the amount of synthesized ACTH. In this manner, an opioid-mediated reduction in HPA axis responsiveness can play a dual role by keeping ACTH and glucocorticoid levels from causing adverse biological responses, and by preventing an extreme core temperature decrease through glucocorticoid-induced antipyresis. Recent work by Brunton *et al.* has shown that HPA axis responses to immune challenge are also blunted (27). $_{I.V.}$ administration of LPS and IL-1β into near-term and virgin rats increased ACTH levels in non-pregnant groups significantly; while it attenuated the response in pregnant animals (27). This trend was also confirmed for plasma corticosterone expression under the same experimental conditions (27). Possible explanations stem from the evidence that pro-opiomelanocortin, a precursor to ACTH production synthesized by the NTS and anterior pituitary corticotropes (160), is enhanced in virgin rats; but is absent in pregnant subjects (27). Subsequent testing utilized pretreatment with naloxone, a potent μ-opioid receptor antagonist to assess the importance of the μ-opioid receptor in the attenuation of the HPA axis. The results showed that μ-opioid receptor pretreatment significantly restored a portion of the ACTH response to IL-1β challenge in pregnant rats, and detailed a significant expression of

noradrenaline and PVN CRH mRNA which is not typically seen in pregnant rats. In addition, expression of proenkephalin-A mRNA tends to increase during gestation (27). This is coupled with a significant increase in expression of the μ-opioid receptor (27), supporting the theory that an increased NTS sensitivity to proenkephalin-A can blunt HPA axis responsiveness. Reduced HPA axis responsiveness may be set in place to counter-balance endogenous antipyresis by lessening the antipyretic impact that glucocorticoids impart on core temperature.

Evidence from Imai-Matsumura *et al*., Malik and Fewell, and Mouihate *et al*. confirm that LPS-challenged near-terms have lower hypothalamic COX-2 and csf $PGE_2$ levels (80; 113; 127). Thus, it has been thought that the near-term attenuated response to pyrogens is partly due to a general reduction of hypothalamic COX-2. Mouihate *et al*. used western blot analysis to detect changes in the COX-2 gene activating pathways NFκB, ERK/MAPK, and STAT5 in rats throughout gestation (128). Immunoblots of phosphorylated-IκB, which represented NFκB activation, showed that an $_{I.P.}$ LPS injection caused a significantly higher expression of POA/OVLT phosphorylated-IκB to unphosphorylated-IκB when compared to saline injections on all gestation days. Other results showed that the constitutive expression of phosphorylated STAT5 was significantly reduced as gestation progressed (128). Therefore, there is evidence suggesting that alterations in these signalling pathways can influence near-term fever suppression.

Recent data have been published showing that neurons containing NO synthase have been identified in the POA hypothalamus (20), and that these neurons increase their production of NOS during the latter stages of pregnancy (205). Begg *et al.* administered

the NO inhibitor $N^G$-monomethyl-L-arginine $_{I.C.V.}$ to near-term rats placed in a thermocline to study the behavioural response to LPS induced fever. The results showed that while the control LPS-challenged near-term group achieved the typical hypothermic response, near-term rats pre-treated with the NOS inhibitor and later administered with LPS, reversed the hypothermia and significantly restored the febrile response (15). Furthermore, these near-term rats pre-treated with the NOS inhibitor consistently relocated to warmer ambient temperature positions within the thermocline, showing a regulated hypothalamic response, and the restoration of the ability to behaviourally thermoregulate—a trait that pregnant rats do not display near the term of pregnancy (15). Thus, NO could be playing a significant antipyretic role in the attenuated febrile response in near-term rats. Evidence in support of this theory lies in that central or peripheral NO donors cause hypothermic responses (69; 183); but central NOS inhibitors not only cause core temperature increases in exercising animals (103), they also reduce the hypothermic effects of AVP (4).

Eliason and Fewell have shown that AVP attenuates the febrile response to $PGE_1$ administration in near-term rats (53). However, it does not similarly attenuate the febrile response to IL-1β (54). Further, it has been reported that PGE levels are not amplified in the OVLT following IL-1β challenge (58). This somewhat weakens the position of AVP as a key antipyretic to the attenuated near-term febrile response, and indicates that the antipyretic mechanism is likely occurring prior to PGE formation.

### 4.3 Consequences of Fever and Febrile Attenuation Near the Term of Pregnancy

Pregnancy is highlighted by numerous physiological alterations. During this period, the maternal body must cope with the hormonal and thermoregulatory changes necessary to support the developing fetus. Fetal temperature remains about 0.5°C above the mother's core (101), creating a fetal-maternal temperature gradient to transfer metabolized heat from the developing fetus, onto the mother for dissipation. Heat transfer is a vital element in pregnancy, as fetal tissue is highly susceptible to heat damage (64). Consequently, chronic elevations in maternal body temperature greater than 4.0°C near the term of pregnancy can lead to adverse effects in the neonate. For instance, the period extending from the third trimester to the first few years of life are a time of great neural elaboration. The reduced fetal-maternal temperature gradient, due to inadequate heat dissipation, caused by a prolonged increase in maternal temperature can lead to neural complications (i.e., autism disorders). In adults, brain damage through polyribosome disaggregation begins to occur at a core temperature of about 40.0 to 41.0°C, and above 41.0°C, irreversible mitochondrial damage occurs (33; 130). Only about 15% of metabolized heat from the fetus is transferred via the surrounding amniotic fluid, with the remainder removed by the incoming umbilical circulation (65). By parturition, fetal metabolic tissue produces more than double the heat per kilogram of body weight than maternal tissue (16). The gestation-dependant temperature drop may be a maternal adaptation to help the fetus cope with the dangers of parturition-linked asphyxiation. A lower core temperature both decreases fetal oxygen requirements and increases hemoglobin's affinity for oxygen, raising oxygen saturation levels (107).

Maternal febrile responses near the term of pregnancy can also be problematic. For instance, fever can cause shunting of the systemic arterial blood flow from the uterine and placental regions, to structures such as muscles and brown adipose tissue in order to drive heat production (23). Blatteis *et al.* used *salmonella enteritidis* endotoxin (LPS, 2 $\mu g \cdot kg^{-1}$, I.V.) to induce changes in colonic temperature and regional blood flow in rabbits on day 30 of pregnancy (23). The results showed a 28% reduction in maternal blood flow to the placenta. A withdrawal of utero-placental perfusion is likely to present a nutrient and gas exchange constraint to fetal development. The Fetal Programming Hypothesis predicts that a low birth weight is associated with health complications such as respiratory distress syndromes, apnea, and hypertension later in adulthood. Preterm birth is a significant factor in perinatal mortality. Increased concentrations of pro-inflammatory cytokines in the amniotic fluid have been linked to preterm labour (157). Cytokines such as IL-1β have been shown to raise the secretion of prostaglandin from the myometrium (9). Astle *et al.* reported that near-term, mPGES-1 is concentrated predominantly in myometrial and vascular smooth muscle cells and showed that IL-1 β increased COX-2 and mPGES-1 mRNA expression and also stimulated the release of $PGE_2$ by human myometrial smooth muscle cells (9). Significant elevations in prostaglandin can quickly stimulate uterine contraction and engage an early activation of the positive-feedback process of parturition.

Into adulthood, the inflammatory and febrile processes become important immunological mechanisms to ward off illness and infection. During infection, animals that cannot invoke these processes adequately are associated with greater morbidity and mortality than those who can (95). Shanks *et al*. have reported that physiological and

behavioural stress in the early stages of life can affect HPA axis responses to noise stress and LPS in adulthood (168; 169). Recent data published by Boisse *et al*. have confirmed this phenomenon in male rats indicating that neonatal exposure to both *Salmonella enteritidis* and *Escherichia coli* derived LPS, which caused febrile responses, display attenuated responses in adulthood to *E. Coli* LPS challenge, but unchanged responses to IL-1β and $PGE_2$ stimulation (24). Furthermore, unlike LPS-stimulated adult controls that display elevated hypothalamic COX-2, neonatally pre-challenged males have higher basal COX-2 levels, but respond to treatment with a reduction in COX-2 (24). This experiment was later replicated with female neonates, who had no elevation in basal COX-2, but expressed the same attenuated responses in adulthood as males (179).

### 4.4 Speculated Mechanism of Febrile Response during Glucocorticoid Inhibition

Our findings suggest that a degree of the cytokine expression that arises following a high-dose LPS challenge in near-term rats, is at least partially modulated by an increased glucocorticoid presence in the circulation. Furthermore, our findings suggest that the differential expression of pyrogenic and antipyretic cytokines also partly modulates the associated febrile response that develops during pyrogenic challenge. The expression of cytokines, along with other putative factors during an LPS challenge might synergistically define the concentration of biologically available IL-1β, which is likely to be the primary factor to engaging the upstream neurological aspects of the febrile response to exogenous pyrogen. IL-1β has been used in a multitude of studies with continual success as an extremely effective fever-inducing agent when administered both

centrally and systemically (22; 42; 151). The results of these experiments suggest that the effective concentration of IL-1β available for biological activity does not change in relation to glucocorticoid concentration following endotoxin challenge. Therefore, this value might depend on plasma levels of IL-1ra and IL-1r II, factors known to attenuate the actions of IL-1β, and could be partly manipulated by corticosterone activity during late gestation. Pillay *et al.* examined the relative basal levels of IL-1ra expression in non-pregnant and near-term women, 80% of the way through their pregnancy (142). They found that concentrations of IL-1ra in the plasma of women in late pregnancy were significantly higher than in the plasma of non-pregnant adults. In addition, the monocytes of pregnant women produced much higher IL-1ra concentrations than the monocytes of non-pregnant adults (142). Mice have also shown to produce a substantially greater count of plasma IL-1ra than IL-1α or IL-1β with the progression of pregnancy (77). During LPS challenge late in pregnancy, it has been documented that IL-1ra expression in rats is elevated (61). A near-term LPS challenge might also induce elevated corticosterone to increase IL-1r II synthesis. IL-1r II varies from its type I counterpart in that it has no known signal transduction capability (26). This receptor variant has a greater affinity for IL-1β than IL-1α, or IL-1ra, and thus preferentially binds with IL-1β. This consequence prevents IL-1β from binding with the type I variant which is capable of intracellular signal transmission (26).

Following pretreatment with metyrapone, the administration of a significant dose of LPS into the bloodstream of a female rat near the term of pregnancy is likely to cause a forceful activation of the immune system's APR. The circulating endotoxin will also begin to interact with the high volume of Kupffer cells in the liver and stimulate their

rapid expression of $PGE_2$ and/or IL-1β. Both of these factors have been shown to bind to and activate hepatic vagal afferents (51; 135). Although often masked in a polyphasic fever, this action could stimulate *Phase I* of the response to LPS by engaging NTS noradrenergic projections into the POA which simultaneously increases the synthesis of hypothalamic $PGE_2$ available to bind to the $EP_3$ receptor, and also directly inhibits the firing of warm-sensitive neurons (22). This shifts the thermoregulatory set-point of the CNS in favour of heat production to bring core temperature back in line with the reduced influence of heat loss effectors. Thus, core temperature is allowed to rise to this new thermoregulatory equilibrium rather quickly.

At the same time, circulating LPS continues to stimulate Kupffer cells of the liver as well as other circulating macrophages and monocytes to enhance the secretion of pyrogenic and antipyretic cytokines, including IL-1β, IL-1ra, IL-6, and TNF-α (94). Glucocorticoid inhibition can alter this expression. Normally, NTS projections reaching into the PVN (perhaps stimulated by innervation of the vagus nerve) induce the expression of CRH (160). The consequential activation of downstream elements of the HPA markedly increases the synthesis of plasma glucocorticoid expression (160). However glucocorticoid synthesis can be diminished somewhat at the PVN by an underlying pregnancy-induced opioid mechanism (27; 28). More importantly, however, diminished synthesis at the adrenal gland can occur by the inhibiting actions of metyrapone, bringing about an alteration in cytokine expression.

The rapid rise in TNF-α noted following LPS, compared to its immediate attenuation in response to MET pretreatment before LPS stimulation could mean that corticosterone expression is directly influencing levels of this hormone. Smyth *et al.*,

reported that murine macrophage cell lines pre-treated with corticosterone can induce significant TNF-α synthesis (177). Barber reported the same amplifying effect on TNF-α in humans pre-treated with hydrocortisone infusion prior to LPS challenge (12). In addition, increased plasma corticosterone has the effect of decreasing IL-6 levels (55; 199). Our results showed that the inhibition of glucocorticoid presence during the response amplifies IL-6 and attenuates TNF-α expression. This is important because although a direct effect of corticosterone on TNF-α expression can be inferred, IL-6 synthesis is noteworthy because it also influences the downregulation of TNF-α (180; 191; 207) and the upregulation of the TNF soluble receptor (191; 207). For instance, Xing *et al*. demonstrated a 300% increase in TNF-α levels from control animals following the systemic delivery of LPS in IL-6 gene knock-out mice (207). A reduction of circulating TNF-α is important, because *in vitro* and *in vivo* reports demonstrate its potential to influence expression of IL-1ra (100; 115; 141; 196). van der Poll *et al*. administered four healthy humans, and eight healthy adult chimpanzees that were stimulated with *E. Coli*, a bolus of $_{I.V.}$ recombinant human TNF-α and reported pronounced increases in plasma IL-1ra in the following hours (196). Furthermore, elevated corticosterone stimulates IL-1ra and IL-1r II upregulation (1; 26). Our data indicated that there was no change in the expression of IL-1β regardless of metyrapone or vehicle pretreatment. Thus, a reduction in plasma corticosterone and consequentially TNF-α might have diminished the occupation of IL-1ra on IL-1r I receptors presumably near the OVLT and other circumventricular organs of the brain. Furthermore, reduced IL-1r II allows a greater percentage of unbound IL-1β to become biologically available. The combined diminished action of IL-1ra and IL-1r II allows more IL-1β to interact with

perivascular/endothelial cells of the BBB, stimulating neural NFκB, ERK/MAPK, or STAT5 signalling pathways to upregulate COX-2 expression, and consequentially PLA/AA elevation. Typically inhibition of both COX isoforms and the antagonizing of AA release also occur via: direct corticosterone influence on the hypothalamus and an elevated corticosterone-induced lipocortin-1 expression (38; 176). A significant reduction in corticosterone may then relieve these inhibitory effects, and further raise hypothalamic COX-2/ PLA/AA expression. All of these factors lead to an increase in the relative levels of POA $PGE_2$ available to bind to $EP_3$ receptors located on warm-sensitive neurons (106). $EP_3$ binding raises warm-sensitive neuron firing threshold, and consequently raises hypothalamic set-point. An elevated set-point reduces efferent signalling to autonomic heat-loss effectors (i.e., peripheral vasodilation), raising the body temperature in the latter *Phase II* and *Phase III* of the response (Figure 4-2).

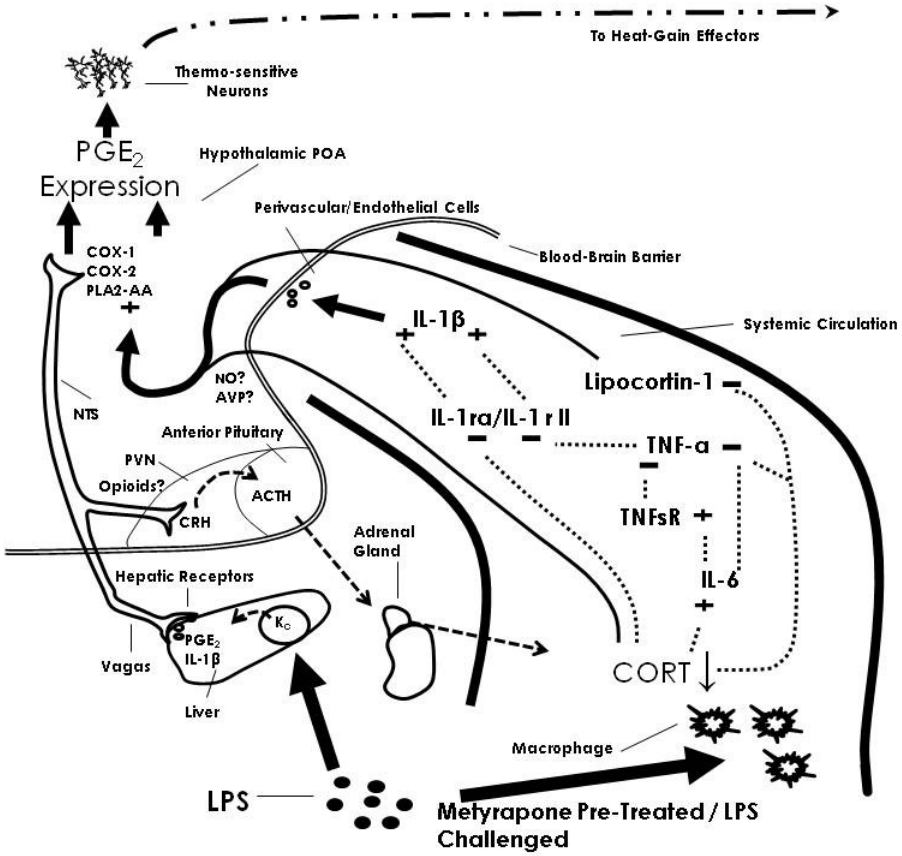


**Figure 4-2: Generalized diagram illustrating some of the key interactions speculated to occur between plasma cytokines and other factors when pretreatment with Metyrapone occurs before a high-dose LPS challenge during late gestation. LPS triggers immune factors to stimulate hepatic vagal innervation, leading to rapid hypothalamic $PGE_2$ expression/warm sensitive neuron inhibition - inducing an initial *Phase I*-like temperature increase. Attenuated CORT expression due to MET pretreatment influences multiple inhibitory effects on IL-1ra/IL-1r II through alterations in associated circulating factors such as TNF-α enabling IL-1β to potently stimulate the release of $PGE_2$ from circumventricular cells. Humorally-mediated hypothalamic $PGE_2$ expression augments thermo-sensitive neuron firing in favour of increased innervation of heat-gain effectors. A dashed line ending in a positive sign extending from one mediator to another indicates an amplifying effect; a dashed line ending in a negative sign indicates an inhibiting effect. Thin lines denote weak expression. Thicker lines indicate strong expression.**

## 4.5 Future Directions

Definite alterations in physiology have been uncovered in the present study, and it is evident that glucocorticoids are not the sole modulating factor of the febrile mechanism near term. Our findings show that corticosterone expression is amplified both in pregnant and non-pregnant animals when stimulated by a significant dose of LPS, suggesting that other physiological factors are also at work at this time. Numerous studies have explored various hormonal, neurological, and behavioural aspects of near-term antipyresis and have added important findings to the literature. Much work is still needed to be done to understand the full significance of the attenuation in core temperature near term, and to fully comprehend the integrative mechanisms that bring about this change. Nevertheless, the results of the experiments outlined in this document provide further evidence of the antipyretic influence that glucocorticoids impart on pregnant rats during late gestation.

The impact of circadian rhythm on body temperature and associated hormonal expression tends to be a recurring aspect of this study. Contrary to diurnal beings, the rat's active period occurs during night. As many studies with rats (including the present study) have occurred during daytime, perhaps further investigation into the involvement of nocturnal versus diurnal thermoregulatory and febrile trends might help our understanding.

This thesis revealed that corticosterone inhibition brings about a significant restoration in the febrile response and evokes changes in the composition of plasma cytokines, yet significant elevations in corticosterone were noted following an infectious-mimicking stimulus in both the non-pregnant and pregnant states. Therefore, near term,

an alteration in the immune system's release of acute phase reactants, with emphasis on the cytokine profile, must be extremely important to the attenuated febrile response. Consequently, there must be another pregnancy-induced factor or cytokine mechanism that is central to antipyresis. Of the cytokines examined, IL-1β expression remained unchanged and IL-6 levels were amplified, but TNF-α was the only protein to show a rapid significant increase in expression following LPS stimulation, as well as a fast reversal to its expression as core temperature similarly reversed due to MET pretreatment. Accordingly, further study of the biological activity of TNF-α and its receptors, along with a deeper examination of its impact on IL-1ra and IL-1 r II expression during gestation may help to unlock the unknown aspects of antipyresis and the attenuated response to pyrogen near term of pregnancy.

## *Chapter Five:*

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