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Quantifying responses to psychological and physiological stress in automotive design

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Quantifying Responses to Psychological and Physiological Stress in Automotive Design.

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ABSTRACT

Background

Attempts to assess psychological stress rely heavily upon subjective techniques which measure changes in perceived mental loading and situational awareness (Hart and Staveland 1988, Reid and Nygren 1988, Lemyre and Tessier 2003, 1998). Although quantitative methodologies do exist, for example monitoring changes in the cardiopulmonary system (Gelfand *et al*. 2004, Harada *et al*. 2006), such parameters are subject to influence by factors other than stress. Psychological stress is known to influence the effectiveness of the innate immune system, leading to an increased risk of infection and immune-related disease (Dhabhar *et al*. 1996, Boscarino *et al*. 1999, Altemus *et al*. 2006). Leukocytes, primarily neutrophils have been identified as an essential component of this mechanism - periods of increased psychological stress have been shown to stimulate neutrophils to release reactive oxygen species into surrounding healthy tissues (Mian *et al*. 2003). The exact biochemical pathways by which this occurs have not yet been fully elucidated. However, this mechanism has become the basis for a novel *in vitro* technique (McLaren *et al*. 2003) which has the potential and sensitivity to rapidly quantify and discriminate between changes in psychological stress, resulting from exposure to short-term low-level everyday life-stressors.

Aims

The overall aim of this research was to further explore the relationship between short-term psychological stress and altered immune responsiveness. Leukocyte coping capacity (LCC) is a luminol-dependent chemiluminescent technique for the assay of reactive oxygen species production in whole blood samples. The feasibility of applying this test as an objective, quantitative, diagnostic measure of altered mental workload (mental stress), in the assessment of ergonomics within automotive research and development was examined.

Methods

Leukocyte activity was determined from whole blood, using a luminol-dependent, *in vitro*, chemiluminescent technique referred to as Leukocyte Coping Capacity (LCC).

The technique measures reactive oxygen species production following phorbol 12-myristate 13-acetate (PMA) stimulation.

Subjective psychological measures, including likert scales and the NASA task load index were employed to assess perceived stress and altered mental workload. Other traditional physiological parameters including heart rate, systolic and diastolic blood pressure and core body temperature were also measured. The ability of each parameter to detect and discriminate between related short-term stressors was investigated, and results were correlated with post-test changes in leukocyte activity.

To investigate the mechanism of stress induced leukocyte activation, standard ELISA was used to assess post-stressor plasma concentration changes in nine mediators including Adrenaline, Noradrenaline, Cortisol, E-Selectin, L-Selectin, Interleukin-1β, Interleukin-6, Endothelin-1, and Tumour Necrosis Factor-α.

All 5 studies involved the use of mental stressors that were associated with either driving or the ergonomics of driving. Participants were moderately fit and healthy, aged between 20 and 65 years. Study one assessed the ability of the LCC technique to objectively discriminate between two closely related stressors (performing a simple manoeuvre in two different vehicles). Study two investigated leukocyte sensitivity, by testing whether a quantifiable response was elicited following exposure to a low-level stressor lasting seconds. The third study was used to explore the mechanism of leukocyte activation following short-term low-level stress. In addition to testing the viability of leukocyte responsiveness as an objective quantitative ergonomic assay for use within the motor industry, study four investigated how the magnitude of leukocyte responsiveness changed following repeated exposure to the same stressor. The final study used leukocyte reactivity to investigate how mental loading was affected during the interaction with three different motor vehicle control interfaces, whilst simultaneously maintaining lane discipline within a simulated driving environment.

Results

This research has shown that leukocyte reactivity is an effective means for objectively quantifying and discriminating between changes in stress levels, resulting from

exposure to short-term low level stressors, encountered as part of daily life. In chapter 3 a significant difference in the magnitude of leukocyte reactivity ($F_{1,38}$ = 5.94, P = 0.02) was observed after the same basic driving manoeuvre was performed in two different motor vehicles (Car A -738.7 ± 185.4 RLU_{adj}; Car B -284.5 ± 96.6 RLU_{adj}). Chapter 4 showed how a mild stressor lasting only seconds resulted in a quantifiable decrease in leukocyte responsiveness, *in vitro*. Subjects adjusted a car electric window, retrofitted with the means to covertly alter its normal operation, to four designated positions. Where modification of the window controls occurred, the decrease in the ability of leukocytes to respond to *in vitro* challenge, via the release of reactive oxygen species, was significantly greater compared to control (Intervention -240.0 ± 56.1 RLU_{adj}, Control -46.2 ± 38.2 RLU_{adj}) ($F_{1,19}$ = 8.23, P = 0.001). The potential of LCC to discriminate between two closely related stressors was tested in chapter 6. The same basic tasks were performed using two different touch screen interfaces from the same motor manufacturer. The magnitude of change in post-test leukocyte responsiveness was significantly different between each interface (Interface A -429.6 ± 177.3 RLU_{adj}, Interface B -86.9 ± 91.9 RLU_{adj}) ($F_{1,39}$ = 2.98, P = 0.04). In chapter 7 the ability of LCC to discriminate between changes in leukocyte responsiveness following interaction with 3 different interface design formats, while simultaneously maintaining lane discipline within a simulated driving environment, was demonstrated. The post-test change in leukocyte activity following the use of Interface A was significantly greater compared to the use of Interface B (P = 0.01 Tukey's *post hoc* procedure) and also Interface C ($P \leq 0.001$ Tukey's *post hoc* procedure). Whereas the use of both Interfaces B and C resulted in post-test changes in leukocyte activity of similar magnitude (P = 0.47 Tukey's *post hoc* procedure).

Studies have shown that repeated exposure to the same stressor results in a habituated response within the cardiovascular system (Veit *et al*. 1997, Bhatnagar *et al*. 2006, Barnum *et al*. 2007). Although evidence exists demonstrating the modification of the immune response during the presence of chronic stress (Altemus *et al*. 2006), there is a paucity of evidence that investigates the effects of short-term mental stress on leukocyte responsiveness. As part of the study described in chapter 6, 4 of the original 15 subjects returned for weekly re-testing on each interface on 2 further occasions. Repeated use of both interfaces resulted in a progressive decrease in the magnitude of post-test leukocyte activity. For Interface A post-test differences proved

significant between week 1 (primary testing) and week 2 (P = 0.03 Tukey's *post hoc* procedure), whereas the magnitude of change was non-significant (P = 0.36 Tukey's *post hoc* procedure) for Interface B. For both Interfaces A and B post-test differences proved non-significant for tests in week 3 (Interface A P = 0.52 and Interface B P = 0.74 Tukey's *post hoc* procedure), suggesting that leukocyte reactivity does exhibit habituation as familiarity to a situation increases. The overall findings of this research have led to the proposal of a novel pathway for eliciting systemic leukocyte activation in response to short-term low-level mental stress (See chapter 8.2 and figure 8.1).

Conclusion

The results presented in this thesis show there is a consistent and significant association between mental stress and leukocyte activity. The findings provide an initial insight into the diagnostic potential of altered leukocyte responsiveness as a quantitative, objective, physiological assay of mental workload. This research applied the technique for assessing ergonomic design within the car industry, however the potential areas for investigation are far reaching.

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1.0 INTRODUCTION

The ability to quantitatively assess stress in an objective and meaningful manner has significant relevance when attempting to evaluate the exceptionally diverse social and environmental interactions in both human and non-human models (Dawkins 1980, Batson and Bradshaw 1997). For example, the ability to quantify the psycho-physiological consequences of domestic livestock husbandry (Tuker *et al*. 2006), assessing the long-term physiological implications associated with extreme psychological stress, as exhibited with post traumatic stress disorder (Altemus *et al*. 2006), also in the evaluation of training regimes in elite athletes (Wolf *et al*. 2007). Of particular relevance, and the basis of this thesis, is the ergonomic evaluation of technologies within automotive design. Current methodologies involve analysis of both the physiological and psychological manifestations of the stress response. These primarily focus upon monitoring characteristics of the cardiopulmonary system, haematological values (Milspaugh *et al*. 2000) and assay of specific stress related hormones including cortisol (Moon and Cho 2001, Hodgson *et al*. 2004, Clow *et al*. 2006, Powers *et al*. 2006) and catecholamines (Brown *et al*. 2003). Evaluating the psychological effects relies upon qualitative assessment of perceived mental loading (Lemyre and Tessier 1988, Syroid *et al*. 2002) and behavioural observation (Rushen 2000). This study aims to both develop and validate a novel technique for quantifying the physiological manifestations of acute, transient psychological stress in humans, using an objective measure for assessing the activation state of neutrophils *in vitro*, which has previously only been applied to animal models.

1.1 STRESS

1.1.1 Definition of Stress

Stress is a fundamental characteristic of life. All animals including humans have a broad range of physiological mechanisms which, when combined, furnish the individual with the ability to endure the challenges they face during life. The stress response is an extremely complex physiological event which results in metabolic, neuroendocrine and behavioural changes.

The concept of stress was first applied to a physiological state of arousal by Canon (1914), who proposed that for an organism to function at its optimum it must possess the ability to maintain a stable internal environment (homeostasis). Any force which acted to perturb the homeostatic state was defined as a stressor; a theory which later resulted in the proposal of the fight-flight response (Cannon 1932). Upon perception of a potential threat the body enters a heightened state of arousal resulting from both endocrine and sympathetic nervous stimulation. The response is characterised by the secretion of catecholamines and increases in cardiopulmonary activity (heart rate, blood pressure and respiration) and fuel mobilisation (blood glucose); all of these serve to prepare the body for a period of strenuous activity (Seeley *et al*. 2003). Under normal conditions once the perceived threat has been resolved, physiological responses return to baseline. Although this is a highly complex topic, fundamentally, stress can be defined as an assemblage of events that begin with a stimulus (stressor), which propagates a reaction in the brain (stress perception), which ultimately results in the initiation and activation of certain physiological systems within the body (stress response) (Dhabhar and McEwen 1997).

As a result of investigating the relationship between psychological stress and the development of disease, Seyle (1956, 1976) provided us with the most influential work on the elucidation of the stress response. In order to explain the potentially harmful effects of prolonged exposure to stress, he proposed a theoretic model referred to as the "General Adaptation Syndrome" (GAS) whereby, the sympathetic nervous system mobilises the organism for action by stimulating physiological systems that are crucial for immediate physical response (e.g. cardiopulmonary system and skeletal muscle), and decreasing energy supply to those organs, which are not so important in a state of emergency (e.g. gastrointestinal system). It was proposed that within its life an organism would be exposed to a myriad of potential stressors, however, the physiological response itself was non-specific with respect to the stressor encountered. Therefore, the individual would manifest the same generic series of physiological reactions, irrespective of the perceived threat. Consequently the impact of repeated or prolonged stressor exposure results in degeneration of the system and formation of certain disease states, including psoriasis, rheumatoid arthritis and asthma.

The GAS model has three main stages: 1) alarm reaction (occurs with both acute and chronic stressors), 2) resistance (at this stage the stressor can be classed as chronic) and 3) stage of exhaustion. In the alarm stage, the body reacts to a stimulus by activating the hypothalamic pituitary adrenal (HPA) axis (Chapter 1.1.3). The resistance stage signals successful adaptation to the stimulus. Exhaustion occurs when exposure to stimuli is prolonged. Selye believed that the body's stores of glucocorticoids (the output of the HPA axis) as well as other physiological resources were depleted. It is now generally believed that the body does not deplete stores of glucocorticoids but that prolonged exposure to a stressor results in suppression of the immune system, which then places individuals at risk of a variety of disease outcomes (Ice and James 2006).

Selye (1956, 1976) proposed the existence of two types of stress. The first was referred to as pleasant stress, which contributed to human well-being. In 2000 Dhabhar demonstrated the beneficial effects of acute stress on immune function, proposing that the acute stress response prepares the immune system for potential challenge in a similar manner as observed for both the cardiovascular and musculoskeletal systems. In contrast, unpleasant stress (now referred to as chronic stress) has been associated with the deregulation of immune function (Dhabhar and McEwen 2001, Saul *et al*. 2005) and is thought to play a role in the aetiology of many disease states including cardiovascular disease, hypertension, arthritis, and immune-related deficiencies (Lovallo 1997). The exact point at which acute stress becomes chronic is still under discussion, however generally any stressor which persists for several hours a day, for weeks, months or years can be classed as chronic (Dhabhar and McEwen 1997, 2001, Saul *et al*. 2005).

It is the panacea-like, non-specific response to both physiological and psychological stressors which is the body's Achilles' heel. Mian *et al*. (2003) reported significant increases in both number and activation of peripheral circulating leukocytes following the observation of an 83-minute fictitious psychologically stressful event (a horror film). While Walsh and Whitham (2006) demonstrated similar leukocyte response trends during exercise within extremes of environmental temperature. The system was highly successful in the time of our ancestors when an individual was likely to encounter a potential predator. However, today we are more likely to voluntarily

expose ourselves to situations which provoke these primitive reactions (Mian *et al*. 2003). It is widely accepted and expected for all of us to exhibit a degree of stress, it has become a part of the modern human psyche. In individuals who are exposed to a high incidence of psychological stress (e.g. such as carers of mentally ill relatives (Keicolt-Glaser 1987), and /or are psychologically ill-equipped to cope with exposure to such stressors (e.g. as with those suffering from post traumatic stress disorder Altemus *et al*. 2006) the non-specific GAS response is to some degree permanently "switched-on", leading to a greater incidence of immune-related diseases (Saul *et al*. 2005).

There are a number of points of contention associated with Selye's GAS model. As previously noted, it does not account for the significant role that psychological factors contribute and places the assumption that all responses to stress are uniform (Hobfoll 1989). It is now believed that psychological appraisal of the situation by the individual is fundamental in the determination of stress (Lazarus and Folkman, 1984a). Furthermore, the stress response can be significantly influenced by an individual's perception, personality, resource availability (internal and external) and biological constitution (Lazarus and Folkman 1984b, Moos 1984). Selye also stated that stress was a product of the GAS, and could, therefore, only exist when the syndrome was operational; confusing the incidence of stress with its end product (Hobfoll 1989). Although severely limited, the GAS model still remains at the foundation of stress research.

The term stress is a highly ambiguous term. Even after 100 years of research, the scientific community is still unable to produce a definitive definition (Segerstrom *et al*. 2004). Throughout the literature the term stress is used to describe both the event and the elicited response to that event. It is also often used in a negative context. For the purposes of this thesis, the term stress will be used to describe an event or events that are perceived by an individual as being a physiological threat or which promotes psychological confusion resulting in increase mental loading that consequently trigger both a psychological and physiological response.

1.1.2 Structure of the Stress Response

The concept of stress can be defined using the terms “Stressor” and “Stress Responses”. Stressors are situations and events that pose an actual or perceived threat or challenge to an individual. These manifest as either physical (where the individual would need to fight or run away) or psychological (promoting emotional distress or melancholic thoughts or feelings). It is more often the case that the stressor is a combination of the two. A typical stress response involves the individual exhibiting a complex interaction of psychological, physiological and behavioural mechanisms. Elliot and Eisdorfer (1982) proposed a taxonomic system where characteristics including the length and magnitude of the stressor were used to categorise the varying forms of stressor into one of five distinct classes.

Class 1 included short-term challenges such as mental arithmetic exercises or addressing an audience which were described as acute time-limited stressors. A brief naturalistic stressor (Class 2) has many of the attributes of class 1, its defining features are that both intensity and duration are greater than is exhibited for a class 1 stressor e.g. a real-life short-term challenge, such as an academic examination. Class 3 stressors include event sequences, including bereavement or involvement in a natural disaster, which results in a series of associated challenges where, although the exact end point of the stressor is unknown, the individual does possess the knowledge that at a future date the stressor will abate. Class 4 stressors relate to chronic stress, where the stressor pervades an individual’s life to such a degree that it forces them to restructure their identity or social role. The primary characteristic of a chronic stressor is its stability i.e. there is no perceived end point. For example, being the carer of a mentally ill relative or being physically disabled as a result of an accident. Distant stressors (Class 5) are often classified as post-traumatic stressors (Altemus *et al*. 2006). These include disturbing events which may have taken place years before, yet due to there emotional and cognitive impact still possess the ability to elicit altered immune functionality (Baum, Cohen, and Hall 1993). Examples include being a prisoner of war, or witnessing the death of a combatant.

1.1.3 Physiology of the Stress Response

Initiation of the stress response, generally referred to as the sympatho-adrenal-medullary response, promotes a significant number of physiological changes; all are designed to prepare the body for "fight or flight", a concept proposed by Canon (1929). When in a serene, unstimulated state, the firing of neurones within the locus ceruleus (a nucleus within the brain stem involved with physiological responses to stress and panic) is minimal. However, when a novel stimulus, such as the perception of danger or panic is perceived, sensory innervation increases. The route of signalling, relayed from the sensory cortex through the hypothalamus, to the brain stem, elicits increased noradrenergic activity within the locus ceruleus, resulting in the individual becoming increasingly alert and attentive to the environment. If a stimulus is perceived as a threat, a more intense and prolonged discharge of the locus ceruleus initiates sympathetic nervous activity (Thase and Howland 1995), which ultimately leads to secretion of catecholamines (adrenaline and noradrenaline) (via innervation by preganglionic sympathetic nerves, activated via acetyl choline) and glucocorticoids, primarily cortisol (via the hypothalamic-pituitary-adrenal axis).

The hypothalamic-pituitary-adrenal axis (HPA axis) is a complex set of direct influences and feedback interactions between the hypothalamus, pituitary and adrenal glands. The HPA axis is a major component of the neuroendocrine system which is responsible for controlling reactions to stress and regulates various systems including digestion, the immune system, mood and sexuality, and energy usage. The paraventricular nucleus of the hypothalamus, consisting of neuroendocrine neurones, is responsible for the synthesis and secretion of corticotropin-releasing hormone (CRH). Which regulates the anterior pituitary gland (adenohypothysis), stimulating the synthesis and secretion of adrenocorticotropic hormone (ACTH) (Rabin *et al*. 1989, Cohen *et al*. 2003). ACTH binds to type-2 g-protein coupled receptors (melanocortin receptor 2 (MCR2) located in the zona fasciculata of the adrenal cortices, stimulating the synthesis and secretion of glucocorticoid hormones (mainly cortisol) (Beuschlein *et al*. 2001). In order to maintain homeostasis, glucocorticoids in turn, act back on the hypothalamus and pituitary (to suppress CRH and ACTH synthesis) in a negative feedback mechanism. It is also true that noradrenaline

stimulates CRH secretion from the hypothalamus, resulting in a positive feedback mechanism during periods of heightened psychological stress and awareness.

Catecholamines (adrenaline and noradrenaline) and glucocorticoids (cortisol) serve to promote the following immediate physical reactions associated with the preparation for violent muscular action (characteristic of the stress response) (Gleitman *et al*. 2004). Increased heart rate and cardiac output results from the association between noradrenaline and cell-surface β_1-adrenergic receptors, causing G-protein-mediated synthesis and accumulation of cyclic adenosine monophosphate (cyclic-AMP) in the cytoplasm of cardiac muscle cells. Cyclic-AMP increases plasma membrane permeability for calcium ions (Ca^{2+}), affecting the rate of cardiac muscle depolarisation, ultimately resulting in increased rate and force of cardiac contraction. The binding of adrenaline with β_2-adrenergic receptors leads to relaxation of cardiac smooth muscle, resulting in vasodilatation. Similar reactions in smooth muscle bring about bronchial dilation (essential for increased oxygen uptake) (Fitzpatrick *et al*. 2004) and also dilatation of arteries to skeletal muscle, in anticipation of increased muscle activity. In contrast, vascular smooth muscle contraction (vasoconstriction) results as a consequence of α_1-adrenergic receptor activation, resulting in the diversion of blood flow away from non-essential organs, e.g. the gastrointestinal system (Sagrada *et al*. 1987) and skin (Schmitz *et al*. 1981). In order to increase glucose metabolism, adrenaline inhibits insulin production and induces glucagon release by binding to α_2-adrenoceptors within the pancreas (Fitzpatrick *et al*. 2004). Cortisol also affects blood glucose concentration by stimulating gluconeogenesis (the synthesis of glucose from non-hexose substrates, including amino acids and glycerol from triglyceride breakdown) (Freeman 2002).

In 1985 Vingerhoets further developed Selye's original revised version of the GAS model by proposing that the initial alarm reaction occurs in two distinct stages; a shock and a counter shock stage (Vingerhoets 1985). The shock stage characteristically consists of an increase in heart rate (β_1-adrenoceptor stimulation), a decrease in both blood pressure and body temperature, and a loss of muscle tone (all associated with β_2-adrenoceptor stimulation). The second stage of the alarm reaction (the counter shock stage) comprises the activation of the pituitary and adrenal axis leading to the secretion of ACTH and adrenocorticoids (as described above). During

the second phase of the model (the resistance phase), increased secretory granule concentration within the adrenal cortex was used as a physical indicator of increased hormone production (Vingerhoets 1985). Vingerhoets (1985) also theorised that during the third and final exhaustion phase, the biological adaptation to the stressor can become diminished or even lost. As a result the system would begin to manifest the characteristics of the shock stage of the alarm reaction.

The psycho-physiological links between stress perception and physiological response, proposed by the GAS model, were further validated when the endocrine system was found to be particularly receptive to psychological influence. Mason (1968) discovered that the presence of psychological stress could be determined through corticosteroid concentration. His studies exposed monkeys to a variety of behavioural conditioning procedures designed to evoke a variety of emotional responses, the resulting emotional stress was accompanied by quantifiable increases in corticosteroid concentration.

Mason (1968) proposed the concept that it was not necessary to look beyond an individual's normal sensory systems for an ancillary mechanism responsible for providing the information necessary to make the determination, if a stimulus is acting as a stressor. This was a key contribution in the elucidation of the stress response. He theorised that the body constantly "senses" the environment in order to interpret the situation. It is this interpretation by the brain which is responsible for perceiving the situation as being a potential threat or challenge.

Both the sympatho-adrenal-medullary and HPA systems are fundamental within stress research. It must, however, be taken into account that neither system exclusively responds to stress, each is constantly operating within the body to maintain homeostasis. For example, cortisol concentration is now one of the primary quantitative measures for assessing the presence of psychological stress. Iellamo *et al*. (2003) investigated the psycho-physiological impact of sports competition on elite pentathletes. He noted significantly increased salivary cortisol concentration during competition compared to normal training days. In 1998, Hucklebridge *et al*. proposed the use of an endogenous inhibitor of Monoamine Oxidase A (MAO-AI) as a means of predicting imminent increased HPA activity and cortisol secretion. MAO inhibitors

interfere with glucocorticoid feedback which increases HPA activity (Kier *et al*. 2005). The circadian pattern of free cortisol and MAO-AI was monitored in saliva, in normal healthy subjects. Hucklebridge *et al*. (1998) showed that MAO-AI concentration was at it highest immediately upon waking and preceded maximum cortisol concentration. In addition, subjects who exhibited persistently elevated MAO-AI demonstrated a more prominent cortisol response. Further research conducted by Clow *et al*. (1999) assessed changes in both cortisol and MAO-AI concentration in prepubertal pigs following two different experimental paradigms of HPA activation. The first involved a period of physical restriction (snared for 15 minutes), whereby post-stressor increases in both the concentration of cortisol and MAO-AI were recorded. The second experiment utilised bacterial endotoxin to stimulate HPA activation. In this instance, although increased cortisol concentration was again recorded, it was not preceded by an increased concentration of MAO-AI. These findings postulated that the pathway utilised during psychological stress-induced MAO-AI HPA activation was different to the mechanism associated with immunological HPA activation.

All objective measures of stress, including alterations in cortisol concentration, and in the parameters measured during the research presented within this thesis (heart rate, blood pressure, body temperature and also leukocyte activity) can identify the presence of a stressor by providing an indication that the normal homeostatic balance has been disrupted, however, none of the techniques are able to confirm what the stressor is.

Knowledge has now progressed regarding the interrelationship between the neural, endocrine and immune systems. Besodevsky *et al*. (1986) was the first to demonstrate the close association between the immune system and HPA axis, proposing the existence of a negative feedback mechanism between the two systems which aided in the control of the immune response. Upon activation, macrophages release the cytokine Interleukin-1 (IL-1), which, in turn stimulates the release of CRH from the hypothalamus. The increase in CRH results in amplified glucocorticoid secretion which serves to down regulate macrophage activity. Additional research has lead to further expansion of the mechanism, demonstrating the ability of both IL-6

and tumour necrosis factor-α in eliciting increased HPA activity during periods of homeostatic imbalance (Turnbull and Rivier 1995).

1.1.4 Effect of Stress on the Immune System

As a consequence of attempting to elucidate the effects of stress on immune function a number of often diametrically opposing conclusions have been formed. One such paradox which has arisen from the current literature is that in some reports stress is described as having a suppressive effect on immune activity (Borysenko *et al*. 1982, Khansari *et al*. 1990, Cohen *et al*. 1991, Kort 1994, Maier *et al*. 1994, Clark *et al*. 2002, Miller *et al*. 2002), whereas others describe how the immune response can become enhanced (Dhabhar 1997, Boscarino and Chang 1999, Viswanathan *et al*. 2007). For the purposes of this thesis acute stress will be defined as stress which exists for a duration of minutes to hours, whereas chronic stress persists for several hours a day for weeks or months.

1.1.5 Acute Stress and the Immune System

Acute stressors often precede or accompany immune challenges and have been shown to be a prerequisite for effective immunoprotection (Viswanathan and Dhabhar 2005). Dhabhar (1997) explained how the psycho-physiological stress response is an important modulator of skin immunobiology in health and disease. It was demonstrated that acute stress had the effect of enhancing cutaneous immune function, through increased leukocyte trafficking to a site of injury. He goes further by explaining that during stress perception, neural stimulation induces the release of neuroendocrine mediators which prepare the immune system to face challenges (wounding or infection) that may be imposed by a stressor (attack by a predator) (Chapter 1.1.3). This increased immune preparedness may be mediated by the pre-emptive trafficking of leukocytes to potential sites of infection (e.g. skin and skin-draining lymph nodes).

These findings were further elucidated by Viswanathan *et al*. (2007) who demonstrated that acute psychological stressors (lasting a period of minutes to hours), induced potent immune-enhancing effects, which were potentially both detrimental (through exacerbation of immuno-pathological disease) and beneficial (promoting immune-protection during wounding or infection).

Acute psychological stressors can also be immuno-suppressive, resulting in an increased risk of infection or disease (Boscarino and Chang 1999, Boscarino 2008). Epidemiological evidence indicates that individuals who are psychologically stressed demonstrate increased susceptibility to opportunistic infection (Galinowski 1997). Clover *et al*. (1989) showed that children belonging to dysfunctional families demonstrated increased susceptibility to influenza A infection.

1.1.6 Chronic Stress and the Immune System

In conjunction with stressor intensity, duration of exposure has now been shown to significantly influence the nature of the immune response. Exposure to chronic stressors (lasting days to weeks) has been shown to suppress immune responsiveness. Miller *et al*. (2002) reported how parents of cancer patients, who exhibited chronic psychological distress, demonstrated impaired immune responsiveness to anti-inflammatory signals, with a reduction in the ability of a synthetic glucocorticoid hormone to suppress *in vitro* Interleukin-6 (a pro-inflammatory cytokine).

A subsequent study demonstrated that the intensity of the chronic emotional stressor need not be as dramatic as exhibited, for example, by the carer of a sick child. The normal daily pressure of a full time healthy student can result in a poor immune response to the administration of the influenza vaccine (Miller *et al*. 2004). Pressman *et al*. (2005) also focused upon the chronic stressors faced within higher education, describing how extended periods of loneliness and small social networks were independently associated with a diminished immune response to a component of the influenza vaccine.

The presence of pre-existing mental or psychological conditions must be taken into account during the evaluation of short-term mental stressors. Blechart *et al*. (2007) demonstrated how an underlying chronic stressor (such as post traumatic stress disorder) significantly influenced heart rate and electrodermal response, during acute psychological stress, leading to blunted responses in both parameters when compared to healthy controls. A study by Bhatnager *et al*. (2006) reported increased body temperature in rats (*Rattus norvegicus*) that had experienced a period of social defeat (psychological stress) compared to controls.

Chronic stressors which persist for years rather than months, such as with post traumatic stress disorder (PTSD) have been associated with the dysregulation of both the HPA axis and sympatho-adrenal-medullary (SAM) system (Boscarino 2004). Both are associated with modulating immune function, suggesting that immune activity would also be affected (Altemus *et al*. 2006). These finding support those of Boscarino *et al*. (2004) who demonstrated how war veterans who had been diagnosed as suffering from PTSD exhibited substantially elevated (50-150% greater) levels of numerous chronic disease states, affecting the circulatory, digestive, mucoskeletal, nervous and respiratory systems. Of those tested, all exhibited abnormally low cortisol levels (suggesting a possible mechanism for the stress / disease relationship). Cohen and Manuck (1995) demonstrated that psychological stress was associated with a greater incidence of upper respiratory infectious illness. While Glaser *et al*. (1999) concluded that psychological stress exerts a negative influence on pro-inflammatory cytokine production within an area of tissue damage, which served to slow or even inhibit wound healing.

The existence of these paradoxical relationships can be accounted for due to the bi-directionality of the effects that stress imparts on immune functionality. This allows for the concept that acute stress can elicit immune enhancement, whereas chronic stress can be immunosuppressive.

1.1.7 Stress and Disease

It has long been hypothesised that stress is a major contributory factor in the aetiology of numerous disease states. Research has provided us with a vast quantity of circumstantial evidence supporting this relationship, however the mechanisms by which stress contributes to the development of certain disease pathologies has not yet been fully elucidated. There are at least 3 distinct physiological systems which can be affected by stress: The sympatho-adrenomedullary (SAM) system, the hypothalamic-pituitary-adrenocortical (HPA) axis, and the immune system (Chapter 1.1.3). The physiological and psychological changes manifested as a consequence of stress exposure can result in the development of illness. Cohen *et al*. (1991) demonstrated a direct relationship between the duration of stress exposure and the probability of developing upper respiratory illness.

In today's society stress is often referred to as being detrimental, however evidence now suggests that upon exposure to brief stressors the immune response can become amplified, causing an increased production and secretion of hormones and locally acting cytokines which aid in the attempt to surmount the physiological changes. In so doing, this form of stress causes adaptation of the immune response, which can be particularly beneficial for an organism (Viswanathan *et al*. 2007). Acute stress can also amplify mental alertness and cognitive ability (Chapter 1.1.3) (Thase and Howland 1995), both of which are essential for coping with environmental stimuli and successful completion of everyday tasks e.g. driving a motor vehicle. If the stress response persists even after the initiating stressor has abated, or if the response remains "switched on" due to the presence of disproportionate levels of stress, then damage due to cumulative changes can result (Altemus *et al*. 2006, Slominski 2007), leading to degeneration of body tissues and even biological systems, especially the cardiovascular system. McEwen and Stellar (1993) referred to the physiological costs of chronic exposure to the neural or neuroendocrine stress response as the Allostatic Load.

It can, therefore, be concluded that both stress and specific stressors can elicit different effects upon an organism depending upon the duration of exposure. A technique capable of quantifying immunocompetence as a consequence of stress

exposure has been developed for this study. In this approach stress-induced decreased immunocompetency is measured, this can be defined as the transient reduction in immune system function that occurs during and after a stressful event.

Evidence exists that links stress with an increased susceptibility to disease in both humans (Dhabhar *et al*. 1996, Viswanathan *et al*. 2005, Boscarino 2008) and animals (Kang *et al*. 1996, Gatien *et al*. 2005). Chronically stressed mice (enduring a two week period of confinement) demonstrated increased susceptibility to UV-induced squamous cell carcinoma compared to controls (Saul *et al*. 2005). High stress levels have been identified as a contributory factor in many disease states exhibited by humans. Altemus *et al*. (2003) described how delayed-type hypersensitivity (DTH) reactions (an antigen specific cell mediated immune response) were enhanced in women with PTSD who suffered childhood abuse. Clow and Hucklebridge (2001) highlighted the association between exposure to periods of physical exertion and increased vulnerability to negative health conditions and susceptibly to injury. Epidemiological studies in humans have provided evidence to suggest that individuals who exhibited a greater incidence of psychological stress were also more susceptible to opportunistic infections (Clover *et al*. 1989, Galinowski 1997). It is the stress-illness relationship that is the reason that pre-existing psychological, as well as physiological vulnerabilities, must be taken into account during the diagnostic process.

Components of the innate (non-specific) immune system (specifically neutrophils and macrophages) have been shown to exhibit adverse changes (both in terms of number and function) following marathon-type exertion. During this period of immune dysfunction, the risk of subclinical and clinical infection is greatly increased (Nieman 2007). Cooper *et al*. (2007) describes how exercise elicits dysregulation of the inflammatory response, resulting in detrimental physiological conditions, such as anaphylaxis, exercise-induced asthma and exacerbation of intercurrent illnesses. Cooper *et al*. (2007) proposed the existence of a pathophysiological mechanism involving exercise modulation of previously activated leukocytes, citing an example of how food-sensitised immune cells remain relatively innocuous until they are redistributed during exercise from gut-associated circulatory deposits (e.g. the spleen)

into the central circulation, where they elicit an acute systemic allergic reaction (exercise anaphylaxis).

Stress also has the ability to indirectly affect a disease state through the modification of an individual's behavioural patterns (primarily those involving health). Evidence for this relationship was identified by Cohen and Williamson (1988). Subjects within their study who reported increased levels of perceived stress reported increased alcohol consumption, interrupted sleep patterns and not wanting to eat breakfast.

This evidence seems to suggest that pre-existing vulnerabilities and behavioural changes may result in the amplification of specific illness precursors (such as symptoms), which ultimately result in the development of illness behaviour or illness itself.

In addition, it was determined that the factors that moderate the stress-illness relationship (age, diet, exercise, personality, sex and smoking) had no effect on the individual's resistance to the disease state. This provides an indication that it is the appraisal mechanism, along with coping resource assessment, which underlies the body's ability to defend against the stressor. The progression of stress into illness exhibits a greater incidence amongst individuals who are regularly exposed to periods of psychological stress, or in those who are less emotionally equipped to deal with the occurrence of psychological stressors.

1.2 LEUKOCYTES

Evidence linking psychological stress with altered immune responsiveness demonstrates how, as with the cardiovascular and mucoskeletal systems, the immune system undergoes neuro-endocrine-immune interactions which serve to prepare it for potential physical challenge (Dhabhar 2003). Leukocytes commonly referred to as white blood cells, play a pivotal role in defending the body from microbial attack, inflammation and healing. To date there is little systemic evidence to elucidate the relationship between stress and leukocyte activation.

Leukocytes are the primary cells of the immune system. In general are larger than erythrocytes and are far fewer in number (1µl of whole blood consists of approximately $5.0x10^6$ erythrocytes, but only $7.3x10^3$ leukocytes (Table 1.1). The principle role of leukocytes in the defence against microbial infection was first described in the nineteenth century when it was discovered that leukocytes would accumulate around sharp thorns which had been deliberately inserted into a mollusc. It was concluded that these cells, which were described as "wandering cells", were associated with the defence of the mollusc (Metchnikoff 1883).

There are 6 main classes of leukocyte, all of which are produced in the bone marrow from stem cells by a process of differentiation referred to as haematopoiesis (Figure 1.1). They are grouped according to their function and morphology. Granulocytes (also referred to as Polymorphonuclear Leukocytes) possess prominent cytoplasmic granules, all leukocytes which belong in this group are named according to the staining properties of their granules. The intensity of granule staining within Neutrophils is particularly low, and is therefore referred to as "neutral", Basophilic granules stain dark blue with basic alkaline dye, and Eosinophilic granules stain pink with Eosin (an acidic dye).

The second group, referred to as Mononuclear Leukocytes, which include Lymphocytes and Monocytes (macrophage precursors), are all agranular in appearance.

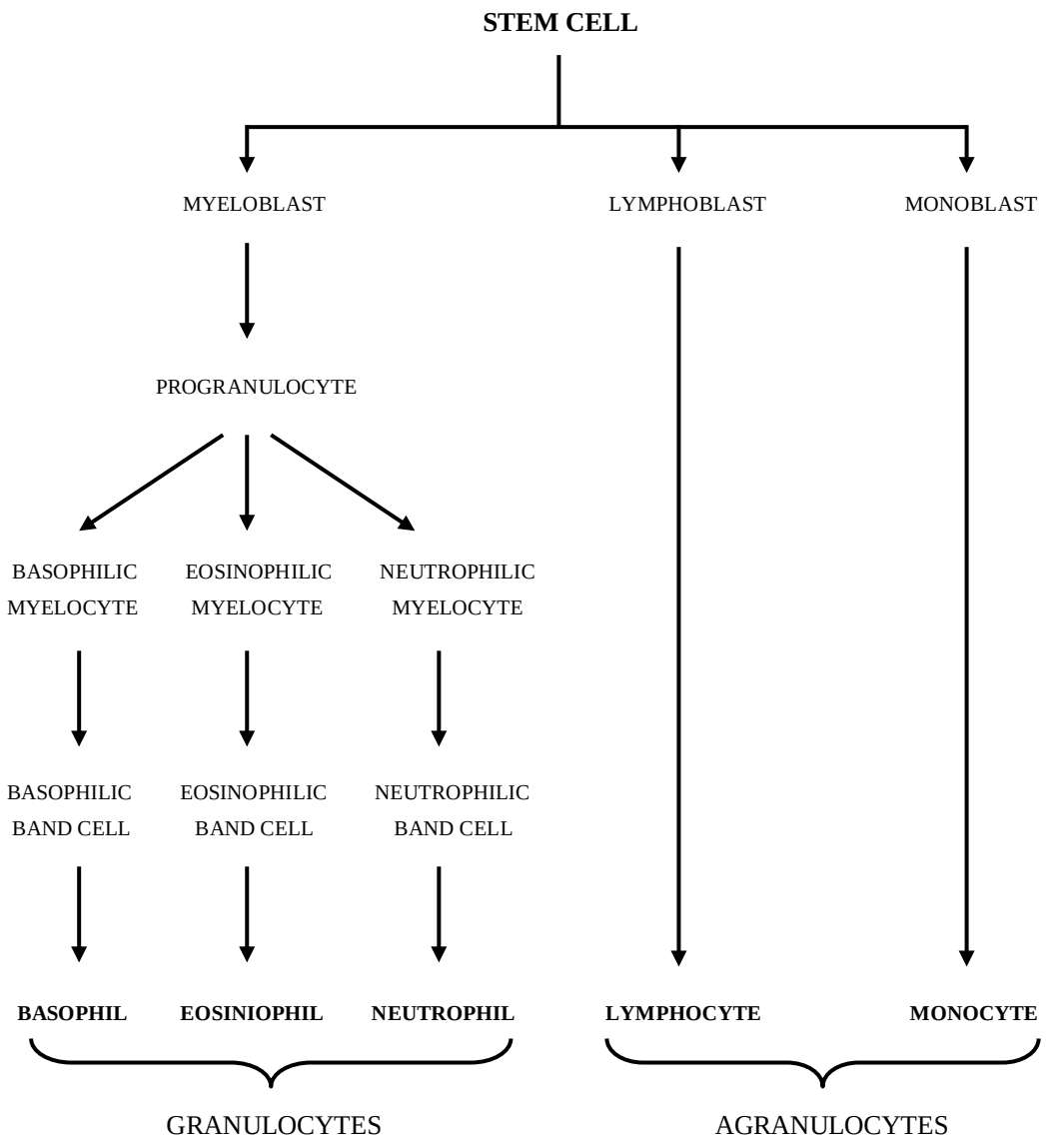


Figure 1.1 Differentiation of precursory hematopoietic stem cell to form various types of leukocyte (Adapted from Seeley *et al*. 2003).

Eosinophils

Eosinophils are easily identified by the bright pink staining granules (with a haematoxylin and eosin stain (H and E stain) within their cytoplasm (Figure 1.2), which contain chemical mediators including, histamine and proteins such as eosinophil peroxidise, RNase, DNases, lipase, plasminogen and major basic protein. All are released by a process called degranulation following activation, and are toxic

to both parasite and host tissues. In addition, eosinophils play a role in fighting viral infections (which is evident from the abundance of RNases they contain within their granules), and in fibrin removal during inflammation. Eosinophils are considered the main effector cells in allergic responses and asthma pathogenesis and are associated with disease severity. They also fight helminth (worm) colonization and may be slightly elevated in the presence of certain parasites. During normal homeostatic functioning very few eosinophils are present in the peripheral circulation, only accounting for between 1 and 3% of the leukocyte population (Table 1.1). Eosinophils have a life-span of only 6-12 hours. Most can be located within medulla and the junction between the cortex and medulla of the thymus, and, in the lower gastrointestinal tract, ovary, uterus, spleen, lymph nodes, and within the connective tissue of the skin (Weater *et al.* 1993). They are only present in the lung, oesophagus and other primary internal organs during disease.

Basophils

Basophils represent about 0.01 to 3% of circulating leukocytes (Table 1.1) and are easily recognised in an H and E stained blood smear by the presence of large, dark blue granules in their cytoplasm (Figure 1.2). Basophils possess commonalities with mast cells, in that they both contain chemicals involved in allergic and immune inflammatory responses (Weater *et al.* 1993) including, histamine and proteoglycans (e.g. heparin). These commonalities caused speculation that they were both the same cell. However, evidence now suggests that they are generated by different precursor cells in the bone marrow. As with eosinophils, basophils are located primarily in the connective tissues of the skin, lungs and gastrointestinal tract.

Monocytes / Macrophages

Monocytes (Figure 1.2) are the pre-cursory cells of Macrophages, totalling between 1 and 6% of the leukocyte population (Table 1.1). Monocytes are formed in the bone marrow and spend on average 8 hours travelling within the systemic circulation to their permanent site of activity within the body's tissues. In leaving the circulation, rounded monocytes enlarge and differentiate into macrophages. Some macrophages spend their lives patrolling body tissue, moving between other cells via amoeboid motion, whilst others remain in fixed locations. Monocytes are responsible for

phagocytosis (ingestion) of foreign substances in the body. Phagocytosis is the cellular process of engulfing solid particles by invagination of the cell membrane to form an internal phagosome (food vacuole). The phagosome is usually delivered to the lysosome, an organelle rich in lytic enzymes which catalyse the breakdown of cellular components, which fuses with the phagosome. The contents are subsequently degraded and either released extracellularly via exocytosis, or released intracellularly to undergo further processing.

Monocytes are larger and more effective that neutrophils, capable of ingesting up to 100 bacteria / antigens during their life span. They also have the ability to remove defective erythrocytes and dead neutrophils (Weater *et al*. 1993).

Macrophages contribute to the development of acquired immunity. Upon ingestion and digestion of molecular or cellular antigens, fragments of the antigenic material are processed and inserted into the macrophage membrane as part of surface protein complexes. The sub-class of macrophage which posses this ability are also referred to as Antigen-Presenting Cells (APCs).

Lymphocytes

Lymphocytes are categorised into two broad groups according to their appearance under the light microscope – large granular lymphocytes, commonly referred to as natural killer cells (NK cells), and small lymphocytes (T and B cells). Lymphocytes are an important component of the immune system, representing between 20-30% of the total leukocyte population (Table 1.1). When viewed, using a Wright's stained peripheral blood smear, a typical lymphocyte has the appearance of a large, dark staining nucleus with little to no basophilic cytoplasm (Abbas and Lichtman 2003). Occasionally, lymphocytes present a clear perinuclear zone (referred to as a halo) around the nucleus or a small clear zone to one side of the nucleus. Another important characteristic of lymphocytes are polyribosomes, these are involved in protein synthesis, and aid in the production of large quantities of cytokines and immunoglobulins (Abbas and Lichtman 2003).

NK cells

NK cells are a part of the innate immune system and play a major role in defending the host from both tumours and virally infected cells. NK cells distinguish infected cells and tumours from normal and uninfected cells by recognizing alterations in levels of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules which then destroy the altered cells. They were named "natural killer" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I (Janeway *et al*. 2001).

T cells and B cells

T cells and B cells are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity whereas B cells are primarily responsible for humoral immunity (relating to antibodies). The function of T cells and B cells is to recognize specific “non-self” antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells. B cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens some T cells, called *helper T cells* produce cytokines that direct the immune response whilst other T cells, called *cytotoxic T cells,* produce toxic granules that induce the death of pathogen infected cells. Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of *memory cell*s. Throughout the lifetime of an animal these memory cells will “remember” each specific pathogen encountered, and are able to mount a strong response if the pathogen reoccurs.

1.2.1 Neutrophils

These are the most abundant class of leukocyte, making up between 50-70% of the total (Table 1.1). They are also the most easily identifiable, consisting of a segmented nucleus of three or five lobes connected by thin strands of nuclear material (Weater *et al*. 1993) (Figure 1.2). This morphological characteristic gives rise to the term polymorphonuclear leukocyte.

Neutrophils are phagocytic cells which have the ability, on average, to ingest and lyse between 5 and 20 bacteria during their programmed lifespan of one to two days. They are normally found in the systemic circulation. However, during the acute phase of inflammation, particularly as a result of bacterial infection, neutrophils leave the vasculature (diapedesis) (Chapter 1.2.4), and migrate toward the site of inflammation in a process called chemotaxis (Chapter 1.2.5). Neutrophils react within an hour of tissue injury and are the hallmark of acute inflammation.

Neutrophils, like all blood cells arise from a progenitor cell called the haematopoietic stem cell. They are synthesised by a process called haematopoiesis within the bone marrow (Figure 1.1). Under specific circumstances, such as infection, the number of neutrophils released from the bone marrow can be increased; a phenomenon known as leukocytosis (Oliveira *et al*. 2008). The number of neutrophils present in blood is often used as a diagnostic tool, for determining the presence of infection (Golob *et al*. 2008).

Taken from Tagliasacchi and Carboni (1997).

Cell Type	Cells mm^{-3}	Percentage Composition
Erythrocytes	5.0×10^6	
Platelets	2.5×10^5	
Leukocytes	7.3×10^3	
Neutrophil		50 – 70
Lymphocyte		20 – 30
Monocyte		1 – 6
Eosinophil		1 – 3
Basophil		< 1

Table 1.1 Composition of Blood: Approximate values for the components of blood in a normal adult (Adapted from Seeley *et al*. 2003).

1.2.2 Neutrophil Pools

The synthesis and circulation of neutrophils, requires the activity of several interrelated "pools" of cells (Cronkite and Fliedner 1964) (Figure 1.3). The most primitive of these is the progenitor pool, consisting of stem cells and other progenitor cells that are not yet fully dedicated to neutrophil production. Committed neutrophil precursors, which still possess the ability to divide, belong to the more mature proliferative pool. These include promyelocytes, myelocytes, and myeloblasts (Figure 1.1). The third storage pool contains neutrophils, including metamyelocytes and band cells. Neutrophils that are within the systemic circulation are grouped within the circulating neutrophil pool. Whereas neutrophils that have adhered to the vascular endothelium, a precursory stage in neutrophil recruitment and activation during the inflammatory response (Chapter 1.2.1), belong to the marginated pool.

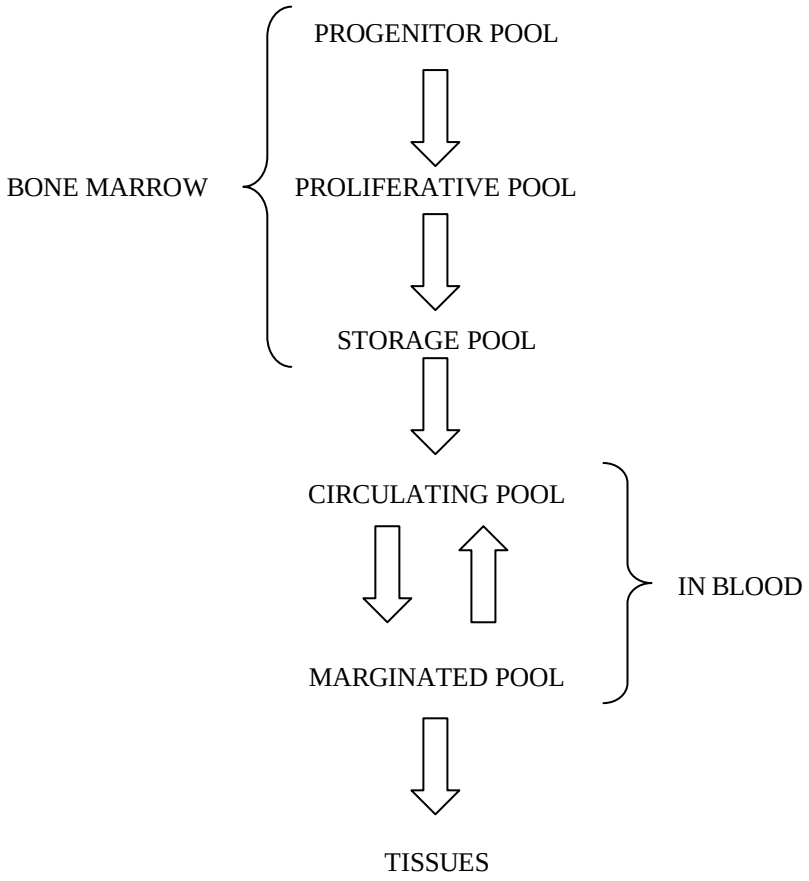


Figure 1.3 Mechanism of neutrophil synthesis – cellular phases.
(Adapted from Cronkite and Fliedner 1964).

1.2.3 Psychological Stress and the Immune Response

The interrelationship between the immune and nervous systems was first proposed by Solomon (1964), leading to the development of a new branch of science referred to as Psychoneuroimmunology (PNI). Ader and Cohen (1991) further expanded the degree of interaction to include not only the immune and nervous systems but also the neuroendocrine system. Primarily research within this field focused upon the physiological mechanisms that interconnected what were previously thought to be discrete, physiological systems. Later two further branches of research developed which attempted to elucidate how the psychological component affects immune activity and conversely how immunity affects psychological disorders and behaviour.

To date PNI has primarily explored the connectivity between the central nervous system (CNS) and immune system, investigating psychological stress-induced neuroendocrine modification and its effects on immune reactivity (Maier *et al*. 1994, Hori *et al*. 1995, Anisman *et al*. 1996). However, evidence now exists that demonstrates that the immune response is activated in response to both physical and also psychological cues. Evans *et al*. (1997) reported that severe or chronic stress could potentially elicit deleterious effects on the immune system and also physical health. Current research has demonstrated that psychological stress can either diminish or enhance the effectiveness of the immune response, dependent upon duration of exposure (Chapter 1.1.6).

Preliminary research postulated that it was the stress hormones (adrenaline, noradrenaline, and cortisol) that were responsible for modulating psychological stress into a physiological disease state (Canon 1932, Seyle 1956) (Chapter 1.1.3). More recently, as well as the secretion of catecholamines and corticosteroid hormones, psychological stressors have been shown to initiate the secretion of prolactin, oxytocin and rennin (Van de Kar and Blair 1999) as well as nitric oxide (Lopez-Figueroa *et al*. 1998).

Although the science behind our understanding of humoral and cell mediated immunity is now fairly well understood, it seems strange that the emphasis has been placed upon investigating the role of natural killer cells, which only constitute approximately 1% of the total leukocyte population. In contrast the mechanisms involved with polymorphonuclear leukocytes (PMN), specifically neutrophils (which make up between 50 and 70% of the total population of leukocytes (Table 1.1) (Seeley *et al*. 2003), have only recently, within the last decade begun to be elucidated.

Neutrophils possess the ability to rapidly respond to chemotactic stimuli such as N-formyl peptides (e.g. fMLP), complement derived C5a, Leukotriene B_4, Interleukin-8 and Platelet Activating Factor (Chapter 1.2.5), causing them to migrate through epithelial cell walls to localised areas of infection or damage (Chapter 1.2.4 and 1.2.6). Neutrophils are the most common form of leukocyte associated with the acute inflammatory response, a short-term process involving the infiltration of leukocytes and plasma into areas of tissue damage, initiated by the secretion of

vasoactive amines (e.g. histamine) and eicosanoids, prostaglandins and leukotrienes (potent paracrine and autocrine mediators). The responsiveness of neutrophils to chemoattractive agents, resulting in increased number and activity, makes them an ideal candidate as a bio-indicator for the presence of stress (physiological and psychological) (McLaren *et al*. 2003).

Studies have demonstrated the potent antimicrobial / bactericidal ability of neutrophils and PMNs during the inflammatory response (Li *et al*. 2008). However, the same response mechanisms have also implicated neutrophil activity in the development of a number of disease states which involve damage of healthy tissue. These include asthma, chronic granulomatous disease, emphysema, myocardial infarction, rheumatoid arthritis and thermal injury (Malech and Gallin 1987, Lublin *et al*. 2002, Fabro and Frenia 2008).

Neutrophils synthesise and release in excess of 50 proteolytic enzymes, products of arachidonic acid metabolism such as eicosanoids (prostaglandins, prostacyclines, thromboxanes and leukotrienes) and toxic oxygen metabolites – including reactive oxygen species (ROS) (Weiss 1989). All have the potential to cause significant damage to healthy tissue. During normal phagocytic ingestion and removal of invading pathogens and bacteria these mediators are safely contained within phagocytic vesicles within the PMN. However, during periods of external stimulation, such as psychological stress, the phagocytic reaction is directed extracellularly which results in damage to surrounding tissues and can lead to development of numerous disease states (Boscarino 2008, Paravicini and Touyz 2008).

It is now accepted that there is an increase in leukocyte number and changes in distribution during periods of physiological stress (Shephard and Shek 1996). A series of related studies conducted by Gleeson *et al*. (1991 and 1993) reported that leukocytosis from the marginated pool occurred following physical exercise. More recently, it has been demonstrated that psychological stressors, such as caring for a sick relative (Kiecolt-Glaser *et al*. 1995), participating in academic examinations (Kang *et al*. 1996, Maes *et al*. 1998), or observing a fictitious stressful event, i.e. a horror film (Mian, Shelton-Rayner and Harkin 2003), have the potential to elicit

similar response mechanisms. These changes were found to be rapid and reversible. The neutrophil is key in the defence of the body against infection and disease; however its phagocytic resources are finite. If activation occurs as a consequence of psychological stress, the body's defence mechanism would be ill-equipped to respond to opportunistic infection (Clover *et al*. 1989, Galinowski 1997), potentially resulting in increased susceptibility to disease.

1.2.4 Neutrophil Recruitment and Adhesion

Neutrophil activity is essential for phagocytosis, as well as the initiation and maintenance of the inflammatory response. Neutrophils pre-synthesize and store many inflammatory mediators in secretory granules within their cytoplasm, which upon activation, are released at the site of inflammation.

Upon arrival at a site of inflammation neutrophils undergo a sequence of morphological changes, mediated via the activation of numerous ligands, receptors and cellular mediators, including, β2 integrins, leukocyte function-associated antigen-1 (LFA-1) and macrophage differentiation antigen-1 (MAC-1). These allow them to adhere to the endothelium (Kuijpers *et al*. 1990). Via diapedesis, a form of amoeboid movement, the neutrophil then migrates through endothelial cell junctions to the site of inflammation (Chapter 1.2.6) using chemoattractant gradients as a guide (Chapter 1.2.5).

The physiological prerequisite for inflammatory leukocyte endothelial transmigration is endothelial activation. In order to occur this requires both transcription and protein synthesis. Activation results in the production of inflammatory mediators, up-regulation of adhesion molecules (Chapter 1.2.6), and secretion of chemoattractants (Chapter 1.2.5) from the endothelium, all these factors contribute to transmigration. *In vivo* analysis has indicated that the most likely stimulus for endothelial activation is the local production of cytokines and other inflammatory mediators including histamine and thrombin, released as a consequence of tissue damage (Ryan and Worthington 1992). Psychological leukocyte activation occurs with the absence of

infection, tissue damage and inflammation, to date there is a paucity of knowledge regarding the mechanisms responsible for this form of activation

There are three main structurally distinct groups that can be used to categorise the molecules known to be associated with leukocyte-endothelium interaction (the initial precursory stage in leukocyte recruitment). The function of each is controlled by a diverse set of signalling pathways: The Integrin Family, the Immunoglobulin (Ig) Gene Superfamily and the Selectin Family (Simons and Green 2005).

The integrin family consists of counter receptors for both Intercellular Adhesion Molecules (ICAMs) and Vascular Cell Adhesion Molecule-1 (VCAM-1) (Hynes 1987, Helmer 1988). It is the function of integrins to mediate both cell to cell and cell to extra cellular matrix interactions. Integrins are a form of plasma membrane receptor, consisting of an α and β polypeptide chain. Integrins are classified by their β chains. Integrins utilising the β_1 gene product belong to the very late activation antigen (VLA) family; Leukocyte Cell Adhesion Molecules (LeuCAMs) exploit β_2. Cytoadhesins employ the β_3 gene product, and have been shown to exist at particularly low concentrations. It is for this reason that the emphasis for research has concentrated on both β_1 and β_2 (Hynes 1987, Phillips *et al*. 1991, Ruoslahti 1991).

The Immunoglobulin (Ig) family (also referred to as antibodies) includes a range of molecules that are associated with the mechanism of leukocyte adhesion and transmembrane signalling. Examples include ICAM-1, ICAM-2, and VCAM-1. Hunkapillar and Hood (1989) describe how these are all expressed by endothelial cells. All the Ig family members bind to ligands present on leukocytes in order to facilitate adhesion and migration. Binding or "coating" an antigen / pathogen with immunoglobulin, specifically IgA, G and E, acts to stimulate phagocytic activity. Immunoglobulins possess a "Y" shaped structure (Figure 1.4). The tips of the Y contain the sites that binds antigen, and therefore, recognises specific foreign objects (e.g. pathogens). This region is referred to as the Fab (fragment antigen binding) region (Putnam *et al*. 1979). While the base of the Y (the Fc region), plays a role in modulating an appropriate immune response i.e. leukocyte recruitment and activation. This configuration allows the Ig molecule to act as a receptor to which the leukocyte

is able to recognise and bind to via a membrane bound Fc receptor (Huber 1980, Anderson 2003).

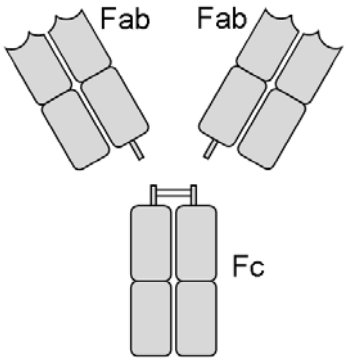


Figure 1.4 Basic immunoglobulin structure.
(Adapted from Seeley *et al*. 2003).

1.2.5 Chemotaxis and Chemoattractants

The ability of being able to rapidly migrate to areas of infection and destroy invading micro-organisms, has labelled the neutrophil as being the immune system's first line of defence. It is the release of chemoattractants, ether released from the bacteria as a by-product of infection, or synthesised by the host itself as a consequence of infection, which act as a biochemical "roadmap" to direct neutrophil migration to the site of infection (Cassimeris and Zigmond 1990). Many chemoattractants also initiate the activation of NADPH oxidase (Chapter 1.2.7).

The migration of neutrophils from the peripheral circulation to an area of inflammation is initiated via the interaction of specific chemoattractants with neutrophil plasma membrane receptors. Numerous cascade reactions are then used to transmit the signal into the cell interior. Once chemoattractant stimulation occurs, neutrophils undergo rapid structural changes from round smooth cells, to elongated cells with discernible pseudopodia (Figure 1.5). The neutrophils then travel in the direction of increasing chemoattractant concentration, via a process of reversible substratum attachment.

Neutrophils are receptive to a number of different chemoattractants, these include: Complement Derived C5a, Interleukin-8, Leukotriene B_4, N-formyl peptides (e.g. fMLP) and Platelet Activating Factor. Chemoattractant receptors belong to the GTPase- coupled receptor superfamily. It is believed that these receptors are coupled to G proteins in order to facilitate intracellular signal transmission. In this manner the G proteins are acting as an intermediary for the cell surface receptors and effector enzymes responsible for second messenger generation.

1.2.6 Leukocyte Migration

The process of leukocyte migration through the endothelium can be sub-divided into 5 distinct stages: Capture, rolling, slow-rolling, firm adhesion and transmigration (Figure 1.5).

Figure 1.5 **Diagram illustrating the progressive leukocyte adhesion and activation cascade** (Biological Research Information Center 2003).

The initial contact between leukocyte and activated endothelium is referred to as "Capture". This process occurs following margination, a process which positions the leukocyte close to the endothelium, away from the central blood flow. Endothelial activation is a prerequisite during the inflammatory response in order to initiate capture. Endothelial stimulation by molecules such as histamine or thrombin, during inflammation, causes the rapid expression of P-selectin on the surface of the activated vascular endothelium. This has the effect of making the endothelium "sticky" to leukocytes. The complementary ligand P-Selectin Glycoprotein Ligand-1 (PSGL-1) or (CD162), expressed on all lymphocytes, monocytes, eosinophils and neutrophils, binds with P-selectin facilitating initial leukocyte capture (Hattori *et al*. 1989, Geng *et al*. 1990, Li *et al*. 1996).

Initial studies into leukocyte-endothelial interaction elucidated cooperative association between integrins and selectins (von Andrian *et al*. 1991). It has now been shown that a continuum of adhesive interactions exists, which utilises the progressive binding of β2-integrins during there conversion from low to high-affinity binding of Intercellular Adhesion Molecule-1 (ICAM-1).

Following successful capture, leukocytes adhere, and begin a process of rolling along the endothelium, at a velocity that is below that of all other free flowing cells. It is the Selectin family, primarily P-Selectin, which mediates the rolling process (Chen and Springer 1999). Lawrence and Springer (1991) and Chen and Springer (1999) describe how the rolling process is achieved through rapid bond dissociation, allowing cells that are subjected to hydrodynamic drag forces, to free themselves at the trailing edge whilst simultaneously forming new attachments at the leading edge. During the initial phases leukocyte integrins remain in their resting conformation, and endothelial immunoglobulin remains at control levels.

In the absence of P-selectin it is E-selectin which becomes responsible for inducing rolling. It has been noted that the average rate of leukocyte rolling for P-selectin is 2 to 5 times greater than with E-selectin mediation: P-selectin 20-50μm/s; E-selectin <5μm/s. Jung *et al*. (1998) suggests that the slower rate of rolling, induced by E-selectin mediation, is necessary to provide opportunities for the rolling neutrophils to interact with chemoattractants during their transit through an area of inflammation.

Selectin bond lifetimes range from seconds to milliseconds. It is speculated that the rate of bond dissociation is essential in establishing the rate at which leukocytes roll via selectins (Simons and Green 2005).

Studies conducted by Simon *et al*. (1993) and Bargatze *et al*. (1994) demonstrate that leukocytes, rolling on activated endothelium, possess the ability to capture free flowing polymorphonuclear (PMN) cells via homotypic interaction (a procedure referred to as secondary capture). This process is mediated by the association of L-selectin (expressed on the free PMN) and PSGL-1 expressed on the adherent PMN.

Phase three of the process is "slow-rolling". This occurs in the presence of cytokines such as Tumour Necrosis Factor-α (TNF-α). As the term suggest during slow-rolling the leukocyte's velocity is reduced. In order for this type of rolling to occur E-selectin on the endothelial cell and CD18 integrins on the leukocytes must be expressed (Kunkel and Ley 1996, Jung and Ley 1999). Neutrophils undergoing E-selectin interaction typically exhibit significantly reduced rolling rates (<5μm/s) compared to P- and L-Selectin.

It is believed that rolling is a necessary phase prior to the initiation of "firm adhesion", allowing adequate time for the association between leukocyte integrins and their counter receptors (Simon *et al*. 1998, Chen and Springer 1999). The conversion from rolling to firm adhesion is thought to be mediated by E-selectin.

A characteristic of cytokine-induced inflammation is that slow-rolling leukocytes exhibit a gradual decrease in their rolling velocity before they become adherent, a process which has been observed to take approximately one minute (Kunkel *et al*. 2000). Evidence exists that indicates the involvement of IL-8 receptor, however, signalling through adhesion receptors (e.g. L-selectin) may also be a contributory factor in the physiological recruitment of leukocytes (Steeber *et al*. 1997).

Leukocytes will migrate across a resting endothelium if there is the presence of an exogenous chemoattractant (Chapter 1.2.5). If a gradient of IL-8 or N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) is detected then "leukocyte driven" or

chemotactic transmigration will be initiated – a process which appears to be dose dependent (Furie and McHugh 1989, Furie *et al*. 1991, Smith *et al*. 1991).

1.2.7 Neutrophils – Mechanism of Action

Neutrophils are the primary leukocytes present at the site of inflammation (Chapter 1.2.3). Neutrophil migration is guided via specific signalling molecules called chemoattractants which are either released by bacteria or are endogenously synthesised by the host itself. Upon arrival at the site of inflammation the primary role of the neutrophil is to identify and eradicate the invading microorganisms. Neutrophil bactericidal activity is panacea-like in nature, in that it is non-specific, unlike the antigen specific mechanism employed by lymphocytes (Goldsby, Kindt, and Osborne 1999). Neutrophils have two distinct mechanisms of attack. The first involves the secretion of lysosomal enzymes, referred to as Non-oxidative killing. The second is Oxygen Dependent killing (the Respiratory Burst).

Baldridge and Gerard (1935) were the first to describe the respiratory burst. They observed a marked increase in oxygen consumption in neutrophils that had been exposed to bacteria. They hypothesized that this was the result of increased mitochondrial oxidative phosphorylation, where NADH is oxidised during the phosphorylation of ADP to ATP – supplying an energy source for phagocytosis.

However in 1959 Sbarra and Karnovsky discovered that the respiratory burst was not affected by cyanide; indicating that mitochondrial activity was not associated with the mechanism. Lyer *et al*. (1961) discovered that the increased oxygen consumption was utilised by stimulated cells in the production of Hydrogen Peroxide (H_2O_2). Later in that decade Klebanoff (1967) reported the existence of a further stage involving the catalysis of hydrogen peroxide and chlorine, by myeloperoxidase, to produce Hypochlorous acid (HOCl), as well as other potent reactive oxygen species. These are released during activation in response to physical challenge (e.g. bacterial infection) and also during periods of psychological / mental stress (i.e. part of the fight flight response in anticipation of potential tissue damage and subsequent infection (Viswanathan *et al*. 2005).

The mechanism is mediated by oxygen metabolites that are generated upon activation of the neutrophil enzyme NADPH oxidase. These metabolites are a form of free radical, the production of which is critical in the host defence mechanism. A free radical is an atom or molecule which contains an unpaired electron (Punchard and Kelly 1996). Upon stimulation the previously inactive NADPH oxidase within the neutrophil is activated via the combination and translocation of at least 6 different discrete subunits to the neutrophil cell membrane, resulting in a multi-component enzyme system. Being membrane bound allows it to interact with NADPH on one side of the membrane and oxygen molecules on the other (Figure 1.6).

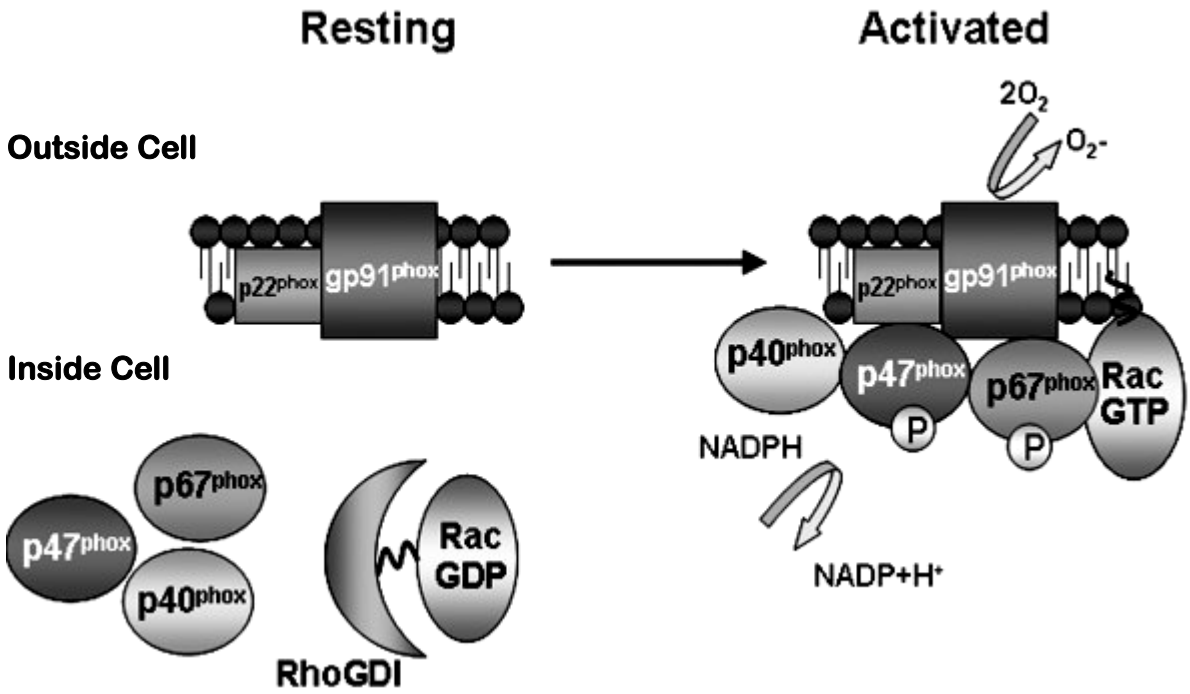


Figure 1.6 The NADPH complex in INACTIVE and ACTIVE form. (Adapted from Wilkinson and Landreth 2006).

Following neutrophil stimulation and parallel activation of oxidase components, induced within the cytoplasm. The mechanism continues via the conversion of Rac into an active GTP-bound form and the phosphorylation of $p47^{phox}$ and $p67^{phox}$. These subunits then translocate to the membrane where they interact with $p22^{phox}$ and $gp91^{phox}$ (NOX2) to initiate reactive oxygen production (Wilkinson and Landreth 2006).

The above process catalyses the following reaction:

Reaction 1

$$NADPH + 2\,O_2 \longrightarrow NADP^+ + 2\,O_2^- + H^+$$

As the equation demonstrates the reaction requires a large input of oxygen, this characteristic causes the process to be referred to as an Oxidative Burst or Respiratory Burst.

The resulting O_2^- species is rapidly converted to Hydrogen Peroxide (H_2O_2) by combining with hydrogen ions (produced as a by-product of reaction 1), in a second reaction which is catalysed by the enzyme Superoxide Dismutase (reaction 2). A final enzymatic conversion occurs (reaction 3), catalysed by Myeloperoxidase, where H_2O_2 is combined with Chlorine to produce the most potent bactericidal of all the oxidants produced by the neutrophil, Hypochlorous acid. It is these toxic oxygen species that are responsible for microbicidal activity and also surrounding tissue damage (Curnutte and Babior 1987, Weiss 1989).

Reaction 2

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Reaction 3

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_20$$

The production of reactive oxygen species by leukocytes is particularly effective at destroying invading micro-organisms at sites of inflammation. However, the same activity can lead to damage of healthy tissue. It has recently been shown that once 'superoxide shooting' commences, the leukocyte initiates a highly coordinated sequence of events, which include fusion and release of several types of granules and activation of antimicrobial enzymes (Bokoch 2002). ROS are effective at killing bacteria, however neutrophils produce ROS in response to stimuli other than bacterial challenge, for example during periods of mental stress within individuals diagnosed with chronic stress. In such cases the individual is predisposed to demonstrate enhanced immune activation and subsequent ROS release, due in part to dysregulation of both the HPA and SAM axis (Chapter 1.1.6) (Dhabhar *et al.* 1999, Altemus *et al.* 2003). Such activation could potentially result in damage to healthy tissue and promotion of the inflammatory response.

1.2.8 Antioxidants

The number and multiplicity of biological antioxidants present within cells provides an indication as to the relevance that oxidative free radicals play within the normal functioning of the cellular system. Examples include vitamin C and E – which as with most antioxidants are important micronutrients. The mechanisms by which these protect the host tissue from free radical attack have not yet been fully elucidated.

1.2.9 Role of Neutrophils in Stress

The process of neutrophil recruitment and activation is an essential stage of the stress response. Although generally accepted as occurring in response to physical stressors where tissue damage and possible infection result, it is now accepted that neutrophil activation occurs in response to altered stress hormone plasma concentration (including catecholamines and glucocorticoids) resulting from psychological and mental stress. A pre-emptive mechanism in anticipation of potential tissue damage (Mian *et al*. 2003, Shephard 2003, Saul *et al*. 2005, Allen 2007). The generic activation of the innate immune response (i.e. neutrophil activation) and inappropriate release of ROS and other lytic agents, following either physical or imagined distress has been postulated as the cause of many physiological disorders and chronic disease states (Dhabhar 2000, Altemus *et al*. 2003, Viswanathan *et al*. 2005).

1.3 EVALUATING PSYCHOLOGICAL STRESS

1.3.1 Qualitative Analysis

Early qualitative psychological stress assessment techniques relied heavily upon measures that were primarily designed to identify specific pathologic disorders, including schizophrenia and major depression (Lemyre and Tessier 2003). Each was validated using dysfunctional clinical populations with abnormal statistical

distributions, which resulted in reduced sensitivity when used to test below intended critical diagnostic thresholds (Lemyre and Tessier 1988), such as during the investigation of acute psychological and physiological laboratory stressors.

These tests are by nature highly subjective due to the reliance of self-assessment and the need for subjects to possess a sufficient understanding of the test. The Buss-Durkee Hostility Inventory was originally intended for the evaluation of patient hostility (Buss and Durkee 1957). However, it is now also used to assess a wider range of psychological factors including, anxiety and depression. Bag *et al*. (2005) used the hostility inventory to assess psychological factors in patients who suffered with migraine and tension-type headaches.

In the 1970s further systems were proposed, Wing (1970) published details of the Present State examination, a semi-structured interview providing an objective evaluation of symptoms associated with mental disorder. Baker *et al*. (2003) assessed the prevalence of depersonalisation disorder within the general population using interviews based on present state examination. Derogatis (1975) (Derogatis and Melisaralos 1983) developed a self report symptom inventory, designed to reflect the psychological symptom patterns of psychiatric and medical patients and non-patients. The Brief Symptom Inventory (BSI) utilised the global severity index (GSI) in order to quantify a patient's severity of illness. Numerous studies still report the use of the BSI in the evaluation of psychological stress. For example, Cucui *et al*. (2006) utilised the BSI for assessing the psychological distress associated with hospital recall in patients with malfunctioning cardioverter-defibrillators, and in so doing, demonstrated the limitations associated with its used in such situations. The study demonstrated a significant limitation of the BSI. It could be assumed that symptomatic shock (being informed of a potentially life threatening situation) would result in a BSI distress profile rating greater than controls. However, in this instance no significant difference in ratings were observed between recall groups (group 1, where standard recall occurred, group 2 where subjects were informed of the potentially dangerous condition). Brosig *et al*. (2007) evaluated the coping and psychological functioning of parents with children who were prenatally and post-natally diagnosed with congenital heart disease. He concluded that although the BSI was unable to differentiate between stress levels according to when the condition was

reported (either pre- or post-natally), it was able to relate disease severity with psychological stress magnitude.

The need for reliable, dedicated measurement techniques for assessing psychological stress and mental loading in non-clinical populations led to the development of a number of diagnostic systems. For example, the Psychological Stress Measure has been used to evaluate health and wellbeing in the workplace (Lemyre and Tessier 1988).

The most widely used psychological tool for measuring stress perception is the Perceived Stress Scale (PSS) (Cohen *et al*., 1983), which assesses an individual's perceptiveness to potentially stressful situations. The system was intended for use with individuals possessing at least junior school level education, which makes it ideal for use with the majority of the general population. It possesses highly generalised questions that are relatively non-specific to any particular population group. Kuiper *et al*. (1986) reported an association between above average PSS scores and increased susceptibility to life-induced depression.

The perceived stress scale demonstrated the need for analytical systems that were capable of assessing the effects of putatively stressful life events, such as driving a motor vehicle. The Subjective Workload Analysis Technique (SWAT) and the NASA-task load index (NASA-tlx) were specifically designed to evaluate alterations in perceived mental workload, and are utilised during the design process to assess ergonomic impact. The NASA task load index is a multi-dimensional rating tool that is used to derive an overall workload rating based upon a weighted average of six workload subscale ratings (Mental, Physical and Temporal Demand and Effort, Performance and Frustration Level). Secondly NASA-tlx employs a paired comparison procedure, involving 15 pair wise combinations. From each combination, the scale that had the greater impact on successful completion of the investigated task was selected (Hart and Staveland 1988). Syroid *et al*. (2002) described how NASA-tlx was used to evaluate changes in perceived workload during trials of a graphical anaesthesia drug display system.

SWAT postulates a multidimensional model of workload comprising three three-point dimensions or factors, time, mental effort, and psychological stress. SWAT involves a two-step procedure that is conducted following stressor exposure. In the first step, the subject ranks, from lowest to highest 27 combinations of the three workload scales. Scores are then calculated for every combination of ratings on the three subscales for each. During the second phase, event-scoring, the subject is asked to provide a rating (1, 2, 3) for each subscale. The researcher then maps the set of ratings to the SWAT score (1 to 100), which is calculated during phase 1. The data is then transformed into an interval scale of workload. The SWAT score is considered the workload value for that activity (Reid and Nygren 1988).

NASA-tlx is widely utilised during design and ergonomic evaluation within the motor vehicle industry. For this reason it was selected for comparison with the leukocyte coping assay within the current studies. Although these systems provide an insight into how an individual reacts to potentially stressful situations, their conclusions are highly subjective, which necessitates the need for accurate quantitative assessment techniques.

1.3.2 Quantitative Analysis

Quantitative measures of psychological stress focus primarily upon monitoring characteristics of the cardiopulmonary system and assay of specific stress hormones including salivary cortisol (Moon and Cho 2001, Clow *et al*. 2006, Powers *et al*. 2006) and plasma catecholamines (Brown *et al*. 2003). Mental stress is a risk factor for cardiovascular disease (Spence 1996). Even short-term mental stressors promote demonstrable alterations in heart rate and blood pressure (Mian *et al*. 2003). Several studies have investigated the influence of mental stress on cardiovascular function (Gelfand *et al*. 2004, Harada *et al*. 2006). Munakata *et al*. (2002) reported that mental stress, induced by a doctor's visit increased blood pressure in patients with essential hypertension. When investigating the influence of mental stress, for example when quantifying the effects of job-related stress it is necessary to be able to quantify the magnitude of the stressor. In this case the number of work hours is often used

(Gelfand *et al*. 2004). However, the physical and mental effects of exposure to mental stressors (e.g. work time) differs significantly between individuals, according to the coping strategies each employ, and can also vary according to environmental conditions (Tochikubo *et al.* 1996). In order to effectively evaluate the influence of mental stress, it is important to determine the quantity of a given stress load.

Energy expenditure, in addition to the use of heart rate and blood pressure, can be employed for assessing mental stress. Energy expenditure (EE) can be defined as the energy utilised to maintain body structures and body temperature and to perform movements for a given task (Garby 1990). Recent studies have demonstrated that energy expenditure is increased in response to mental stress, allowing its use as a measure of mental stress (Seematter *et al.* 2000, Seywert *et al.* 2002, Delarue *et al*. 2003). Energy expenditure is measured by calculating the subject's oxygen uptake per minute, adjusted according to the individual's body weight (adjusted VO_2) (Hosoya *et al*. 2002, Kuga *et al*. 2002, Tamura *et al*., 2002).

Evaluating the physiological effects of short-term psychological stressors is more problematic, and is generally attempted by measurement of physical characteristics including respiration rate and skin conductance (Oetting 1966, Sher *et al.* 2007) in addition to heart rate, BP and body temperature (Moon and Cho 2001, Hodgson *et al*. 2004, Brown *et al*. 2006, 2003). Although these methods return rapid results, all are subject to considerable biological variation, introducing uncertainty during comparison between individuals and populations.

Blood pressure, heart rate and catecholamine and cortisol concentrations are often used as indicators of the quantity of stress received (Hiramatsu *et al*. 1981 Holbrook *et al*. 1984, Jern 1991, Tochikubo *et al.* 1996, Seematter *et al.* 2000, Munakata *et al.* 2002). However, blood pressure, heart rate and sympathetic activity possess feedback systems for stabilisation and tolerance to stress (homeostasis), which can alter their effectiveness as measures of mental stress (Delarue *et al*. 2003). A study investigating the effect of a mental stressor (mental arithmetic test) on cardiovascular activity reported that repeat testing resulted in habituation of systolic blood pressure (Sawai *et al*. 2007). The physical condition and the existence of underlying conditions, including hypertension, can also result in inaccurate assessment of the

effects of mental stressors. Several studies have reported that the mental stress–induced increase in BP is greater in subjects with essential hypertension than in subjects with normal BP (Perkins *et al*. 1986, Lenders *et al*. 1989, Sawai *et al*. 2007).

The use of plasma catecholamine and cortisol concentration as indicators of mental stress can also cause inaccurate values, as measurement by blood sampling can increase the overall physiological stress response (Sawai *et al*. 2007). Many studies report the use of salivary cortisol as a useful indicator of mental stress (Hiramatsu *et al*. 1981, Kuga *et al*. 2002, Clow *et al*., 2006). Hodgson *et al*. (2004) reported significant increased cortisol concentration in care home patients who had undergone relocation to a new facility compared to residents who had not yet moved. However, its use can be problematic and may therefore be deemed unsuitable, due to the degree of variation in concentration exhibited diurnally and also between subjects, and that factors such as eating can also affect results. Hormone analysis may also be deemed unsuitable as many techniques require several hours before results are known, and the initial procurement costs of the ELISA kits can itself be prohibitive.

Although numerous techniques for assessing psychological stress exist, all rely on indirect assessment of parameters that are subject to influence by other physiological factors, or are based on subjective evaluation. There is a definite need for a reliable objective means of assessing psychological stress and mental workload, that rapidly provides results, is relatively inexpensive, and which eliminates inter-individual variation, allowing direct comparison of results.

1.4 BIOLOGICAL MEDIATORS OF INTEREST

This section provides additional information and the reasoning regarding why each mediator was selected for analysis.

Stress hormones, in particular cortisol and adrenaline (Bateson and Bradshaw 1997, Ouellet-Morin *et al*. 2008) have been used as indicators of stress. Neutrophil activity, including reactive oxygen species production, can be modified by both cortisol

(Kurogi and Iida 2002) and adrenaline (Bergmann and Sautner 2002). The mechanism by which the brain modulates the immune system involves the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (Chapter 1.1.3). Measurements of the status of the HPA axis, in particular the production of cortisol, provide important data on the stress response. However, the magnitude of the HPA response depends on basal hormonal concentration (Milde *et al*. 2003). Cortisol levels can vary widely between individuals, obscuring the effects of stress (Montané *et al*. 2002). The degree of variation between results for adrenaline, noradrenaline and cortisol can be high, as secretion of these hormonal mediators is highly variable depending on factors such as environment and time of day i.e. Seasonal and Diurnal variation. As a consequence the significance of the results, as an accurate measure of stress in an individual, can be questionable. It is unknown as to whether LCC is regulated by cortisol or adrenaline. Studies conducted by McLaren *et al* (2003) on badgers (*Meles meles*), have indicated that transport and handling stress causes increased cortisol production, and cortisol is known to have immunosuppressive effects.

The adhesion of neutrophils to the endothelium is a component of activation during stress (Jean *et al.* 1998) and modification of the receptors on either endothelial cells or neutrophils can dramatically alter the number of adherent (and thus the number of free flowing) neutrophils (Ley 1996). Endothelial and neutrophil derived adhesion molecules serve important roles in properly orienting neutrophils temporally and spatially for activation along the endothelium (Park and Lucchesi 1999) (Chapter 1.2.6). Important regulators in this process are TNF-α, and ICAM- 1. TNF-α up-regulates ICAM- 1, resulting in increased neutrophil-endothelium interaction (Menger *et al.* 1999). The selectin family of adhesion molecules, which includes E- and L-selectin, mediate the first contact of neutrophils with the endothelium (Ley 1996). E-selectin is expressed on the surface of endothelial cells and L-selectin on the surface of neutrophils. L-selectin can also influence the production of reactive oxygen species (Nagahata *et al.* 2000).

Endocrine Factors:

Cortisol and Adrenaline.

Cortisol is a steroid hormone synthesized from cholesterol. It is a primary glucocorticoid produced and secreted by the adrenal cortex, and can exist in the blood in two forms – free cortisol or bound to corticosteroid-binding globulin (CBG). Cortisol has both metabolic and immunological actions (Chapter 1.1.3). The concentration of cortisol present within plasma follows a diurnal trend, being highest in the morning, progressively diminishing throughout the day.

Stress hormones, in particular cortisol and adrenaline have been used as indicators of stress (Bateson and Bradshaw 1997, Clow *et al*. 2006, Ouellet-Morin *et al*. 2008, Thorn *et al*. 2009). Neutrophil activity, including reactive oxygen species production, can be modified by both cortisol (Kurogi and Iida 2002) and adrenaline (Bergmann and Sautner 2002). The mechanism by which the brain modulates the immune system involves the HPA axis and sympathetic nervous system (Chapter 1.1.3). Measurements of the status of the HPA axis, in particular the production of cortisol provides important data on the stress response.

Non-Endocrine Plasma Factors:

Interleukin-1 (IL-1), Interleukin-6 (IL-6)

Interleukin 1 is a group of associated proteins comprising IL-1α / IL-1ß / IL-1Rα (receptor antagonist) which are referred to as the original multifunctional cytokine. IL-1ß is a 31KD precursor expressed by numerous cell types including neutrophils, macrophages, monocytes and platelets. IL-1ß utilises inducible transcription factors to start a pro-inflammatory cascade of cytokines and proteins necessary for leukocyte recruitment and mobilisation (Hamon *et al.* 1997) (Chapter 1.2.6).

Interleukin-6 (IL-6) is a cytokine critical to the regulation of the immune system. It is a 212 amino acid glycoprotein expressed by cell types such as mast cells, endothelial cells, monocytes, and macrophages, which appears to take part in acute phase

reactions in response to injury and inflammation (Kishimoto 1989, Hirano *et al*. 1990). Levels of IL-6 have been shown to increase rapidly in serum with sepsis and burn trauma (Van Snick 1990). IL-6 has been implicated in conditions including auto-immune diseases and polyclonal B-cell abnormalities.

Non-endocrine plasma-borne factors could potentially modify the sensitivity of circulating neutrophils to PMA, therefore leading to an altered LCC response. In particular the cytokines IL- 1 & IL- 6, these are known to be released from activated endothelial cells, and affect neutrophil activity (Joseph *et al*. 1992).

Leukocyte Cell Adhesion Molecules:

Tumour Necrosis Factor – α (TNF-α), E-Selectin, L-Selectin, P-Selectin.

Tumour Necrosis Factor – α (TNF-α) is a 17.5 K Dalton, 157 amino acid protein that is a potent lymphoid factor. It exerts cytotoxic effects on a wide range of tumour and other target cells (Zhang *et al*. 1988). It has been suggested that TNF-α has a pro-inflammatory role and that it is the primary mediator of immune regulation (Waage, *et al*. 1989) (Chapter 1.2.6). Biosynthesis is highly controlled, where extremely small amounts are synthesized in quiescent cells, whereas TNF-α is a major secreted factor in activated cells (Hida *et al*. 2009). This factor could lend itself as being an effective diagnostic indicator of leukocyte activation and psychological stress detection.

The Selectin family of adhesion molecules consisting of E Selectin, Endothelial Leukocyte Adhesion Molecule (ELAM-1), L-Selectin (LECAM-1) and P-Selectin (GMP-140), guide non-activated polymorphonuclear cells (PMN's) to areas of inflammation by creating initial loose contacts with the endothelial layer (Chapter 1.2.6). L-Selectin mediates the rolling of PMN's on endothelial cells (Shimizu *et al*. 1991, Tozeren *et al*. 1992, Von Andrian *et al*. 1992), while E-Selectin is expressed on cytokine-activated endothelial cells, promoting the adhesion of leukocytes to the endothelium via the functional receptor L-Selectin. This initial binding event is a pre-requisite for the activation of the immune cells via inflammatory mediators (Kyan-

Aung *et al*. 1991, Lawrence *et al*. 1991). The circulating form of E-Selectin attracts neutrophils and activates ß2-integrins in preparation for cell migration.

It was hypothesised that monitoring post-stressor changes in concentration of these mediators would provide an indication of the leukocyte activation state, thus providing an insight into how subjects have responded to the putative psychological stressor.

1.5 PSYCHOLOGICAL STRESS AND THE ERGONOMICS OF DRVING

There are many potential in-vehicle sources of distraction that have the capacity to alter the driver's mental loading and situational awareness (SA). SA was a term originally coined for military ergonomics and was first cited in civilian literature 10 years later by Spiker *et al*. (1986). The definition of SA has had numerous revisions, however, generally SA can be defined as "a cognitive state or process associated with the assessment of multiple environmental cues in a dynamic situation" (Isaac 1997). The motor vehicle industry employs a diverse array of quantitative and qualitative methods which serve to assess every aspect of the ergonomic relationship (Rubio *et al*. 2004). Considerable interest has been placed upon the perceptual and cognitive demands of using mobile phones while driving, as well as other advanced electronic driver aids, such as satellite navigation (Reed and Green 1999). However, it is also the case that relatively simple low-tech tasks such as tuning a vehicle radio or adjusting environmental controls also have series safety implications. Indeed, adjusting the radio or CD player was found to be one of the major causes of distraction-related crashes by Stutts *et al.* (2001). These factors have led to the development of technologies which aim to reduce the degree of mental loading associated with in-car systems, therefore, limiting the need for driver's attention to be directed away from the external environment.

A direct relationship exists between situational awareness, driver performance and mental loading (Young and Stanton 2007). In fact any stimulus which distracts the driver from the road ahead will affect both mental workload and driving performance (Funke *et al*. 2007, Ma and Kaber 2007). It has been shown that with high feed back vehicles (those employing the latest driver aids and interface technologies) situational awareness was improved and was coupled with lower perceived workload (Walker *et al*. 2001). Basic techniques for assessing situational awareness involve monitoring aspects of driver performance. Horberry *et al*. (2006) monitored mean vehicle speed and the ability to maintain a constant speed, whilst being distracted by a mobile telephone conversation and during attempts to operate an in-car entertainment system. In both cases significant fluctuations in set speed resulted, suggesting that situational awareness was compromised.

Reaction/response time has been shown to be impaired during periods of reduced situational awareness and increased mental loading. The speed at which an individual is capable of moving from the accelerator to the brake pedal in response to a red light stimulus has been shown to decrease when the individual is engaged in conversation either with somebody within the vehicle (Consiglio *et al*. 2003) or via mobile telephone (Hendrick and Switzer 2007). Similar results were found in a study by Golden *et al*. (2003), whereby a reduction in situational awareness resulted during mobile phone and in-person conversations compared to control (no interaction with driver). In this case situational awareness was appraised using Connor's Continuous Performance Test (CPT). In its basic form CPT is a computerised, 14 minute, visual performance task in which the subject must respond repeatedly to non-target figures, and then inhibit responding whenever the infrequently presented target figure appears. This system can be easily incorporated within both simulator and test track on-road scenarios.

The technology now exists that allows monitoring of both eye movement and pupil diameter – both of which are indicators of attention focus (Sodhi *et al*. 2002, Strayer *et al*. 2003). These can be related to altered mental loading during completion of secondary tasks. Takahashi *et al*. (2006), used eye tracking to monitor the situational awareness of drivers during their attempts to navigate a specific route with and without the aid of a developmental 2D real-time geographical landmark navigational

system. Dukic *et al*. (2005) utilised eye tracking to investigate the visual behaviour of drivers in a motorway environment, while attempting to locate specific button controls. These were either located on the centre stack or close to the gear stick. It was noted that as the angle between the normal line of sight (road ahead) and the button location increased, eyes-off road time also increased. This indicates that dashboard ergonomics can severely influence driver safety. It, therefore, follows that interface design must allow ease of operation so as to minimise the time spent looking away from the road ahead and the overall impact on total mental workload.

Technologies such as eye tracking provide quantitative indications of an individual's situational awareness, which can be related to mental loading. However, the data requires interpretation before meaningful results can be produced. Zhang *et al*. (2006) suggests that eye tracking systems require further development before they can be classed as a real-time diagnostic measure. The motor industry does employ other measures, including instantaneous heart rate (Muhlberger *et al*. 2007) and skin resistance level (SRL) (Morel *et al*. 2005), for rapid assessment of altered mental loading. However, these parameters are liable to influence by other physiological mechanisms.

The automotive industry relies heavily upon the use of qualitative evaluation of mental loading and situational awareness. The situational awareness rating technique (SART) is one such example (Taylor *et al*. 1993). During a comparative investigation of high (those with higher responsiveness and richer levels of instrumentation) and low (basic instrumentation and reduced responsiveness) feedback vehicles, SART, in conjunction with the NASA-tlx, was used to evaluate situational awareness (Walker *et al*. 2001). NASA-tlx is commonly used within the automotive industry (Chapter 1.3.1). A study by Horberry *et al*. (2006) used NASA-tlx in the evaluation of driver performance and response to roadway hazards, whilst attempting to hold a mobile telephone conversation and also operate the in-car entertainment system, within a simulated environment. While Liu (2001) used subjective workload ratings to assess the best sensory format to present navigational commands.

The main problem associated with qualitative subjective work load evaluation is that it is, by definition, highly subjective, and can be influenced via a diverse array of

psychological and diagnostic factors. All quantitative assessment techniques must demonstrate their reliability for consistently and accurately reflecting mental workload. The diagnostic sensitivity of the test must allow for the detection of changes in task difficulty. Rubio *et al*. (2004) demonstrated that as task complexity increased, NASA-tlx demonstrated an ability to detect the resultant increases in mental loading. In contrast SWAT, lacking the complexity of NASA-tlx, demonstrated reduced detection sensitivity. Intrusiveness (the measure should not interfere with the primary task under evaluation) and Implementation (time, instrumentation and software for data collection and analysis) should be considered. Techniques including SWAT rely on the use of card sorting as a means of ranking the importance of each scale dimension (time load, mental effort load and psychological stress load). This method could be deemed more intrusive when compared to NASA-tlx which utilises a computer interface to facilitate the ranking process. Finally, in order for the tool to be completely effective, the subject must be willing to accept and understand the measure as being a valid and useful means of assessing the desired parameter (Rubio *et al*. 2004).

Within ergonomics and automotive development there is a requirement for a diagnostic tool with the ability to provide rapid near real-time quantitative measures of mental workload and psychological stress. This thesis aims to explore and validate the suitability of altered leukocyte responsiveness, using the leukocyte coping capacity (LCC) technique, for assessing the psychological stresses associated with exposure to unfamiliar situations and the evolution of new automotive design and technologies.

1.6 QUANTIFYING STRESS USING WHOLE BLOOD CHEMILUMINESCENCE

The ability to quantify the stress response in humans is confounded by the existence of both physiological and psychological stress, and their associated interactivity. As explained in chapter 1.3.2 there are numerous physiological responses including

changes in HR, BP, body temperature and skin conductivity, and biochemical responses which can be quantified and used to assess changes in stress level. However, such responses are mostly used to assess the effect of stressors of far larger intensity and magnitude compared to the stressors utilised during this research, and therefore may lack the ability to detect such subtle physiological changes. As explained below the Leukocyte Coping Capacity (LCC) technique may be a useful means of quantifying the physical effects of short-term, low intensity psychological and / or physical stress. The measure was developed using animal models, involving short-term confinement of wild badgers (*Meles meles*) (McLaren *et al*. 2003, Montes *et al*. 2003, 2004) and has been preliminarily assessed for use with human models in quantifying the difference in the magnitude of stress exhibited following elective or acute coronary bypass surgery (Ellard 2003). To date, LCC has been used to assess the physical response to high magnitude stressors (Ellard 2003, McLaren *et al*. 2003, Montes *et al*. 2003, 2004). This research aims to use the LCC technique with humans to investigate the physical effects resulting from interaction with stressors, associated with automotive ergonomic design, of lower intensity and duration to those previously investigated. At present, the automotive industry relies heavily upon subjective psychological self assessment techniques for evaluating new and developing technology (Chapter 1.3.1 and 1.5) (Kramer *et al*. 1987, Hankock and Caird 1993, Yung-Hui Lee and Bor-Shong Liu 2003), the findings from this thesis will aim to demonstrate the benefits of using an aspect of the immune response to objectively quantify stress and mental workload.

1.6.1 Leukocyte Responsiveness as a Measure of Psychological Stress – the Leukocyte Coping Capacity Technique

Recent advances in knowledge have led to the development for direct analysis of an aspect of the innate immune system in order to quantitatively assess stress, the Leukocyte Coping Capacity (LCC) technique (Ellard 2003, McLaren *et al*. 2003, Mian *et al*. 2003, Montes 2003, 2004). The aim of this thesis was to apply the LCC protocol to human models, in order to investigate via the use of controlled experimentation, the ability of the technique to objectively quantify and differentiate

between the responses observed for a variety of different low intensity, short duration stressors which relate to automotive ergonomic design. Attempts to elucidate the mechanisms responsible for eliciting changes in immune activity, specifically leukocyte activation, were also explored.

The technique is based on an individual's ability to mount a challenge-induced immune response after exposure to a defined and potentially stressful event. Each individual's capacity to respond to immune challenge (the individual's immunocompetency) is compared with their own baseline level of immune system activity – leukocyte responsiveness. Following exposure to the putatively stressful event, the ability of an individual's leukocytes to mount a quantifiable immune response (the respiratory burst) is measured (McLaren *et al*. 2003). A characteristic of the respiratory burst is the increase in oxygen uptake by leukocytes in order to synthesise oxygen free radicals, which destroy the invading micro-organism (Halliwell and Gutteridge 2000). With the correct type and level of stimulation, leukocytes will extracellularly synthesise highly reactive oxygen species (ROS) (Chapter 1.2.7). Examples of these species include: O_2, H_2O_2, $^{\bullet}O$, $^{\bullet}OH$. Reactive oxygen species have the ability to elicit many biological effects, including the destruction of bacterial cells, parasites and tumour cells, and the promotion and modulation of the immune response. The concept that leukocytes produce oxygen free radicals when in the presence of specific agonists is well documented. Examples include, adhesion of bacterial peptides to cell membrane receptors (Dietert *et al.* 1996), activation of protein kinase C with Phorbol 12-Myristate 13-Acetate (PMA) (Hu *et al.* 1999).

Additionally, a similar response has been observed with specific neuropsychiatric disorders (Zhou *et al.* 2007), suggesting immune activation occurs following exposure to psychological stressors. Thompson *et al.* (1993) provided further evidence that the respiratory burst can be affected by stressors that do not possess specific antigens. He found that leukocytes extracted from the kidney of salmon (*Salmo salar*) exhibited a reduced respiratory burst of approximately 40% after the fish had been exposed to 2 hours of confinement stress.

The response of leukocytes to PMA challenge after stress exposure is termed the Individual's Coping Capacity (ICC). Individual's who exhibit greater coping capacity have an increased potential to mount an effective respiratory burst, from a physiological viewpoint; allowing them to respond to a greater degree when subjected to bacterial challenge after stress. Therefore, coping capacity is an *in vitro* assessment of an individual's current *in vivo* status (McLaren *et al*. 2003).

1.6.2 The Theoretical Basis of Chemiluminescence

Role of Reactive Oxygen Species

An important component of the immune response is the ability of leukocytes to produce highly cytotoxic, non-specific oxidants called reactive oxygen species (ROS), including superoxide ($\bullet O_2^-$), nitric oxide ($\bullet NO$) and their particularly reactive products, hydrogen peroxide (H_2O_2) and peroxynitrite ($OONO^-$) (Nathan and Shiloh 2000). ROS gain their reactive properties due to the presence of unpaired valence shell electrons. The non-specificity of ROS means that it will damage almost every part of the pathogenic target cell (Rice-Evans and Gopinathan 1995) and that it is released whenever leukocytes are activated. As previously discussed leukocyte activation can occur as a result of expose to chemotactic stimuli such as complement derived C5a, Interleukin-8, Leukotriene B_4, N-formyl peptides (e.g. fMLP) and Platelet Activating Factor (Chapter 1.2.5). However, it has also been shown that a mechanism for central nervous activation, via stimulation by the HPA and SAM pathways also exists (Dhabhar and McEwen 1997) (Chapter 1.1.3), which acts as a pre-emptive measure allowing the innate immune system to become primed in response to the perception of potential physical harm.

Reactive Oxygen Species Synthesis

Leukocyte ROS synthesis occurs via NADPH oxidase activity (Chapter 1.2.7). Activation can occur firstly, following detection of a foreign body (such as a bacterium or parasite) through receptor activation, and subsequent pathogen vacuolar isolation, via the process of invagination. Secondly, resulting from sympathetic nervous stimulation via the HPA and SAM biomediator pathways (Chapter 1.1.3).

The exact mechanism by which nervous stimulation (resulting from mental stress) elicits leukocyte NADPH activity and ROS production is not yet fully understood. It has, however, been demonstrated that during periods of mental stress leukocyte ROS production increases (McLaren *et al*. 2003). Subsequently, cessation of the psychological stressor results in a reduction in ROS production (Atanackovic *et al*. 2002).

Measuring Reactive Oxygen Species

Specific soluble substances exist that lead to the activation of membrane bound NADPH oxidase, which do not require the presence of a phagocytic vacuole. These include: activated complement fragment C5a, formyl-leucyl-methionyl-phenylalanine (fMLP) (this is a chemotactic peptide derived from the bacterial cell wall), bioactive lipids (e.g. platelet activating factor (PAF), leukotriene B4, and neutrophil activating proteins such as interleukin 8 (IL-8).

Kopprasch *et al*. (1996) states that fMLP can be used as a stimulant in whole blood chemiluminescence studies, in order to explore the phagocytic abilities of the cells present within an *in vitro* sample, via receptor mediated activation. It was concluded that the stimulant must remain engaged with the receptor in order for the oxidase enzymes to remain active. Oxidase activity is also dependent upon the process of phosphorylation and dephosphorylation. Therefore, exposure to tyrosine kinase inhibitors (e.g. erbstatin) would lead to inhibition of receptor mediated phagocytic activity, such as that by fMLP (Channock *et al*. 1994).

It was initially believed that receptor stimulated NADPH oxidase activation was calcium-dependent (Naccache *et al*. 1990), with initial activation occurring within 2-5 seconds. Reaching maximum superoxide production at t=60 seconds, where it remained at an optimum level for a maximum of 5 minutes. Baggiolini *et al*. (1990) found evidence to suggest that receptor-independent NADPH oxidase activation is not calcium dependent, and occurs via direct activation of Protein Kinase C (PKC). Phorbol-12-myristate-13-acetate (PMA) is an agonist for PKC. Upon migration into the cell, PMA acts as a diacyl glycerol analogue resulting in PKC activation. The activated PKC then migrates into the phagocytic cell membrane where it initiates the activation of the NADPH oxidase system and subsequent ROS production. This

mechanism allows PMA to act as a stimulant in whole blood chemiluminescence assays.

In order to amplify the extent of luminescence to allow more accurate analysis, lumigenic substrates are added. These compounds react with ROS to form products which lead to the production and emission of photons. The extent of photonic emission is then quantified using a Luminometer, which contains a photo-multiplier. The most common lumigenic substances employed for this technique are Luminol (5-amino-2,3 dihydro-1,4-phtalazinedion) and lucigenin (bis-N-methylacridium-nitrate) with emission at 460nm and 510nm respectively.

Using the chemiluminescence technique, the formation of ROS and the resultant photonic production allows ROS production to be monitored over time. The resulting kinetic profile allows the biological functionality of the cells under evaluation to be calculated and monitored. "Native" chemiluminescence is particularly weak and therefore cannot be easily used in analytical systems as a means of quantifying the phagocytic process. For the purposes of this research Luminol will be used to amplify the degree of light emission. Studies by Erskine *et al*. 1994, Carulli *et al*. 1995, Kukovetz *et al*. 1997, and Romaschin *et al*. 1998, have all concluded that whole blood chemiluminescence is a highly effective means for quantifying leukocyte (neutrophil) function.

1.6.3 The Luminometer

Chemiluminescence relies on non-thermal molecular excitation via a chemical reaction frequently involving an oxidative process, which leads to the emission of "cold" light – light produced by a method other than incandescence. There are two different forms of reaction kinetics associated with chemiluminescence reactions; Glow-type and Flash-type (Tannous *et al*. 2003).

Luminol enhanced chemiluminescence, as used throughout this investigation, exhibits glow-type characteristics. The advantages of this form of reaction are that as reagent

injectors are not required the instrumentation is therefore much simpler, resulting in lower experimental costs. Chemiluminescence provides greater sensitivity with a larger dynamic range compared to fluorescence techniques (Roda *et al*. 2000, 2004). It yields lower background interference and is also non-radioactive. It also eliminates the need for protein quenching as is necessary in fluorescence reactions.

The photonic pulses produced as a result of the interaction between reactive oxygen species released by leukocytes and luminol are detected using a Photo Multiplier Tube (PMT) (Figure 1.7). When light hits the cathode of the PMT it is converted into electrical energy called photoelectrons (Engstrom 1980). These then interact with a series of dynodes (up to 14) which act to amplify the photoelectric effect. For every photoelectron which enters the dynode, 4-6 secondary electrons are emitted. These secondary electrons are then detected by an anode which results in the production of an electric pulse, which can then be analysed (Zambra *et al*. 2004).

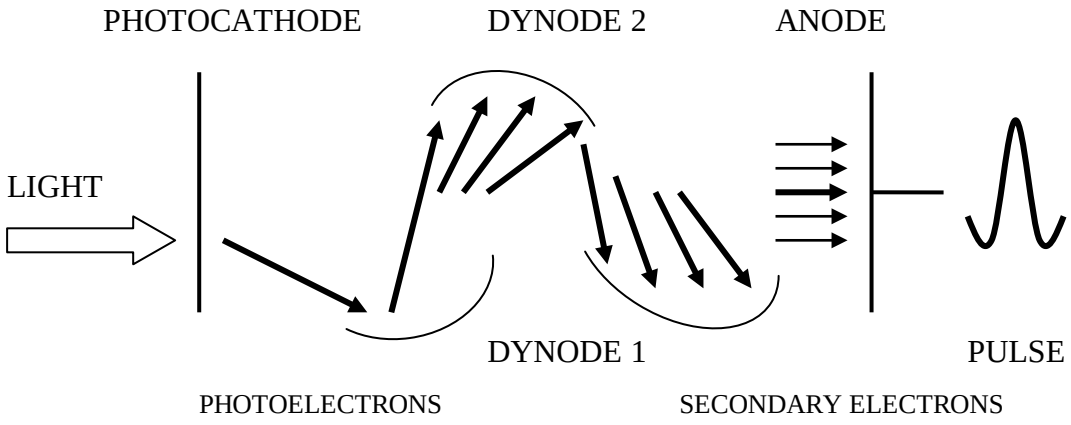


Figure 1.7 Illustrative Representation of the Photo Multiplier Tube
(Adapted from Berthold Technologies 2006).

The photons are counted via the formula:

Released Photoelectrons (Counts Per Second)	=	Photons Hitting the Cathode	x	Quantum Efficiency (QE)

Quantum Efficiency is defined as the number of photons detected by the PMT compared to the number of photons which actually hit the photo cathode. This value is different for each PMT and also depends on the wavelength of the photon (Castelletto *et al*. 2005).

The sensitivity of the luminometer is dependent upon the response time of its PMT – referred to as its "dead time". The dead time is the time that the PMT is unable to register another photon after the first has hit the cathode. The typical dead time for the type of luminometer used in this study is 10 nano seconds (Berthold Technologies Junior LB 9509 – illustrated in Figure 1.8). This means that the equipment is capable of responding to photonic rates of $1x10^9$ per second. These values are then converted using an arbitrary scale referred to as Relative Light Units (RLUs). The RLU value is the photon count divided by 10. This allows the use of slower and more accurate electronics in the final quantification. Therefore ultimately the luminometer is capable of measuring photon rates in the order of $1x10^8$ photons / second.

Figure 1.8 The Berthold Technologies Junior LB 9509 Luminometer used throughout this research.

In this series of studies the number of photons released over a 30 second period is measured. The advantage of utilising longer counting periods is in the statistical accuracy of the values obtained. During the 30 seconds all the photons are integrated and the reported value is total RLU / measuring time expressed as RLU / second. In

this way accuracy is a function of the total RLU accumulated, therefore 10 000RLU/s measured in one second will have the same accuracy as 500RLU/s measured over a sample period of 20 seconds.

1.7 CONCLUSION

The physiological responses to stress are extremely complex, involving a diverse range of biological systems. Considerable evidence exists, describing the effects of chronic stress, however, little is known regarding the effect of acute, short-term low-level everyday stressors. The primary reason for this is the inability of traditional techniques, such as heart rate and blood pressure, to objectively quantify the response to transient low-level stressors. The development of techniques, primarily LCC, provides a possible means to not only detect but also objectively quantify the presence of short-term low-level stressors. It is hypothesised the technique will also allow discrimination between related stressors. The ability to quantify stress following environmental and social interaction is important within countless areas of scientific research. This thesis is concerned with how ergonomic design of in-car technologies facilitates interaction within the automotive industry.

2.0 GENERAL METHODOLOGY

This chapter presents general principles and techniques utilised throughout this thesis and can be referred to as one reads the following chapters. Each study within this thesis will have its own distinct methodology section, explaining the specific protocols used.

2.1 LEUKOCYTE COPING CAPACITY PROTOCOL

This thesis utilises a novel technique for the quantitative analysis of leukocyte activity *in vitro*. The biological mechanisms involved have already been described (Chapter 1.6.1).

2.1.1 Reagent Preparation

Phosphate Buffered Saline (PBS)

PBS (Sigma, catalogue number P4417) (providing mineral concentrations of 0.01M phosphate buffer, 0.0027M Potassium Chloride, 0.137M Sodium Chloride, at pH 7) was prepared by adding 1 tablet to 200ml distilled water. PBS was used during the serial dilution of both luminol and PMA stock solutions.

99ml aliquots of PBS were stored at -20°C for a maximum of 2 months and were used for serial dilution of stock luminol (Refer to luminol preparation for details). 10ul of stock PBS was also used during leukocyte activity analysis (Chapter 2.1.2).

Luminol (5-amino-2,3 dihydro-1,4-phthalazinedione) ($C_8H_7N_3O_2$ FW 177.16).

Figure 2.1 Structural formula of 5-Amino-2,3-dihydro-1,4-phthalazinedione (Luminol) (Sigma-Aldrich 2008).

Stock Luminol (Sigma, catalogue number A8511) (concentration 10^{-2}M) was produced by dissolving 0.0177g of luminol in 1ml of Dimethyl Sulfoxide (a polar aprotic solvent) (C_2H_6OS) (Sigma, catalogue number D5879) and 9ml of fresh PBS (See PBS preparation), using a magnetic hotplate stirrer. As Luminol is photosensitive the glass beaker containing the solution was wrapped in foil to occlude all light during preparation. 1ml aliquots were stored, in foil to minimise light contact, at -20°C for a maximum of 1 month.

A working solution of 10^{-4}M was produced fresh every day from stock solution by dilution with 99ml stock PBS buffer (See PBS preparation), and was stored on ice in the dark until required.

Phorbol 12-Myristate 13-Acetate (PMA) ($C_{36}H_{56}O_8$ FW 616.8).

Figure 2.2 Structural formula of Phorbol 12-Myristate 13-Acetate (Sigma-Aldrich 2008).

Stock PMA (concentration 10^{-3}M) was produced by adding 8.106ml Dimethyl Sulfoxide (Sigma, catalogue number D5879) to PMA (Sigma, catalogue number P8139). 1ml aliquots were produced and stored at -20°C for a maximum of 2 months (to minimise loss of activity).

A working solution of 10^{-5}M was produced daily, by adding 99ml of fresh PBS buffer to 1ml of stock 10^{-3}M PMA.

Lithium Heparin ($C_{12}H_{19}NO_{20}S_3$ FW 593.5)

Each blood solution contained 10µl of whole blood which required addition of 0.1 I.U. heparin to inhibit clotting. Heparin was supplied pre-dissolved at a concentration of 10 units per ml (Hepsal™, CP Pharmaceuticals Ltd, Ash Road North, Wrexham, LL13 9UF United Kingdom). Therefore 10µl of lithium heparin was added to each blood solution to achieve a final working concentration of 10 I.U. per ml.

2.1.2 PMA Challenge and Measurement of Leukocyte Coping Capacity (LCC)

LCC assessment involves the simultaneous testing of two whole blood samples, obtained using subcutaneous capillary finger prick sampling (Chapter 2.2.3), per time point; an unstimulated control (in order to establish baseline leukocyte activity – Sample A) and a stimulated challenge (where Phorbol 12-Myristate 13-Acetate (PMA) is added, which acts as a Protein Kinase C (PKC) agonist – Sample B). The subsequent activation of PKC causes it to migrate to the leukocyte cell wall where it stimulates the activation of the NADPH oxidase pathway, ultimately leading to the production of reactive oxygen species (ROS) (Chapter 1.2.7 and Figure 1.6). The ROS then combine with the luminol present in the sample causing a conformation change which leads to the emission of photons. It is this photonic emission that is used as a quantitative determination as to the state of leukocyte activation within the sample. In order to gain a true representation of leukocyte activity, the base control activity readings (Sample A – non-stimulated) are subtracted from the challenge values (Sample B – PMA stimulated) (See Table 2.1 for reagent concentrations and quantities). The resultant activity is expressed as Adjusted Relative Light Units (RLU_{adj}) (Chapter 1.6.3).

To measure the unstimulated blood chemiluminescence levels (Sample A), 10µl whole blood was transferred into a silicon anti-reflective tube (Lumivial, Berthold® Technologies, Hertfordshire, AL3 7LZ United Kingdom) to which 90µl of 10^{-4} mol/l luminol (5-amino-2, 3-dihydrophthalzine; Sigma A8511) was added. A further 10µl of PBS was added to account for the absence of PMA. The tube was then shaken gently.

To measure the chemiluminescence produced in response to challenge (Sample B), a second tube was prepared as above, substituting the 10µl of PBS solution for 10µl phorbol 12-myristate 13-acetate (PMA; Sigma P8139) at a concentration of 10^{-5} mol/l.

For each tube chemiluminescence was measured every 5 minutes in a portable chemiluminometer (Junior™ LB 9509, Berthold® Technologies, Hertfordshire, AL3 7LZ United Kingdom) for a total of 45 minutes.

When not in the chemiluminometer, tubes were incubated, in darkness at 37°C (water bath - JB1™ Grant Instruments, Cambridge, United Kingdom).

	Luminol (10^{-4}M)	Heparin (10 units / ml)	PMA (10^{-5}M)	PBS	Whole Blood
Control (Sample A)	90µl	10µl	-	10µl	10µl
Challenge (Sample B)	90µl	10µl	10µl	-	10µl

Table 2.1 Reagent summary for control (sample A) and challenge (sample B) blood solutions for LCC assay.

2.2 SAMPLING TECHNIQUES

2.2.1 Ethical Approval and Informed Consent

Before the initiation of any form of physical sampling, detailed ethical approval, in accordance with the declaration of Helsinki (World Medical Association 2004), was obtained from Coventry University Ethics Committee. In addition, each volunteer gave full informed consent.

A complete and detailed medical history was taken from each volunteer (See Appendix 1 for standard medical questionnaire), in order to ensure that there were no underlying acute and long term medical conditions present which may have been exacerbated as a result of the sampling protocol (Chapter 2.2.2).

2.2.2 Subject Recruitment

Throughout this research subjects were recruited using the following standard demographic. Healthy, moderately fit males and females aged between 21 and 65 years. People were excluded if they suffered from psychiatric illness, respiratory or cardiovascular disease, were smokers, had taken prescription medicine within the previous month, or if they possessed prior knowledge of the test equipment. Subjects were recruited primarily from within the university population (including lecturers, senior management as well as the general student population). In total, a pool of 40 volunteers was recruited. Generally each subject was allocated for inclusion within only one of the five studies, thus minimising the possibility of habituation to the selected stressors. Only a small proportion of volunteers participated in more than one of the studies, where this occurred it was ensured that the stressor protocol was sufficiently different to minimise habituation (See Table 2.2 for details of percentage of subjects within each study who had participated within more than one study).

Table 2.2 Number of Subjects Associated With Each Study

	Number of subjects			Number of subjects who had participated in another study		
	Male	Female	TOTAL	Male	Female	TOTAL
Study 1 (Chapter 3.0)						
Phase 1	12	9	21	4	8	12
Phase 2	8	10	18	2	4	6
Study 2 (Chapter 4.0)	10	10	20	1	3	4
Study 3 (Chapter 5.0)	15	15	30	2	3	5
Study 4 (Chapter 6.0)	7	8	15	1	4	5
Repeat Testing	2	2	4	2	2	4*
Study 5 (Chapter 7.0)	7	8	15	2	4	6

Studies are presented in chronological order. The total number of subjects (male and female) involved with each study is presented, along with the number of individuals who had participated in at least 1 other study.

* The 4 repeat test subjects within Study 4 had not participated in any of the other 4 studies. Their only previous experience was with the initial test phase of study 4.

2.2.3 Multi-Capillary Subcutaneous Finger Prick Blood Sampling – For LCC Analysis

This is a method for obtaining small quantities (up to 50µl volume) of capillary whole blood. The technique involved the use of a lancing device (Roche® Soft-Clix Pro™, Roche Products Ltd. Hertfordshire, AL7 1TW United Kingdom) consisting of a disposable lancing needle placed in a spring loaded mechanism. The mechanism permits rapid and accurate puncturing of the cutaneous tissue to an exact depth to allow for harvesting of the blood. The finger was "milked" to obtain a large droplet of blood, using a micropipette, 10µl of blood was added directly to each of the pre-prepared reagent solutions (Samples A and B).

2.2.4 Intravenous Blood Sampling – For ELISA Analysis

Most quantitative methods for determining stress involve assay of blood-borne bio-mediators (e.g. cytokines) and hormones (e.g. adrenaline, noradrenaline and cortisol). For this to be achieved venous blood samples were taken using the evacuated tube method (BD Vacutainer™ Systems, Oxford, OX4 4DQ United Kingdom), before and immediately after exposure to the putative stressor. Samples were harvested through venepuncture, where relatively large quantities of blood (compared to the amount required for LCC analysis) were collected from the anticubital fossa region of the inner arm, where the median cubital, cephalic and basilica veins lie close to the surface of the skin and are most prominent. To become proficient in the venepuncture technique, a phlebotomy training course was undertaken at Coventry and Warwickshire hospital. Blood samples were then centrifuged into there constituent parts; plasma and cells. The appropriate constituent was selected according to the assay used (following manufacturers guidelines).

Vacutainer™ tubes were supplied pre-coated with heparin or ethylenediaminetetraacetic acid (EDTA) at the correct concentration. The requirements and sensitivities of the assays to be employed were determining factors regarding which anticoagulant was used. The evacuated technique permits multiple

samples to be taken allowing both types of anticoagulant to be used, from a single needle. This negates the need for repeat sampling at a specific time point, therefore reducing the overall stress inflicted on the volunteer. In order to avoid cross contamination, the samples were harvested in the following sequence, blank (no anticoagulant present), lithium heparin, EDTA (following manufacturer's guidelines – BD Vacutainer™ Systems, Oxford, OX4 4DQ United Kingdom).

2.2.5 Intravenous Sample Preparation and Storage

Standard Enzyme Linked Immunosorbant assay (ELISA) was used to determine the post-stressor changes in concentration of mediators believed to be associated with leukocyte activation and the stress response (Chapter 1.4) . ELISA is unable to analyse whole blood, it must first be fractionated, isolating plasma or serum (dependent on mediator assaying and protocol). The fractionation protocol was the same for both anticoagulants (heparin and EDTA). It was essential that all samples were treated within 30 minutes of harvesting and stored on ice until ready to begin. Samples containing heparin and EDTA were centrifuged at 1000x g for 30 minutes at room temperature. The supernatant (plasma) was transferred to 300µl aliquots and stored at -70°C. For the assay of L- and E-Selectin, blood samples were harvested with the absence of an anticoagulant. The samples were permitted to clot at room temperature (20-22°C) for 60 minutes and then centrifuged at 1000x g for 10 minutes at 4°C. The supernatant (serum) was transferred to 300µl aliquots and stored at -20°C, in order to minimise bio-reactivity degradation.

2.3 ELISA METHODOLOGY

The basic principles utilised during ELISA testing remain constant for all biological mediators under investigation (Figure 2.3).

Antibodies to the mediator requiring detection are bound to the walls of a micro-titre plate (Stage 1). The mediator present in the plasma sample binds to the immobilised

antibodies. The excess sample is then washed with buffer solution (Stage 2). A second antibody for the bioactive mediator requiring detection, which has undergone conjugation with an enzyme, is added to the wells and binds to the mediator already captured by the first antibody (Stage 3). After a second wash with buffer, in order to remove any unbound antibody-enzyme conjugate, a substrate for the enzyme is added which leads to the formation of a coloured product (Stage 4). The intensity of the coloured product observed is proportional to the amount of antigen (bioactive mediator) present. Colour intensity is measured using a micro-plate colorimeter. Using standard solutions of known concentrations a calibration curve is produced, which is used to calculate the concentrations of the unknown samples.

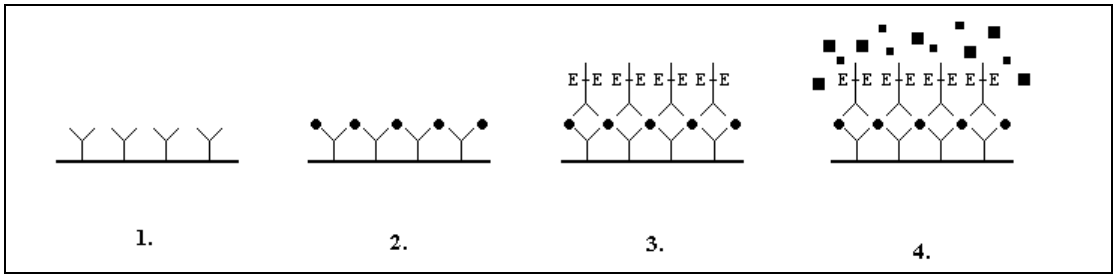


Figure 2.3 Basic stages in the methodology employed for the Enzyme Linked Immunosorbant Assay.

2.4 HEART RATE, BLOOD PRESSURE AND CORE BODY TEMPERATURE

Systemic blood pressure and heart rate were measured using an oscillometric, wrist-mounted, combined blood pressure and heart rate monitor (Omron RX-3, Omron Healthcare Inc. Illinois, 60015. U.S.A.). The equipment calculated heart rate in beats per minute by sampling the rate over a 15 second period and multiplying the value by a factor of four. Prior to obtaining resting measurements, subjects were required to sit quietly and to breathe orthonasally for 15 minutes. Core body temperature (CBT) was measured using an infra-red ear thermometer (Braun® Thermoscan™, P and G Brooklands, Waybridge, AT13 0XP United Kingdom). The equipment sampled the infrared heat radiated from the ear drum 8 times per second and presented the mean

value. The stated parameters were measured at three defined points during the test protocol, 45 minutes pre-stressor (for baseline measurement), immediately post-stressor, and 45-minutes post-stressor.

2.5 DATA ANALYSIS

This section describes the standard statistical methodology common to all studies. SPSS (statistics package for social sciences) statistical software (release 15.0 Lead Technologies Inc.) was used throughout.

2.5.1 Leukocyte Activity

For all treatment groups each subject's leukocyte activity was adjusted to compensate for baseline activity via subtraction of Sample A (absent of PMA) from Sample B (stimulated sample containing PMA) (Chapter 2.1.2). Five specific attributes of the leukocyte luminescence profiles produced for 45 minutes pre- and immediately post-stressor were used to assess the effect of the putative stressors (Figure 2.4).

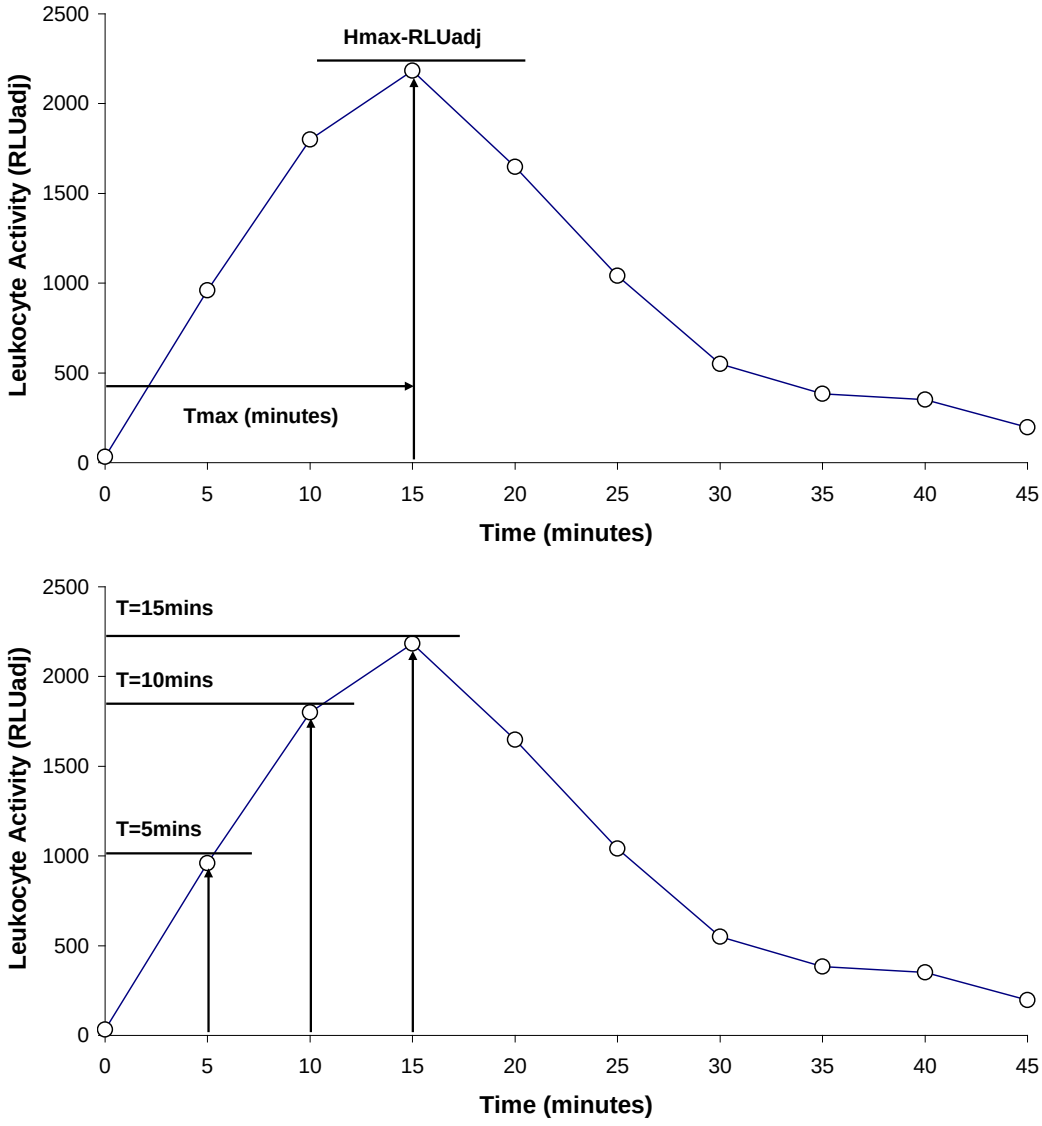
1) Maximum Leukocyte Activity (Hmax-RLU_{adj}) – the maximum adjusted response exhibited during the 45 minute sampling period.
2) T-max (minutes) – the time taken to reach maximum adjusted leukocyte activity (Hmax-RLU_{adj}).

From previous studies (McLaren *et al*. 2003) it was noted that the most significant changes in leukocyte responsiveness occurred up to and including 15 minutes post-stressor, therefore analysis of measurements within this time period were made.

3) T=5 minutes (RLU_{adj}) – the adjusted response in leukocyte activity recorded 5 minutes into the 45 minute activity profile.
4) T=10 minutes (RLU_{adj}) – the adjusted response in leukocyte activity recorded 10 minutes into the 45 minute activity profile.

5) T=15 minutes (RLU_{adj}) – the adjusted response in leukocyte activity recorded 15 minutes into the 45 minute activity profile.

Figure 2.4 Typical Luminescence Profile Demonstrating Measurement of Attributes of Interest



The diagrams illustrate a typical leukocyte activity luminescence profile and how the profile was interpreted to calculate the following 5 responses, maximum adjusted leukocyte response observed during 45 minute time profile (Hmax-RLU_{adj}), the time taken to reach maximum leukocyte activity (T-max) (on first diagram), and adjusted leukocyte activity at 5, 10 and 15 minutes into the 45 minute time profile (on second diagram).

To minimise the effect of variation among subjects (inter-individual variation), data for each of the 5 foregoing attributes of leukocyte activity are expressed as differences between leukocyte activity at 45 minutes pre- and immediately post-test (RLU_{adj}) ± standard error of mean (S.E.M.). Individual data were then combined and presented as mean post-test changes in leukocyte activity for each treatment group. In the case of T-max (the time taken to reach maximum adjusted leukocyte activity), as leukocyte activity was measured at 5 minute intervals for a total of 45 minutes, the data is discontinuous unlike the other selected responses (e.g. Hmax-RLU_{adj}) which were measured using a continuous scale. Therefore the median ± standard error of mean (S.E.M.) rather than the mean is presented for T-max (minutes).

Blood samples taken 45 minutes post-stressor were used to establish the time point by which leukocyte responsiveness returned to pre-stressor baseline activity, and is included within the luminescence profiles for each treatment group.

Single factor analysis of variance (ANOVA) was used to test the effect of each putative stressor on leukocyte activity. Each of the five attributes of leukocyte activity (e.g. Hmax-RLU_{adj}) were used in-turn as the dependent variable, with treatment as the fixed factor.

To individually test the effect of the putative stressor on each treatment group, mean adjusted leukocyte activity at 45 minutes pre-, immediately post, and 45 minutes post-stressor was applied to a single factor ANOVA model, where each of the five response attributes (e.g. Hmax-$RLU_{adj,}$ T=5 minutes) were used in-turn as the dependent variable, with time as the fixed factor. Tukey's honestly significant difference test for multiple comparisons was used as *post hoc* tests for time.

2.5.2 Heart Rate, Blood Pressure and Core Body Temperature

At each sampling point in addition to taking blood samples for leukocyte activity analysis, heart rate, core body temperature and systolic and diastolic blood pressure measurements were collected. As with leukocyte activity the differences between 45 minutes pre- and immediately post-stressor for each parameter were calculated for

each subject. The mean ± S.E.M. were calculated for each treatment group. The effect of the stressor on each parameter was investigated using single factor ANOVA, where post-test changes in heart rate, core body temperature and diastolic and systolic blood pressure were used in-turn as the dependent variable, with treatment as the fixed factor.

To test the effect of the stressor on each treatment group, heart rate, core body temperature and blood pressure 45 minutes pre- and immediately post-test were individually tested using single factor ANOVA, where heart rate, core body temperature and blood pressure were used in-turn as the dependent variable, with time as the fixed factor. Tukey's honestly significant difference test for multiple comparisons was used as *post hoc* tests for time.

2.5.3 Relationship between Leukocyte Activity and Heart Rate, Blood Pressure and Core Body Temperature

Bivariate correlation was used to assess the relationship between post-test changes (difference between 45 minute pre- and immediately post-stressor) in responses 1-5 with post-test changes in heart rate, core body temperature and systolic and diastolic blood pressure. To correct overall *P*-values for the use of multiple comparisons, the Truncated Product Method was applied (Zaykin *et al*. 2002). This method combines the set of *P*-values for each multiple comparison (i.e. the correlations between each leukocyte activity response and physiological indicator of stress) to evaluate the probability of obtaining a given product of *P*-values by chance alone.

2.5.4 Perceived Stress

Likert scales (employing a continuous scale where 1 represented relaxed and 10 stressed) were used to subjectively assess perceived stress 45 minutes pre- and immediately post-stressor exhibited by each subject. 2-way analysis of variance, where perceived stress rating acted as the dependent variable, and treatment and time acted as fixed factors, assessed the significance of the change following exposure to

each stressor. The relationship between post-test change in leukocyte activity and perceived stress ratings for 45 minutes pre- and immediately post-test, along with the difference between 45 minutes pre- and immediately post-test were assessed using bivariate correlation, correcting the overall *P*-values for the effect of multiple comparisons via the Truncated Product Method.

3.0 Pilot Study: Validation of the Leukocyte Coping Capacity Technique: Investigating the Physiological Effects of Psychological Stress Associated with Driving an Unfamiliar Vehicle.

3.1 INTRODUCTION

Chemiluminescence allows the exploration of *in vivo* neutrophil functional ability, within whole blood *in vitro* samples. Studies conducted by McLaren *et al*. (2003) and Montes *et al*. (2003, 2004) show the potential benefits for the use of luminol-enhanced chemiluminescence as a simple rapid assay which, when applied in conjunction with physiological and/or psychological stimulants/stressors, can investigate the phagocytic capacity of neutrophils (leukocytes), providing an indirect quantitative means for assessing changes in psychological stress and mental loading, in both human and animal models.

The evaluation of psychological stress exhibited as a consequence of ergonomic design, as well as for stressors in general, relies heavily upon qualitative methodologies. Subjective mental workload (SWAT) and the NASA task load index are the most common examples (Hart and Staveland 1988, Reid and Nygren 1988, Rubio *et al*. 2004) (Chapter 1.3.1). Quantitative techniques include measurement of changes in heart rate (Blechert *et al*. 2007), blood pressure (Richman *et al*. 2007, Manikonda *et al*. 2008), body temperature (Bhatnager *et al*. 2006, Barnum *et al*. 2007) and concentration of specific bio-mediators including cortisol (Allen 2007, Nij *et al*. 2007). All are influenced by a diverse array of factors other than psychological stress and therefore do not correlate well (Chapter 1.3.2). Psychological stress reduces leukocyte responsiveness to PMA induced reactive oxygen species production, *in vitro* (McLaren *et al*. 2003). Using chemiluminescence ROS concentration can be measured, providing rapid assessment of an individual's psychological state (Chapter 1.6.1).

This study was designed to investigate the possible applications for luminol-enhanced chemiluminescent leukocyte analysis within a commercial environment. The aim was

to determine if the leukocyte coping capacity protocol could provide an indirect quantitative assessment of altered mental loading, induced through exposure to a short-term unfamiliar psychologically disturbing event, in this instance, being asked to drive an unfamiliar motor vehicle. At present the motor industry uses a combination of basic physiological monitoring including, altered cardiopulmonary responses (Blechert *et al*. 2007, Richman *et al*. 2007, Manikonda *et al*. 2008), body temperature (Bhatnager *et al*. 2006, Barnum *et al*. 2007) and concentration of specific bio-mediators including cortisol (Allen 2007, Nij *et al*. 2007), all are influenced by a diverse array of factors other than psychological stress, resulting in inaccurate measurement (Chapter 1.3.2). Subjective workload analysis (through self assessment questionnaires) e.g. the NASA task load index (Lee *et al*. 2001) (Chapter 1.3), and analysis of driver situational awareness, either qualitatively, via the Subjective Awareness Rating Technique (SART) (Taylor *et al*. 1993) or quantitatively through the use of eye tracking technologies (analysis of where the driver's attention is focused during key events) (Young and Stanton 2007) (Chapter 1.5). In contrast LCC would provide the industry with detailed physiological information as to how a subject is responding to a situation at a cellular level. Psychological stress reduces leukocyte responsiveness to PMA induced reactive oxygen species production, *in vitro* (McLaren *et al.* 2003). Using chemiluminescence, ROS concentration can be measured providing rapid assessment of an individual's psychological state (Chapter 1.6.1). It was hypothesised that the sensitivity of the LCC technique was such, that it would be capable of differentiating between the mental loading (psychological stress) associated with performing the same basic driving related tasks in two different motor vehicles.

3.2 METHODS

The Subjects

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained from each subject before commencement of the study, in accordance with the declaration of Helsinki (World Medical Association 2004).

The study was unable to accommodate a randomised cross over design, as concurrent use of both test vehicles was not possible. Instead the study consisted of two phases, separated by a 2 month interval (to accommodate test vehicle availability). Phase 1 consisted of 21 subjects (12 male and 9 female). With the aim of reducing the possibility of habituation to the novel test scenario 18 different subjects (8 male and 10 female) were recruited for phase 2. The process of being taken to a facility not normally accessible to the public may have provoked changes in emotional status which could either serve to inhibit or amplify the leukocyte response. Either way it was decided that in the absence of a cross over design, the use of two different subject groups would serve to reduce the possibility of this occurring. All subjects were moderately fit and healthy, aged between 21 and 65 years. Potential subjects were excluded on the following criteria: suffering from psychiatric illness, suffering from respiratory or cardiovascular disease, smokers, had taken prescription medicine within the previous month, and if they possessed any prior knowledge or experience regarding the test equipment.

Design

The experimental protocols were rigorously standardised, and testing occurred between two time slots (11.00-12.00 hours and 14.00-15.00 hours) which were again determined as a consequence of test vehicle availability. Subjects were required to avoid any strenuous activity for at least 2 hours prior to testing (as periods of exertion may have influenced leukocyte reactivity (Montes *et al*. 2003, 2004).

Prior to obtaining resting heart rate, BP, and core body temperature following the standardised procedure outlined in chapter 2.4, subjects sat quietly and were instructed to breathe orthonasally for 15 minutes. The first capillary blood samples were then taken 45 minutes before exposure to the test apparatus (45 minutes pre-test) (see below).

During the 45 minute Pre-Test period subjects were taken from the laboratory (Coventry University) to Jaguar Cars Ltd. research and development centre at Whitley, Coventry, United Kingdom – a 5 minute car journey. In an attempt to

minimise the possibility that the events associated with subject transport influenced leukocyte reactivity, subjects were instructed to sit quietly in the researcher's vehicle for 10 minutes. This time was also used to set up the test equipment in the test vehicle.

Two minutes prior to entering the test vehicle (Phase 1 – Car A, Phase 2 – Car B) the examiner explained the test protocol. The test lasted a maximum of 20 minutes. Upon successful entry into the test vehicle the subject was to adjust correctly the driving environment so as to meet British Motoring Standards, this was achieved by making the following adjustments.

1. Driver's seat (including distance from pedals, angle of back rest, seat height)
2. Position of steering wheel (rake and reach)
3. Near and offside wing mirrors
4. Rear view mirror
5. The subject was required to then start the vehicle, select the correct gear and release the handbrake. Then perform a simple manoeuvre involving moving the car out of a pre-determined car parking space and travelling 40 metres along an access road at 3 mph, stop, select the correct gear and reverse back down the road re-entering the original parking space.
6. Finally, safely stop the engine and hand the examiner the key.

It was explained that once the test had begun no further verbal communication was allowed, and that assistance would only be provided after 3 minutes of attempting to complete the task, followed by the next instruction. Immediately after completing the test the second pair of blood samples BP, heart rate and core body temperature measurements were taken (Immediately post-test).

Following a 45 minute recovery period, where the subject remained in the test vehicle and either read quietly (reading content was of a non stimulating content and was usually in the form of a local daily newspaper) or talked with the examiner, a further pair of blood samples and BP, heart rate and core body measurements were taken (45 minutes post-test).

Determining Leukocyte Activity

Two 10µl blood samples were taken using a finger lancing device (Roche® Products Ltd, Hertfordshire, AL7 1TW United Kingdom) at each of the time periods specified (Chapter 2.2.3 for sampling methodology).

Leukocyte responsiveness to *in vitro* PMA stimulation was assessed for each pair of whole blood samples (Chapter 2.1.2 and Table 2.1 for full description of LCC protocol), at each of the specific time points.

Data Analysis

Standard statistical methodology (Chapter 2.5) was applied to investigate and compare the effect of both psychological stressors (Car A n=21, Car B n=18) on leukocyte activity (Chapter 2.5.1) and also heart rate, systolic and diastolic blood pressure and core body temperature (Chapter 2.5.2). The relationship between post-stressor changes in leukocyte activity and heart rate, systolic and diastolic blood pressure and core body temperature, was analysed using Bivariate correlation (Chapter 2.5.3). Perceived psychological stress was qualitatively assessed using likert scales pre- and immediately post-stressor. The significance of the post-stressor change between treatment groups was investigated, followed by their correlation with the post-stressor changes in leukocyte activity (Chapter 2.5.4).

3.3 RESULTS

Leukocyte Activity

LCC profiles are displayed on Figure 3.1, with post-test changes in activity for the 5 attributes of the luminescence profiles given in Table 3.1. Data are expressed as mean differences between leukocyte activity at 45 minutes pre-and immediately post-test (RLU_{adj} ± standard error of mean (S.E.M.). As data for time to maximum leukocyte activity (T-max (minutes) is discontinuous, median values ± S.E.M. are presented.

Pre-stressor activity for both treatment groups followed the same trend with regard to both the rate and magnitude of reactive oxygen species (ROS) release (Figure 3.1). Following the test, the mean LCC response for both treatment groups showed decreases in leukocyte activity, with the most pronounced post-test change occurring at T=15 minutes (RLU_{adj}) (adjusted response in leukocyte activity recorded 15 minutes into the 45 minute activity profile) for Car A (Car A 1198.6 ± 478.3 RLU_{adj}; Car B 511.5 ± 150.6 RLU_{adj}). These differences were found to be significant between treatment groups for T=5 minutes (RLU_{adj}) ($F_{1,38}$ = 5.94, P = 0.02), T=10 minutes (RLU_{adj}) ($F_{1,38}$ = 4.35, P = 0.04), and for T-max (minutes) (the time taken to reach maximum adjusted leukocyte activity (Hmax-RLU_{adj}) ($F_{1,38}$ = 7.86, P = 0.008), (Table 3.1). These findings show that the decrease in leukocyte activity following exposure to vehicle A is significantly greater than for vehicle B, suggesting that the emotional / psychological response provoked as a consequence of performing the same basic driver related tasks in vehicle A was greater in magnitude compared to vehicle B.

Perceived Stress

Trends in post-test qualitative perceived stress (assessed using likert scales employing a continuous scale where 1 represented relaxed and 10 stressed) paralleled those in LCC scores, with subjects exhibiting a significant post-test increase of 2.0 ± 1.0 Units ($F_{1,41}$ = 7.61, P = 0.009) in response to Car A, whilst showing no significant post-test change in response to Car B (0.0 ± 0.0 Units ($F_{1,35}$ = 0.009, P = 0.93). As with leukocyte activity, post-test differences were significant between treatment groups ($F_{1,77}$ = 4.0, P = 0.05).

Core Body Temperature

No significant difference was shown between post-test increases in core body temperature between treatment groups ($F_{1,38}$ = 0.54, P = 0.47) (Table 3.2). The response of subjects to Car A showed a significant increase between 45 minutes pre- and immediately post-test of 0.4 ± 0.1°C (P = 0.04 Tukey's *post hoc* procedure). Whilst subjects exposed to Car B showed no significant change (0.3 ± 0.1°C

P = 0.29 Tukey's *post hoc* procedure). No significant correlation between leukocyte activity and core body temperature was reported.

Heart Rate, Blood Pressure

There were no significant post-test differences in either heart rate or systolic or diastolic BP between 45 minutes pre- and immediately post-test, or between treatment groups (Table 3.2).

FIGURE 3.1a and b. Luminescence profiles showing adjusted mean leukocyte activity (RLU_{adj}) (subtraction of baseline leukocyte activity from PMA stimulated challenge activity) ± S.E.M. for blood samples taken 45 minutes pre-, immediately post- and 45 minutes post-test for treatment group A (n=21) and treatment group B (n=18) respectively. * indicates significant difference in activity between 45 minutes pre- and immediately post-test ($P < 0.05$).

Figure 3.1a Treatment Group A (car A) (n=21)

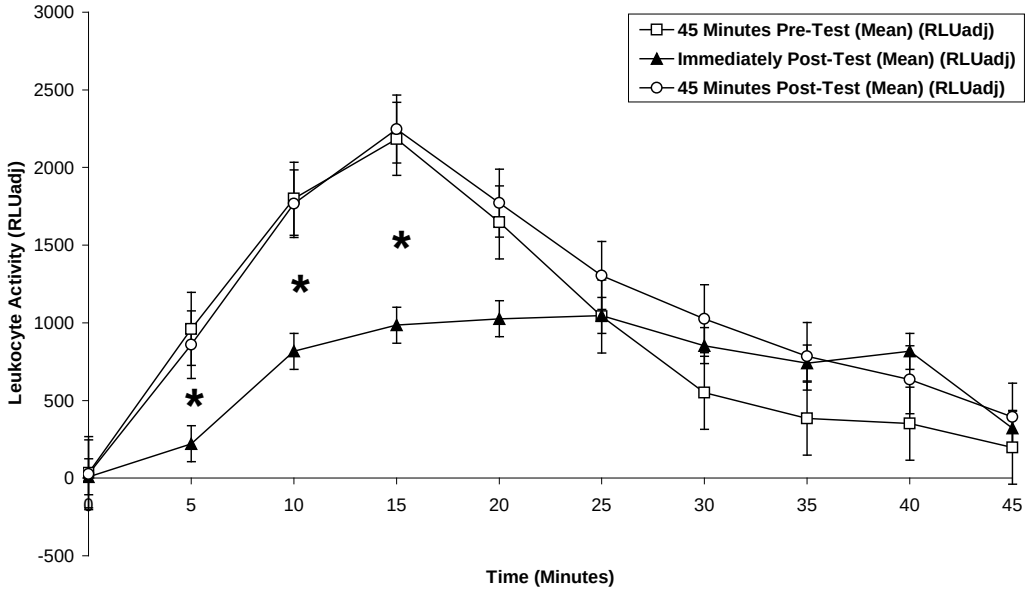


Figure 3.1b Treatment Group B (car B) (n=18)

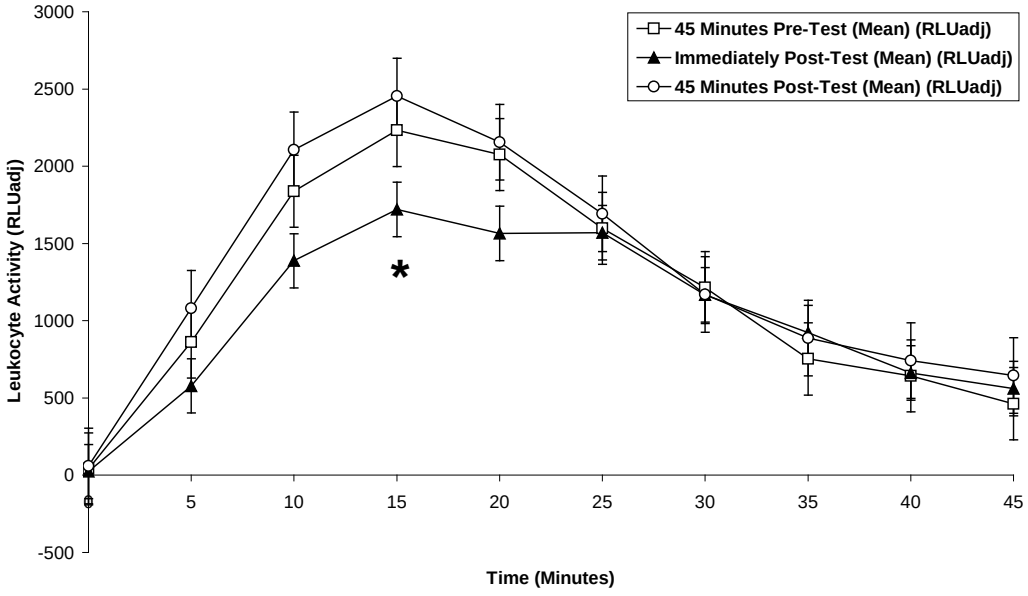


TABLE 3.1 Effect of stressor on leukocyte activity

	CAR A	CAR B	*P* (F)
Δ Hmax (RLUadj)	-899.9 ± 412.0	-368.1 ± 188.5	0.13 (2.43)
Δ T-max (minutes)	5.0 ± 2.0 •	0.0 ± 0.8	0.008* (7.86)
Δ T=5 minutes (RLUadj)	-738.7 ± 185.4 •	-284.5 ± 98.6	0.02* (5.94)
Δ T=10 minutes (RLUadj)	-969.6 ± 280.4 •	-449.9 ± 169.7	0.04* (4.35)
Δ T=15 minutes (RLUadj)	-1198.6 ± 478.3 •	-511.5 ± 150.6 •	0.09 (3.05)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) for each of the four specific attributes of the leukocyte luminescence profiles for both treatment groups (car A n=21, car B n=18) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}). Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}) For the difference in the time taken to reach maximum adjusted leukocyte activity (T-max) (minutes) the median is presented along with S.E.M. Single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (*P*) (*d.f.* = 38).

- Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

* Difference between Car A and Car B ($P < 0.05$)

TABLE 3.2 Effect of stressor on heart rate, core body temperature and blood pressure

	CAR A	CAR B	*P* (F)
Δ Heart rate (bpm)	3.0 ± 2.0	0.0 ± 1.0	0.26 (1.31)
Δ Core Body Temperature (°C)	0.4 ± 0.1 •	0.3 ± 0.1	0.47 (0.54)
Δ Systolic Blood Pressure (mmHg)	1.0 ± 2.0	3.0 ± 3.0	0.75 (0.1)
Δ Diastolic Blood Pressure (mmHg)	1.0 ± 3.0	4.0 ± 2.0	0.17 (2.01)

Mean and standard error of the mean (S.E.M.) are presented for the post-test change (Δ) in heart rate, core body temperature and blood pressure (difference between 45 minutes pre- and immediately post-test samples) for each treatment group (Car A n=21, Car B n=18). Single factor ANOVA was used to investigate the effect of treatment on each of the stated parameters (*P*) (*d.f.* = 38). No significant difference between treatment groups was shown for any of the listed parameters.

- Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

3.4 DISCUSSION

The results reveal that exposure to mild short-term psychological stress evoked rapid and reversible changes in immune responsiveness, permitting the quantitative differentiation between two closely related psychological stressors. In contrast, no significant changes in heart rate, systolic or diastolic blood pressure or body temperature were noted between treatment groups (Table 3.2). This ability demonstrates the importance of psychological immune-competency as a quantitative measure of mental workload, and suggests that the activation threshold of leukocytes may be lower than is required for the manifested changes of increased heart rate and blood pressure, that are normally associated with, and used to monitor the presence of psychological stress. The post-stressor change in PMA-induced leukocyte activity was significantly greater for car A compared to car B (Table 3.1 and Figure 3.1a and b). Indicating that the ability of leukocytes to produce a respiratory burst in response to *in vitro* PMA stimulation, following completion of the stressor protocol in car A, was significantly reduced compared to car B. As qualitative data concluded that subjects perceived completion of the protocol using car A more psychologically stressful, the degree of physiological stimulation, through HPA axis activity would have resulted in a greater *in vivo* respiratory burst for treatment group A. This would have resulted in the observed reduced *in vitro* PMA-stimulated respiratory burst exhibited by treatment group A. These findings support those of Atanackovic *et al*. (2002), whereby following exposure to a putatively stressful event, a significant reduction in ROS production is observed, compared to control.

Naccache *et al*. (1990) and McLaren *et al*. (2003) both showed that the maximum rate of superoxide production was reached 15 minutes after PMA introduction, the reaction would then plateau before gradually decreasing to baseline activity. Consistent with these findings, this experiment showed a peak output between 15 and 20 minutes, with the rate of increase and magnitude of response decreasing according to psychological stressor intensity.

The efficacy of the Junior™ LB 9509 luminometer (Berthold® Technologies, Hertfordshire, AL3 7LZ United Kingdom) in detecting luminol-enhanced chemiluminescence in whole blood, has been proven by this research and by studies

conducted by McLaren *et al*. (2003) and Montes *et al*. (2003, 2004). However, it does possess a number of limitations which may reduce and restrict the devices ability to quantify and follow the reaction kinetics in real-time; such as an inability to continuously monitor samples, due in part to the lack of automatic temperature control. The LB 9509 luminometer has the facility to adjust the duration of photonic detection from 1 to 999 seconds, presenting the overall result in RLU (Chapter 1.6.3). However, the sampling process is unable to indicate when, within the specific sampling time, the reactions took place. In this experiment each sample was measured for 30 seconds every 5 minutes, which, although only provided a glimpse of the total reaction kinetics, was deemed sufficient for the purposes of this research, to provide adequate detail for comparison between stressors, allowing them to act as a diagnostic measure of psychological stress.

One possible limitation associated with the LCC protocol, used throughout this research, is associated with the concentration of Dimethyl Sulfoxide (DMSO), utilised as a solvent in the preparation of both luminol and PMA stock solutions. The ability of DMSO to act as an antioxidant (a scavenger of free radicals) has been demonstrated in animals (Itoh and Guth 1985). Although it has been shown that DMSO is most effective at concentrations between 90 and 70%, with activity diminishing with reducing concentration, it has still proven capable of crossing the cellular membrane at a 15% concentration (Herschler and Jacob 1980). The effective working concentrations of DMSO within the current LCC protocol are 0.1% for luminol at (10^{-4}M) and 1% for PMA (10^{-5}M) stock. After the addition of heparin and whole blood the dilution factor of DMSO was further increased. Within the challenge LCC sample (Sample B) (Chapter 2.1.2), DMSO was at a total working percentage concentration of 0.155% (i.e. 10ul 10^{-5}M PMA contains DMSO at a percentage concentration of 0.08% and 90ul 10^{-4}M Luminol contains DMSO at a percentage concentration of 0.075%), within the control LCC sample (Sample A) with the absence of PMA the final working percentage concentration of DMSO was 0.075% (DMSO present in 90ul 10^{-4}M luminol). At these concentrations it was unlikely that DMSO would still possess sufficient antioxidant ability to affect the chemiluminescence results following PMA-induced leukocyte challenge. In that a percentage of the leukocyte respiratory burst would be scavenged and effectively neutralised before it has been detected by the luminometer, possibly leading to

inaccurate assessment of the extent of the reduction in leukocyte activity resulting from the psychological stressor under investigation. It may however be the case that as DMSO concentration differed between samples (sample A 0.075% DMSO, sample B 0.155% DMSO) the antioxidant effect of DMSO within the Challenge sample (sample B) would be marginally greater than that of the control (sample A). The resulting observed reduction in reactive oxygen species production within sample B may not have therefore been solely attributed to a reduction in leukocyte responsiveness. In order to remove this effect the total percentage concentration of DMSO within sample A should have equalled sample B.

Unlike leukocyte activity, physical measurements such as heart rate and blood pressure did not return significant post-test changes between treatment groups, this may suggest that the psycho-physiological threshold for leukocyte reactivity is less than for the other parameters, allowing LCC to detect particularly subtle and transient changes in mental loading. The possibility exists that the stressors' influence was diminished due to anticipation of experiencing an environment not normally accessible to the public (a motor vehicle testing facility). Therefore although subjects still consciously perceived the test as being stressful, sub-consciously the novel environment and subsequent excitement lessened the stressors overall impact. The ability of emotion to influence physiological responses is well documented, Manikonda *et al*. (2008) demonstrated how contemplative meditation reduced ambulatory blood pressure and stress induced hypertension. A similar mechanism may also be present for the sub-conscious.

Due to restrictions placed upon test vehicle availability (concurrent use of both vehicles was not feasible) a randomised cross over study design was not logistically feasible. In an attempt to reduce the possibility of habituation occurring in response to the novel test scenario, 18 different subjects were recruited for the second phase of the study (Car B). This process meant that paired data comparison was not possible and that despite subjects being randomly recruited to each teat group, a truly randomised cross-over study design was not possible.

A further limitation of the study design was associated with the collection of pre-test measurements and subsequent transportation of volunteers to the test site. Ideally the

45 minute Pre-Test measurements, used to establish baseline leukocyte activity, should have been conducted once the subject had reached the Whitley test facility. As both the journey to and entry into the facility, which involved a number of security protocols may have provoked an emotional response which could have influenced baseline leukocyte activity. Access to the test vehicles was the main determining factor as to why this protocol was not followed. Demand was such that vehicles were only available during the periods of 11.00 to 12.00 hours and 14.00-15.00 hours, which made it logistically impossible to take both pre- and immediately post-test measurements as well as conduct the actual stressor test within the available time. It was therefore decided to conduct baseline measurements within the laboratory and then to ensure that once volunteers had been transported to the test site that they were allowed a 10 minute period of relative relaxation (sitting quietly in the researcher's vehicle) prior to exposure to the stressor.

In conclusion, the aims of the experiment were achieved in that the leukocyte coping capacity protocol at the stated reagent concentrations, despite the possible inhibitory effects of DMSO, displayed the ability to differentiate between two similar psychological stressors. Providing a quantitative indication of the degree of psychological stress (mental loading) exhibited as a result of performing the same basic driving related tasks in two different motor vehicles employing different ergonomic design principles.

4.0 Quantifying LCC Sensitivity – Window Lift Polarity Reversal Study.

4.1 RATIONALE

The pilot study in section 3.0 demonstrated the capability of the LCC protocol to differentiate between changes in leukocyte responsiveness resulting from exposure to a short-term (as classified by Elliot and Eisdorfer 1982) psychological stressor, lasting on average 10 minutes. The purpose of this study was to explore the sensitivity of the LCC protocol. It was hypothesised that LCC at the reagent concentrations stated in the previous study would possess sufficient sensitivity to detect the physiological changes in immune responsiveness manifested as a consequence of a psychological stressor lasting a maximum of 30 seconds. The selected "acute" stressor was designed to evoke a subtle level of psychological confusion and increased mental loading. Although the same sample population demographic, as used with the previous study was utilised (healthy, moderately fit males and females aged between 21 and 65 years). Subjects were recruited on the basis that they had not participated in the previous study, and therefore possessed no prior knowledge regarding the stressor protocol, this action removed the possible occurrence of stressor habituation (Chapter 2.2.2).

4.2 INTRODUCTION

Numerous studies have shown links between mid to long term psychological stress and altered immuno-competency (Boscarino and Chang 1999, Glaser *et al*. 1999, Boscarino 2004, Altemus *et al*. 2006). Examples include the effect of post traumatic stress disorder (PTSD) exhibited by females who experienced physical and psychological childhood abuse (Altemus *et al*. 2003 and 2006), and elevated leukocyte counts and depressed systemic cortisol concentration (Boscarino 1999) (Chapter 1.1.6). Evidence highlighted by Okutsu, *et al*. (2005) demonstrates how acute physiological stress, such as thermal and traumatic injury, surgery, and acute myocardial infarction can elicit modification of leukocyte trafficking through altered

glucocorticoid secretion. A characteristic of such a modification appears to be associated with the development of a variety of immune related disease states. It is also widely accepted that short term stressors including physical exercise and increased mental loading (e.g. academic examinations) can modify leukocyte (primarily neutrophils) distribution (Shinkai *et al*. 1996) and activation state (Mian *et al*. 2003). In contrast, evidence also exists which demonstrates the existence of an immuno-enhancing effect during acute physiological and psychological stress, part of the fight flight stress response which serves to prepare the immune system for potential physical harm and subsequent infection (Dhabhar 2003, Viswanathan 2005) (Chapter 1.1.5).

The often unwarranted activation of leukocytes and inappropriate release of a limited store of lytic agents (e.g. ROS, nitric oxide), in response to periods of acute psychological stress, can result in altered leukocyte distribution, damage to healthy tissue and an inability to respond to a genuine physical threat (e.g. pathogen) (Maes *et al*. 1998, Dhabhar 1998, 2003, Mian *et al*. 2003, Viswanathan 2005). Epidemiological studies indicate that individuals who experience greater degrees of psychological stress are more susceptible to opportunistic infection (Clover *et al*. 1989, Galinowski 1997). Clover *et al*. (1989) linked stress associated with an unstable family environment to an increased incidence of upper respiratory tract infection and influenza B. Similarly, accumulating stress has been linked to chronic reduced immuno-competency and increased susceptibility to opportunistic infections in elite athletes (Gleeson 2000). Rodriguez-Galan *et al*. (2001) demonstrated how the opportunistic fungal disease *Candida albicans* proliferated in stressed rather than non-stressed individuals.

There is however, little evidence which demonstrates the effect on immune function, particularly leukocyte activation of extreme short term psychological stress or even simple psychological confusion lasting seconds rather than hours or days. Using leukocyte coping capacity, this study aimed to, firstly, investigate leukocyte ability to respond to particularly mild "acute" alterations in mental loading (psychological stress) lasting seconds rather than minutes or hours. Secondly, we aimed to validate the ability of the LCC technique to detect such transient alterations in leukocyte responsiveness.

The selected stressor involved adjustment of a motor vehicle electric window to a number of designated positions. The window mechanism had been modified so as to allow covert adjustment of the normal functioning protocol.

4.3 METHODS

Subjects

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before commencement of the study, in accordance with the declaration of Helsinki (World Medical Association 2004)

10 male and 10 female healthy, individuals aged between 27 and 53 years were recruited from a pool of 40 volunteers. People were excluded if they suffered from psychiatric illness, respiratory or cardiovascular disease, were smokers, had taken prescription medicine within the previous month, or if they possessed prior knowledge of the test equipment (Chapter 2.2.2).

Design

Subjects were assigned to one of two treatment groups, group A - control and group B – Intervention. It was ensured that both groups contained equal numbers of each sex. The experimental protocols were rigorously standardised, and testing was confined to between the hours of 10.30am and 3.00pm. Subjects were required to avoid any strenuous activity for at least 2 hours prior to testing (e.g. they were instructed to take the lift to the laboratory, rather than climbing the stairs).

At the beginning of the experiment each subject sat quietly and was instructed to breathe orthonasally for 15 minutes prior to obtaining resting heart rate, BP and core body temperature (Chapter 2.4 for standard protocol). A resting blood sample was taken (Chapter 2.2.3) 45 minutes before exposure to the test apparatus (45 minutes

Pre-Test). During the 45 minute pre-test period subjects were asked to sit quietly or read (reading material was of non stimulating content (local newspaper).

Approximately 2 minutes before the start of the trial, the subject was ushered to an isolated, previously unseen, area of the lab, where they were seated comfortably, and the test protocol explained. The test lasted a maximum of 1 minute. During which time the subject was requested to open the car electric window to a designated level. After a 5 second pause the subject was then to open the window fully, then, after a further 5 seconds, partially closing the window to the designated halfway mark before, after a final 5 second pause, closing the window fully. It was explained that once the test had begun no further verbal communication was allowed, although the researcher would state when each 5 second pause had elapsed.

For the test, a Jaguar X-Type passenger door was secured in a wooden stand simulating the correct height and position a passenger would experience within a normal car (Figure 4.1). The electric window was powered by a 20 Volt D.C. power supply. The door had been retro-fitted with a switch which allowed the researcher to alter, unseen, the polarity of the electric window mechanism, so that when the subject pressed the up-button the window lowered and vice versa (Figure 4.2). The door was unveiled to the subject immediately before the test started.

Figure 4.1
Jaguar X-Type Door in Stand.

Figure 4.2
Location of Electric Window Motor Polarity Reversal Switch.

During the test, the function of the window mechanism was as indicated by the arrows on the switch for group A (control), however the polarity of the window was reversed after each 5 second pause for group B (intervention).

Immediately upon completion of the task, and again 45 minutes afterwards, heart rate, BP and core body temperature were recorded and further blood samples taken.

Determining Leukocyte Activity

Two 10µl blood samples were taken using a finger lancing device (Roche® Products Ltd, Hertfordshire, AL7 1TW United Kingdom) at each of the time periods specified (Chapter 2.2.3 for sampling methodology).

Leukocyte responsiveness to *in vitro* PMA stimulation was assessed for each pair of whole blood samples (Chapter 2.1.2 and Table 2.1 for full description of LCC protocol), at each of the specific time points.

Data Analysis

Standard statistical methodology (Chapter 2.5) was applied to investigate and compare the effect of the psychological stressor (n=10 for each treatment group) on leukocyte activity (Chapter 2.5.1) and also heart rate, systolic and diastolic blood pressure and core body temperature (Chapter 2.5.2). To minimise the effect of inter-individual variation the differences between 45 minutes pre- and immediately post-test for each of the above parameters were calculated for each treatment group. Using 2-way analysis of variance, the effect of treatment group and gender (both used as fixed factors) on post-test changes in leukocyte activity and also heart rate, core body temperature and systolic and diastolic blood pressure (all used as dependent variables), was investigated. The interaction between treatment group and gender was also investigated for each of the stated parameters.

The relationship between post-stressor changes in leukocyte activity and heart rate, systolic and diastolic blood pressure and core body temperature, was analysed using Bivariate correlation (Chapter 2.5.3).

Perceived psychological stress was qualitatively assessed using likert scales (where 1 represented relaxed and 10 stressed) pre- and immediately post-stressor. The significance of the post-stressor change between treatment groups was investigated, followed by their correlation with the post-stressor changes in leukocyte activity (Chapter 2.5.4).

4.4 RESULTS

Leukocyte Activity

LCC profiles are displayed on Figure 4.3, with post-test changes in activity for the 5 attributes of the luminescence profiles given in Table 4.1. Data are expressed as mean differences between leukocyte activity at 45 minutes pre- and immediately post-test (RLU_{adj}) ± standard error of mean (S.E.M.). Following the test, the mean change in LCC response (Hmax-RLU_{adj} and T=10 minutes) between 45 minutes pre- and immediately post-test for group B was greater than that of group A (control) (Table 4.1). In general, LCC scores were depressed following the test in group B, but not in group A (Figure 4.3). Most strikingly, at T=10 minutes the post-test change in leukocyte activity for the Control group (group A) exhibited a decrease of 46.2 ± 38.2 RLU_{adj} which was significantly different to the larger decrease observed within the group B (240.0 ± 56.1 RLU_{adj}) ($F_{1,19}$ = 8.23, P = 0.001). A similar trend was noted at maximum leukocyte activity (Hmax-RLU_{adj}) where group A demonstrated a post-test increase of 41.6 ± 20.7 RLU_{adj}, compared to a decrease between pre- and immediately post-test for group B (150.7 ± 82.0 RLU_{adj}) ($F_{1,19}$ = 5.3, P = 0.04).

Gender and Leukocyte Activity

A significant difference in post-test leukocyte activity was noted for the interaction between genders and treatment group at T=5 minutes ($F_{2,59} = 4.6$, $P = 0.04$). Both males and females within group A (control) exhibited post-test decreases in activity which proved to be statistically different ($F_{1,59} = 8.47$, $P = 0.008$) (males: 9.4 ± 21.91 RLU_{adj}, females: 40.8 ± 60.59 RLU_{adj}), For group B, males demonstrated a more dramatic decrease between pre-and immediately post-test of 262.4 ± 122.06 RLU_{adj} while conversely the females demonstrated a post-test increase of 68.0 ± 257.58 RLU_{adj} ($F_{1,59} = 0.01$, $P = 0.93$) (Table 4.2).

Perceived Stress

No significant change in post-test qualitative perceived stress was shown by either treatment group (Group A post-test increase of 1.0 ± 1.0 Units ($F_{1,19} = 0.012$, $P = 0.87$), Group B post-test increase of 2.0 ± 2.0 Units ($F_{1,19} = 0.01$, $P = 0.9$). Similarly post-test differences between treatment groups also proved non-significant ($F_{1,39} = 0.009$, $P = 0.93$).

Core Body Temperature

A significant difference in post-test core body temperature was found between treatment groups ($F_{1,39} = 6.38$, $P = 0.02$). Group B demonstrated a post-test decrease of 0.2 ± 0.1°C while group A did not show any change post-test (0.0 ± 0.1°C) (Table 4.3 and Figure 4.5).

Post-test differences in core body temperature were found to be significant for the interaction between gender and treatment group ($F_{2,39} = 4.9$, $P = 0.03$) (Table 4.4). Both males and females within group A did not show any post-test change. For group B, males showed a post-test increase of 0.2 ± 0.1°C while females showed a statistically different decrease of 0.2 ± 0.1°C ($F_{1,29} = 21.34$, $P < 0.001$).

Heart Rate and Blood Pressure

Post-test differences in heart rate were found to be significant for the interaction between treatment group and gender ($F_{2,39}$ = 5.23, P = 0.02) (Table 4.4). Within group A, males demonstrated a post-test increase of 1.0 ± 2.0 bpm, while females showed a statistically different post-test decrease of 2.0 ± 3.0 bpm ($F_{1,29}$ = 7.97, P = 0.009). Within group B both genders demonstrated statistically similar post-test increases in heart rate (males 1.0 ± 3.0 bpm vs. females 4.0 ± 2.0 bpm ($F_{1,29}$ = 0.53, P = 0.47).

The interaction between treatment group and gender was also proved significant for post-test differences in systolic BP ($F_{2,39}$ = 5.16, P = 0.02) (Table 4.4). Both treatment groups demonstrated statistically significant post-test changes in systolic BP from baseline. Group A: males 8.0 ± 6.0 mmHg vs. females 2.0 ± 2.0 mmHg ($F_{1,29}$ = 9.56, P = 0.005). Group B: males 1.0 ± 3.0 mmHg vs. females 3.0 ± 5.0 mmHg ($F_{1,29}$ = 0.02, P = 0.87).

Although the interaction between treatment group and gender was proved non-significant for diastolic BP ($F_{2,39}$ = 0.55, P = 0.46), both treatment groups demonstrated statistically different changes between genders (Table 4.4). For group A both genders exhibited statistically different post-test decreases (males 3.0 ± 3.0 mmHg vs. females 2.0 ± 2.0 mmHg ($F_{1,29}$ = 5.73, P = 0.02). While the males within group B followed the same trend, showing a post-test decrease of 3.0 ± 4.0 mmHg, females reported a significantly different post-test increase of 5.0 ± 3.0 mmHg ($F_{1,29}$ = 4.23, P = 0.05).

Leukocyte Activation and Heart Rate, BP and Core Body Temperature

Due to the effect of multiple comparisons the significant correlation observed between post-test change in time required to reach maximum adjusted leukocyte activity (T-max) and the post-test change in heart rate (Bivariate correlation P = 0.01 (Pearson correlation 0.38 n = 20) cannot be classed as significant (Truncated Product

Method $P = 0.97$). There were no significant correlations between adjusted post-test changes in leukocyte activation and any of the traditional physiological indicators of stress. (Table 4.5).

FIGURE 4.3a and b. Luminescence profiles showing mean adjusted leukocyte activity (RLU_{adj}) (subtraction of baseline leukocyte activity from PMA stimulated challenge activity) ± S.E.M. for treatment group A (control) and treatment group B (intervention) (n=10 for each) respectively. To obtain an activity profile, leukocyte activity was measured at 5 minute intervals for a total of 45 minutes. * indicates significant difference in activity between 45 minutes pre- and immediately post-test ($P < 0.05$).

Figure 4.3a – Treatment Group A (Control)

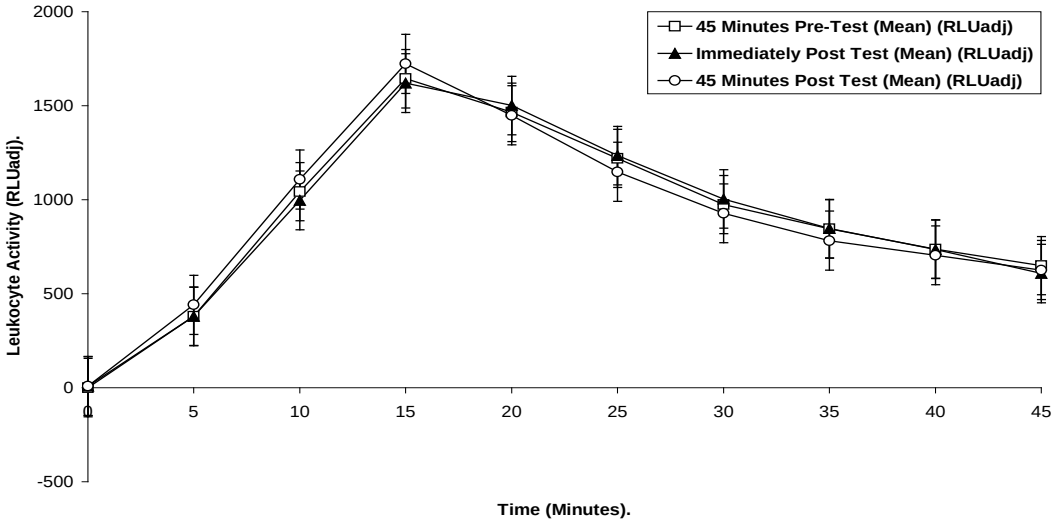


Figure 4.3b – Treatment Group B (Intervention)

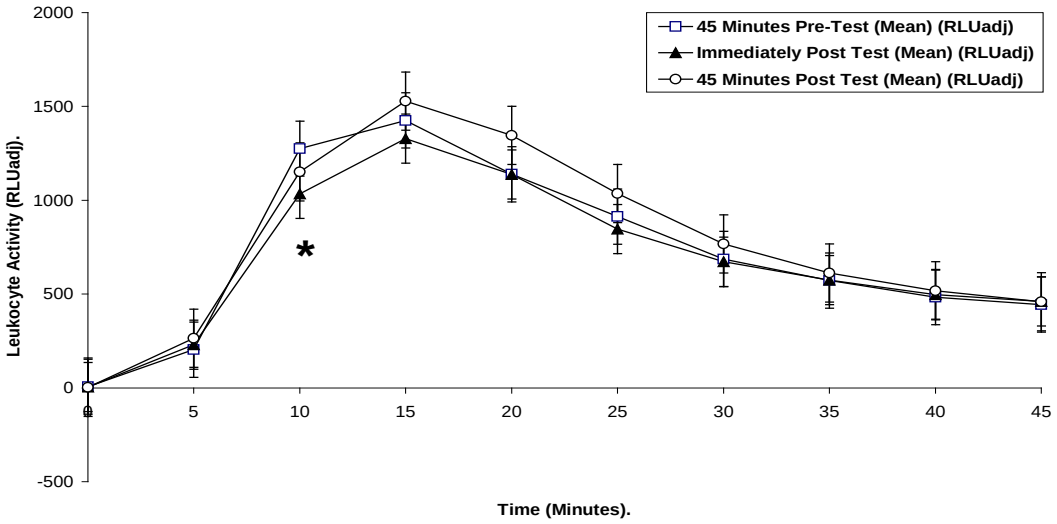


TABLE 4.1 Effect of stressor on leukocyte activity

	GROUP A	GROUP B	*P* (F)
Δ Hmax (RLUadj)	-41.6 ± 20.7	-150.7 ± 82.0	0.04* (5.3)
Δ T-max (minutes)	0.0 ± 1.1	0.0 ± 0.8	0.48 (0.53)
Δ T=5 minutes (RLUadj)	-0.1 ± 30.8	-25.7 ± 63.2	0.72 (0.13)
Δ T=10 minutes (RLUadj)	-46.2 ± 38.2	-240.0 ± 56.1 •	0.001* (8.23)
Δ T=15 minutes (RLUadj)	-25.1 ± 30.8	-97.0 ± 145.0	0.63 (0.24)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) for each of the four specific attributes of the leukocyte luminescence profiles for both treatment groups (n = 10 for each) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}). Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}) For the difference in the time taken to reach maximum adjusted leukocyte activity (T-max) (minutes) between samples taken 45 minutes pre- and immediately post-test, the median is presented along with S.E.M. Single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (*P*) (*d.f.* = 19).

- Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

* Difference between Group A and Group B ($P < 0.05$)

TABLE 4.2 Comparison of the effect of stressor on leukocyte activity between genders

	GROUP A	*P*1 (F)	GROUP B	*P*1 (F)	*P*2 (F)
Δ Hmax (RLUadj)					
Male	68.8 ± 34.88	0.73 (0.13)	-245.4 ± 107.77	0.14 (2.35)	0.42 (0.66)
Female	14.4 ± 18.36		-56.0 ± 121.29		
Δ T-max (minutes)					
Male	2.0 ± 2.0	0.19 (1.8)	1.0 ± 1.0	0.74 (0.19)	0.59 (0.29)
Female	-1.0 ± 1.0		2.0 ± 1.22		
Δ T=5 minutes (RLUadj)					
Male	-9.4 ± 21.91	**0.008*** (8.47)	-262.4 ± 122.06	0.93 (0.01)	**0.04*** (4.6)
Female	-40.8 ± 60.59		68.0 ± 257.58		
Δ T=10 minutes (RLUadj)					
Male	-98.6 ± 54.6	0.59 (0.29)	-195.2 ± 85.04	0.13 (2.44)	0.36 (0.95)
Female	6.2 ± 47.04		-285.8 ± 76.9		
Δ T=15 minutes (RLUadj)					
Male	-40.2 ± 47.93	0.62 (0.25)	-43.6 ± 55.04	0.17 (1.93)	0.54 (0.39)
Female	40.4 ± 33.91		95.0 ± 112.08		

Mean and standard error of the mean (S.E.M.) are presented for the change in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}) for each of the four attributes of the leukocyte luminescence profiles for each gender within both treatment groups (n = 5 males 5 females for each). Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}). For T-max - the time taken to reach maximum adjusted leukocyte activity (minutes) the median ± S.E.M. is presented. For each treatment group, 2-way ANOVA was used to investigate the effect of gender on post-test changes in leukocyte activity (difference between pre- and post-stressor) (*P*1) (*d.f.* = 29 for each). The significance of the interaction between treatment group and gender on post-test changes in the 5 responses of the leukocyte activity luminescence profiles (*P*2) are reported using 2-way ANOVA (*d.f.* = 59).

* $P < 0.05$ Statistically Significant

Figure 4.4 Comparison of leukocyte activity between genders for each treatment group

Figure 4.4a Control Group A

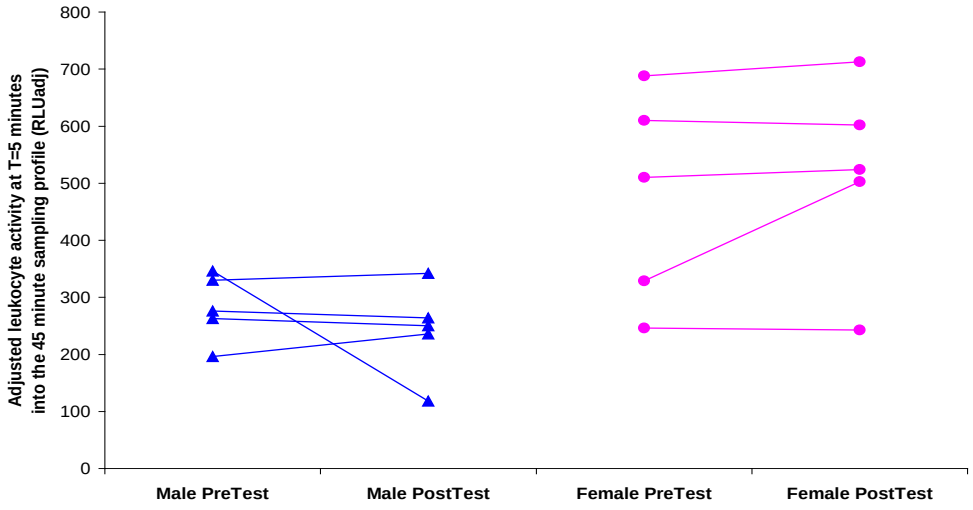
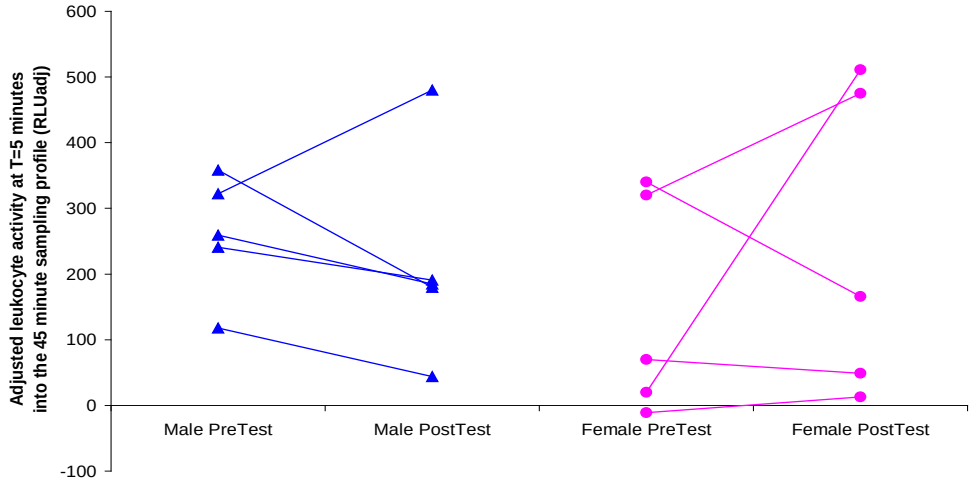


Figure 4.4b Intervention Group B



Comparison of leukocyte activity between gender (closed triangles represent males, closed circles represent females) for each treatment group (n=5males and 5 females for each) for blood samples taken 45 minutes pre- and immediately post-test, measured at T=5 minutes into the 45 minute sampling profile.

TABLE 4.3 Effect of stressor on heart rate, core body temperature and blood pressure for both treatment groups

	GROUP A	GROUP B	*P* (F)
Δ Heart Rate (bpm)	0.0 ± 2.0	2.0 ± 2.0	0.94 (0.01)
Δ Core Body Temperature (°C)	0.0 ± 0.1	-0.2 ± 0.1	0.02* (6.38)
Δ Systolic Blood Pressure (mmHg)	5.0 ± 3.0	2.0 ± 3.0	0.13 (2.38)
Δ Diastolic Blood Pressure (mmHg)	-2.0 ± 2.0	1.0 ± 2.0	0.79 (0.07)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in heart rate, core body temperature and blood pressure (difference between 45 minutes pre- and immediately post-test samples) for both treatment groups (n = 10 for each). 2-way ANOVA was used to investigate the effect of treatment on each of the stated parameters (*P*) (*d.f.* = 39). For each treatment group the differences between pre- and post-test values for all parameters proved non-significant (Tukey's *post hoc* procedure).

* Difference between Group A and Group B ($P < 0.05$)

TABLE 4.4 Comparison of the effect of stressor on heart rate, core body temperature and blood pressure between genders

	GROUP A	*P*1	GROUP B	*P*1	*P*2
Δ Heart Rate (bpm)					
Male	1.0 ± 2.0	0.009* (7.97)	1.0 ± 3.0	0.47 (0.53)	0.02* (5.23)
Female	-2.0 ± 3.0		4.0 ± 2.0		
Δ Core Body Temperature (°C)					
Male	0.0 ± 0.1	0.46 (0.55)	0.2 ± 0.1	< 0.001* (21.34)	0.03* (4.9)
Female	0.0 ± 0.1		-0.2 ± 0.1		
Δ Systolic Blood Pressure (mmHg)					
Male	8.0 ± 6.0	0.005* (9.56)	1.0 ± 3.0	0.87 (0.02)	0.02* (5.16)
Female	2.0 ± 2.0		3.0 ± 5.0		
Δ Diastolic Blood Pressure (mmHg)					
Male	-3.0 ± 3.0	0.02* (5.73)	-3.0 ± 4.0	0.05* (4.23)	0.46 (0.55)
Female	-2.0 ± 2.0		5.0 ± 3.0		

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test samples) for each gender within both treatment groups (n = 5 males 5 females for each). For each treatment group, single factor ANOVA was used to investigate the effect of gender on post-test changes in each of the stated parameters (*P*1) (*d.f.* = 29 for each). Also the significance of the interaction of treatment group and gender on the post-test difference in HR, BP and core body (*P*2) are reported using 2-way ANOVA (*d.f.* = 39)

* $P < 0.05$ Statistically Significant

TABLE 4.5 Relationship between post-test changes in leukocyte activity and changes in heart rate, core body temperature and blood pressure

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes
Δ HR	-0.12 (0.61)	0.38 (0.01)	-0.24 (0.3)	-0.36 (0.11)	-0.05 (0.82)
Δ Core Body Temperature	-0.09 (0.71)	-0.17 (0.47)	0.01 (0.96)	-0.04 (0.87)	0.02 (0.93)
Δ Systolic BP	-0.37 (0.1)	-0.36 (0.12)	-0.11 (0.63)	0.05 (0.82)	-0.39 (0.09)
Δ Diastolic BP	0.18 (0.45)	-0.15 (0.54)	0.41 (0.07)	-0.26 (0.26)	0.32 (0.16)

Combined data from both treatment groups (n=20) was tested using bivariate correlation to investigate the significance of the relationship between post-test changes (Δ) in leukocyte activity and in heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test) (Pearson correlation with *P*-values in brackets). The Truncated Product Method (Zaykin *et al*. 2002) was used to correct for the use of multiple comparisons, no significant values were returned, indicating the absence of any significant relationships between leukocyte activity and any of the other stated parameters.

FIGURE 4.5 Core body temperature, heart rate and systolic and diastolic blood pressure pre-, immediately post and 45 minutes post-stressor.

Figure 4.5a

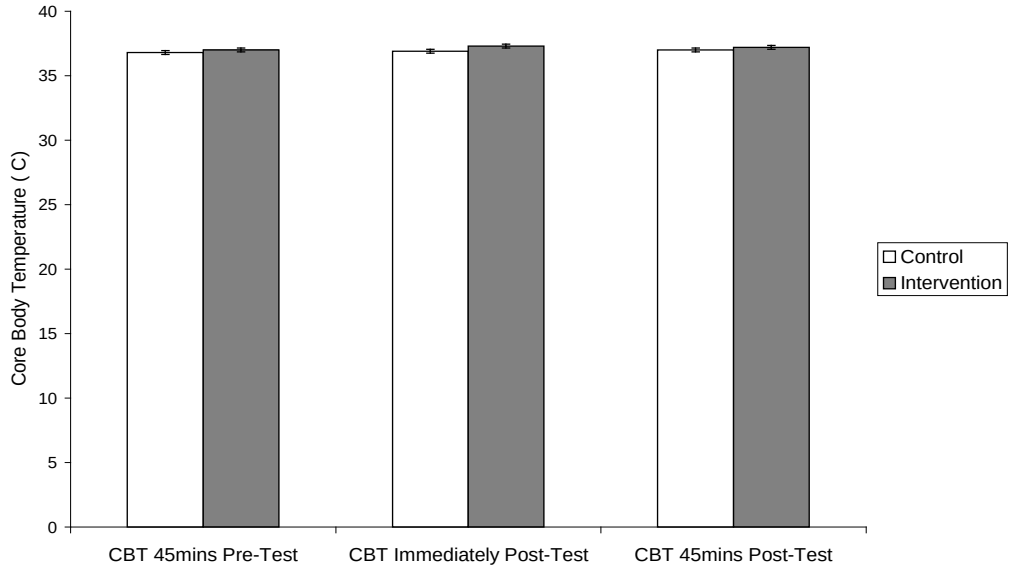
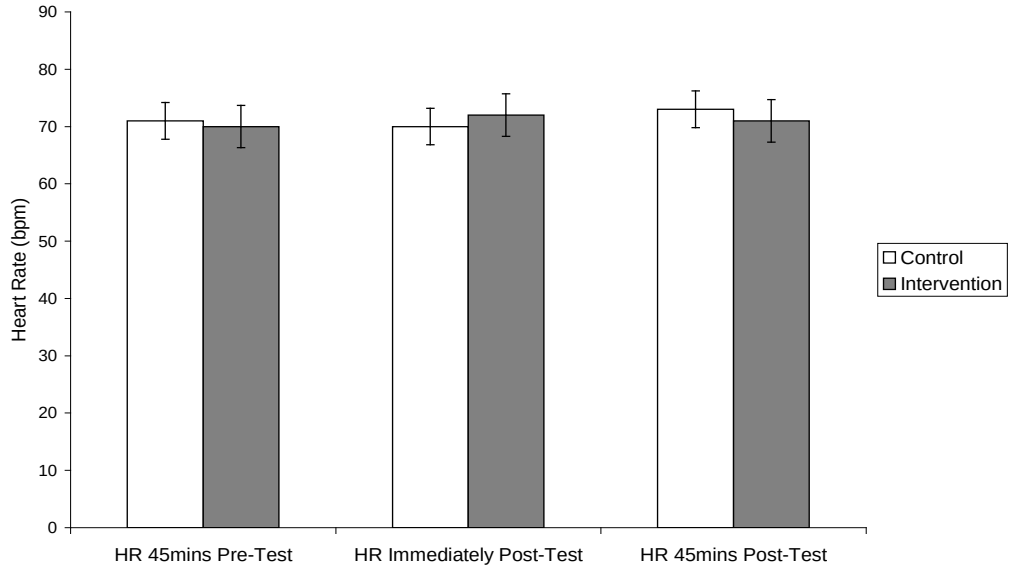


Figure 4.5b



Mean core body temperature (figure 4.5a) and heart rate (figure 4.5b) ± S.E.M. are presented for 45 minutes pre-, immediately post and 45 minutes post-stressor for treatment group A (Control) (open bars) and group B (Intervention) (closed bars) (n=10 for each).

Figure 4.5c

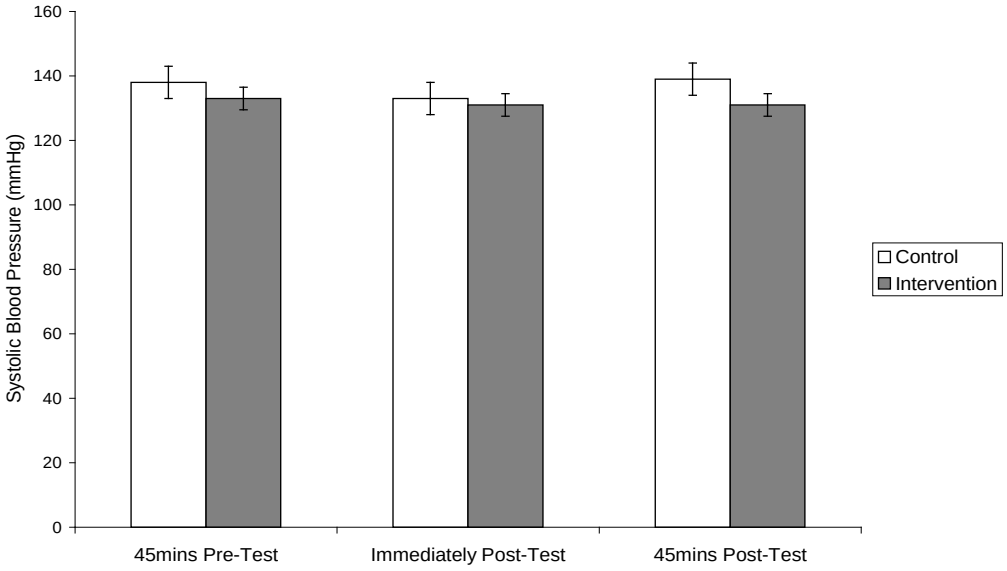
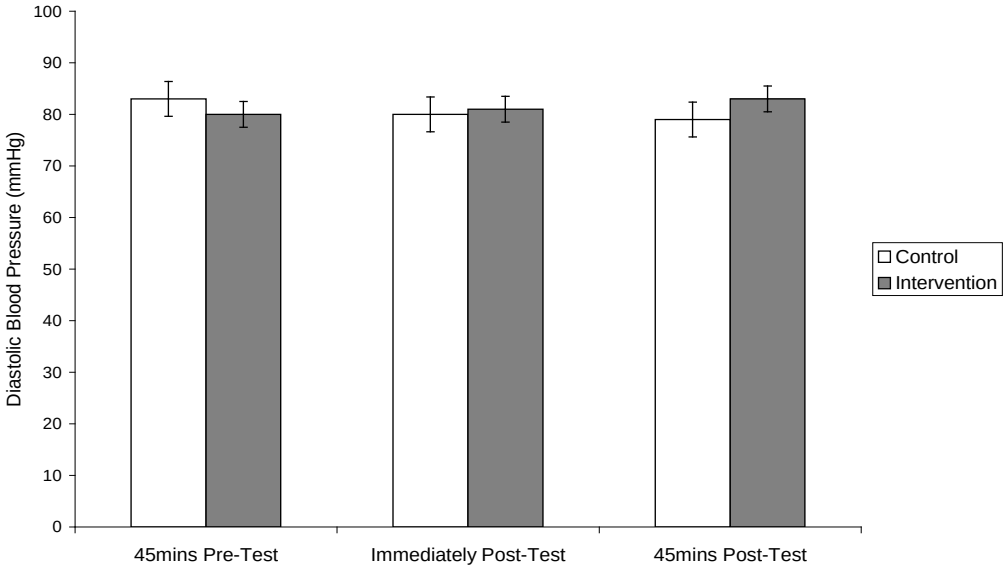


Figure 4.5d



Mean systolic (figure 4.5c) and diastolic blood pressure (figure 4.5d) ± S.E.M. are presented for 45 minutes pre-, immediately post and 45 minutes post-stressor for treatment group A (Control) (open bars) and group B (Intervention) (closed bars) (n=10 for each).

4.5 DISCUSSION

The results revealed a marked increase in leukocyte activation in response to the short-term acute psychological stressor, compared to the control, where little or no change was observed. Consequently, these findings support the hypothesis that a short duration acute psychological stressor, as provided by essentially little more than a moment of mental confusion, was sufficient to increase the activation state of leukocytes. As with the previous study, detection occurred 10 minutes into the 45 minute sampling period (T=10 minutes). However, unlike the previous study significant results were not obtained until 10 minutes (rather than 5 minutes, as with the previous study) into the 45 minute sampling profile. This suggests that the magnitude and rate of the leukocyte response is proportional to both stressor intensity and duration of exposure.

When questioned, approximately 30% of subjects within group B did not consciously realise that the polarity of the window switch had changed (until they were told, once the test and sampling was completed), yet they were still capable of accomplishing the task. This could suggest that such trifling everyday tasks are not initiated at a conscious level, instead it is the sub-conscious that is responsible. This leads to the hypothesis that the sub-conscious mind is capable of manifesting physiological and quantifiable responses, that the individual is, too some extent, unaware of there existence. This factor could signify that the driver perceives the machine (car) as an extension of their own body, and as such relies on the sub-conscious to control its basic functions (a form of autonomic control), definitely an area which requires further study.

It was expected that exposure to a psychological stressor would elicit demonstrable changes to both heart rate and systolic and diastolic blood pressure. However, in this instance no significant post-stressor changes were shown for any of the stated parameters (Table 4.3). Interestingly, a comparison of these parameters between genders showed significant differences for the control group (Group A) but not for group B (where intervention of the window control mechanism occurred), with the exception of diastolic BP where males showed a 3.0 ± 4.0 mmHg decrease whereas females reported a statically different 5.0 ± 3.0 mmHg increase ($F_{1,29} = 4.23$, $P =$

0.05). This trend suggests that as the observed changes occurred within the control group, they were due to other factors (such as biological variation) and not as a result of the investigated stressor.

Exposure to periods of social and environmental stress has been shown to affect body temperature in animal models (Olivier *et al*. 2003, Bhatnager *et al*. 2006). This study demonstrates that humans react to short-term stressors in a similar fashion. During testing, the environmental temperature was monitored and maintained at, or as close to 20°C as was possible with the equipment available (including electric hot air room heater, fan and window ventilation). Therefore it can be assumed that any observed change in body temperature was as a result of exposure to the stressor and not in reaction to changing environmental temperature. A significant difference in post-stressor change in core body temperature was recorded between treatment groups, with group A remaining unchanged and group B showing a post-test increase of 0.2 ± 0.1°C (Table 4.3). Within group B body temperature did significantly differ between gender with males showing an increase of 0.2 ± 0.1°C, while females showed and decrease of the same magnitude (Table 4.4).

Although the expected changes in heart rate and blood pressure were not observed, the above changes in core body temperature demonstrate the well known fact that anticipation of a stressor can elicit a physiological response, and that the manifested response is different according to gender. Hughes (2007) demonstrated how cardiovascular reactivity differed, specifically in terms of habituation, between genders. An earlier study by Burns and Katkin (1993) demonstrated how anger expression in males elicited the greatest incidence of cardiovascular reactivity during a wide variety of social and environmental situations, a trait that was not observed among women. It therefore follows that the physiological coping mechanisms associated with psychological stressors may also differ between genders. It could be argued that the selected stressor was gender specific, in that, traditionally compared to females, males express a more enthusiastic response to motor vehicle orientated tasks. Within the intervention test group (Group B) all males (with 1 exception) (Figure 4.4b) demonstrated a post-test decrease in leukocyte activity, whilst females generally reported an increase (Figure 4.4b). This observation may be due to behavioural

attitude and differing coping strategies employed by each gender, further studies would be required to fully investigate these effects.

Dhabhar *et al*. (1996) suggested the existence of specific receptors expressed on the surface of neutrophils, which may be responsible for increased cell numbers in peripheral blood, associated with changes in the expression of adhesion molecules and / or other receptor types, during periods of challenge. Neutrophils have a number of receptor types expressed on their surface, in particular β-adrenergic, which may be associated with increased leukocyte activation. The physiological response resulting from a psychological stressor may differ, compared to the response provoked by a physical stressor, as each is elicited via a different class of adrenoceptor (Gregg *et al*. 1999). As part of the acute stress response β-adrenergic stimulation elicits increases in both the rate and force of contraction of cardiac muscle, and relaxation of coronary smooth muscle (leading to vasodilation), in order to prepare the body for sudden and rapid exertion. As a consequence, both heart rate and blood pressure are used to identify the presence of psychological and physiological stress. As neutrophils also possess β-adrenergic receptors, it is therefore reasonable to infer that they are involved in their activation, during a stress situation. As leukocyte activity showed a significant post-stressor change, whereas heart rate and systolic and diastolic blood pressure did not, it could be suggested that the β-adrenergic stimulus threshold for leukocyte activation is less than for heart rate and blood pressure. Making leukocyte activation a more sensitive and accurate means for detecting periods of short-term mental and physiological stress.

It has been reported that exposure to periods of mild psychological stress (arithmetic stress) lasting only minutes elicits an up-regulation in catecholamine synthesis (Reims *et al*. 2004). The results from this study suggest that the mechanism is even more sensitive and allows a physiological response to be initiated following a psychological stressor lasting only a few seconds.

In contrast to catecholamines, cortisol has an inhibitory effect on the synthesis of Superoxide Anion and Nitric Oxide. Yamaguchi *et al*. (2001) showed that carp macrophages treated with cortisol exhibited suppressed phagocytosis. Cortisol also inhibits the up-regulation of both Intercellular Adhesion Molecule-1 (ICAM-1) and

Vascular Cell Adhesion Molecule-1 (VCAM-1) (Ihm *et al.* 1996). As ICAM-1 expression is necessary for the binding of β2 Integrin in the process of neutrophil emigration / migration – leukocyte activation would be suppressed or inhibited. Plasma cortisol concentration is widely used as a means of quantifying physiological and psychological stress in both human and non-human models (Clow *et al*. 2006, Takai *et al*. 2007). Research has shown significant gender differences in cortisol concentration following exposure to identical psychological stressors (Takai *et al*. 2007). LCC monitors ROS production (the end product of leukocyte activation) as a means of assessing psychological stress, rather than one of its constituent mediators (in this case, cortisol). In effect LCC looks at the overall stress equation, rather than focusing on a single variable, therefore reducing the possibility of inaccurate interpretation.

This study demonstrates that the LCC technique is sensitive enough to detect the subtle change in immune-competency manifested as a result of exposure to a transient situation designed to cause psychological confusion. A significant difference in leukocyte activity following exposure to the stressor was demonstrated between treatment groups, with post-stressor LCC scores generally being depressed in group B, but not in group A (Table 4.1). Despite confirmation of the presence of psychological stress, no significant post-test differences in heart rate, systolic and diastolic blood pressure were recorded between treatment groups (Table 4.3). This would suggest that the innate immune system (specifically leukocytes) possesses a greater sensitivity to subtle changes in emotional and psychological status, when compared to cardiovascular responsiveness. This suggests that its assessment should be used in preference to more traditional measures as a means of detecting the presence of, and quantifying, increased mental loading and psychological stress.

In conclusion, this research is the first reported study on the effect of an acute short-term psychological stressor (lasting only seconds) on polymorphonuclear leukocyte activation. It was observed that exposure to a psychological stressor lasting only seconds, was sufficient to activate the most abundant leukocyte in the body, the neutrophil. The number of blood-borne mediators that can alter leukocyte responsiveness *in vivo* is vast, making elucidation of the exact mechanism by which an emotional response stimulates leukocyte activation extremely complex.

Surrounding leukocytes with these diverse mediators may influence the production of ROS and may well explain the alteration in responsiveness to PMA *in vitro*. By analysing post-stressor changes in plasma concentration of a selection of mediators known to be involved with aspects of the stress response, the following chapter will aim to further explore the mechanisms involved.

5.0 Biochemical Changes and the Effect on Circulating Leukocyte Activation - Hazard Perception Study.

5.1 RATIONALE

Chapter 4 demonstrated the ability of the leukocyte coping capacity assay (LCC) to detect changes in leukocyte responsiveness, following exposure to a mild acute psychological stressor lasting less than 1 minute. In this chapter the aim was two fold. Firstly to investigate the biological mechanisms involved in psychological stress induced leukocyte activation, using ELISA to analyse post-stressor concentration changes of specific stress hormones and bio-mediators, believed to be associated with leukocyte recruitment and activation. Secondly, to compare LCC with an automotive industry standard quantitative psychometric tool for assessing altered mental loading and psychological stress during ergonomic assessment, called the NASA-task load index.

5.2 INTRODUCTION

One of the challenges of quantifying psychological 'stress' – a threat which would not require a physiological response which elicits physiological consequences (Segerstrom and Miller 2004), is that it is multifaceted affecting numerous biological systems. These include plasma endocrine factors, changes to blood biochemistry, haemoglobin concentration and haematocrit, cytokines, and factors released from other cells both circulating and non-circulating (Maes *et al*. 1998) (Chapter 1.2.6). In addition, the concentration of biomediators known to be associated with the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) including glucocorticoids (cortisol) and catecholamines (adrenaline and noradrenaline) (Vingerhoets 1985, Rabin *et al*. 1989, Beuschlein *et al*. 2001, Cohen *et al*. 2003, Gleitman *et al*. 2004), have been shown to alter following exposure to psychological stress (Mian *et al*. 2003) (Chapter1.1.3).

Chapters 3 and 4 have demonstrated that psychological stress has an effect on leukocyte activity. This study poses the question of whether this characteristic could be applied to provide a quantitative alternative to the traditional quantitative, yet highly subjective methods of assessing psychological stress and altered mental workload (e.g. subjective workload assessment (Reid & Nygren 1988) and the NASA-task load index (NASA-tlx) (Hart and Staveland 1988). Although both are utilised during ergonomic analysis within the motor industry, NASA-tlx was selected for comparison with LCC, at the sponsor's request (Chapter 1.3.1).

There are many forms of psychological stressor to which people knowingly expose themselves daily, from the stringent training regimes of elite athletes (Malm 2006), participating in academic examinations (Kang *et al*. 1996, Maes *et al*. 1998), or even watching an emotionally disturbing fictitious event (horror movie) (Mian *et al*. 2003). All have been shown to evoke demonstrable changes in the cardiovascular and immune systems, e.g. in heart rate and in the distribution and activation state of leukocytes. Detection of potentially hazardous situations, especially when driving involves considerable mental loading and can promote psycho-physiological arousal comparable to Canon's fear flight defence reaction, the so called "stress response" (Canon 1932, Folkow 1982). This chapter investigates whether changes in leukocyte responsiveness can be detected following attempts to identify potentially hazardous driving situations within a selection of pre-recorded real-life roadway scenarios, displayed as part of a computer training program.

Leukocytes possess an impressive array of receptors, in excess of 150, for a vast number of chemicals including, integrins, immunoglobulins, cytokines and selectins (Kuijpers *et al*. 1990, Martin *et al*. 2002, Moretta *et al*. 2006), and respond to an equally diverse range of biological variables (Chapter 1.2.5). Much of the literature places emphasis upon elucidating the biochemical pathways responsible for the physiological component of leukocyte activation. Whereas little evidence pertaining to the mechanism responsible for inducing activation following psychological stimulation could be found within the literature. To investigate whether the same mediators are involved in the psychological induction of leukocyte activation, as during physiological activation, the post-stressor changes in plasma concentration of nine mediators (Adrenaline, Noradrenaline, Cortisol, E-Selectin, L-Selectin,

Interleukin-1β, Interleukin-6, Endothelin-1, and Tumour Necrosis Factor-α) known to be associated with various stages of leukocyte activation, were assessed using standard ELISA.

5.3 METHODS

The Subjects

As with all studies, local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before commencement, in accordance with the declaration of Helsinki (World Medical Association 2004).

Subjects were 30 (15 male and 15 female) moderately fit and healthy individuals, aged between 26 and 62 years. Potential subjects were excluded on the following criteria: suffering from psychiatric illness; suffering from respiratory or cardiovascular disease; smokers; had taken prescription medicine within the previous month, and if they possessed any prior knowledge or experience regarding the test equipment (Chapter 2.2.2).

During recruitment volunteers were informed of the possible need to obtain venous blood samples. All 30 subjects consented to venous sampling which eliminated the problem of including individuals who were nervous regarding the use of needles. To verify this, 15 subjects were randomly selected for capillary finger prick blood sampling only (Group A). In addition to finger prick sampling, the remaining 15 subjects (8 female and 7 male) also provided venous blood samples pre- and immediately post-stressor, allowing plasma concentrations of Adrenaline, Noradrenaline, Cortisol, E-Selectin, L-Selectin, Interleukin-1β, Interleukin-6, Endothelin-1, and Tumour Necrosis Factor-α to be monitored (designated Group B). The post-test change in leukocyte activity between treatment groups was then compared.

Design

The experimental protocols were rigorously standardised, and testing was confined to between 10am and 2pm. Subjects were required to avoid any strenuous activity for at least 2 hours prior to testing (e.g. they were instructed to take the lift to the laboratory, rather than climb the stairs).

Prior to obtaining resting heart rate, BP, and core body temperature following the standardised procedure (Chapter 2.4), subjects sat quietly and were instructed to breathe orthonasally for 15 minutes. The first capillary and venous blood samples were then taken 45 minutes before exposure to the test apparatus (45 minutes pre-test) (capillary samples from both groups A and B, venous samples were taken from group B only) (see below).

During the 45 minute pre-test period subjects were asked to sit quietly or read (reading material was local daily free newspaper).

It was explained that once the test had begun no further verbal communication was allowed. Two minutes prior to testing, the subject was directed to an isolated, previously unseen area of the laboratory, where he or she was instructed to sit in front of a notebook pc displaying a blank screen. Ensuring the subject was comfortable, and could access the computer's mouse controls, the examiner explained the test protocol.

Each subject had a maximum of 6 minutes to identify (via a left mouse button click) developing hazards within 6 randomly selected driving scenarios (from a selection of 100). Typical developing hazards include identifying a cyclist wearing dark clothing riding an unlit bicycle at dusk, and an approaching vehicle having to enter oncoming traffic to avoid a parked car.

Immediately after completing the test, capillary (both groups) and venous (group B) blood samples, BP, heart rate and core body temperature measurements were taken (Immediately post-test).

After sitting quietly or reading for 45 minutes, a final capillary blood sample was taken, along with BP, heart rate and core body temperature measurements (45 minutes post-test), to investigate whether base line activity had re-established.

Blood Samples

Both Groups A and B underwent capillary sampling, only Group B submitted to venous sampling.

Capillary Sampling

Two 10µl blood samples were taken for LCC analysis using a finger lancing device (Roche Products Ltd. Hertfordshire, AL7 1TW United Kingdom) at each of the specified time periods, following the standard procedure outlined in Chapter 2.2.3.

Venous Sampling

45 minutes pre- and immediately post-test 3 x 7ml blood samples were taken from the anticubital fossa region of the inner arm using single venepuncture (with the needle being placed into a different region, and being removed between each of the two sampling periods) and the evacuated tube method (BD Vacutainer™ Systems, Oxford, OX4 4DQ United Kingdom) (Chapter 2.2.4). Two of the containers contained lithium heparin (17 I.U/ml) or ethylenediaminetetraacetic acid (EDTA) (1.8mg/ml) as an anticoagulant. The third container did not contain an anticoagulant to allow the blood to clot in order to isolate the serum. Samples were harvested in the following order (as per manufacturer's guidelines - BD Vacutainer™ Systems, Oxford, OX4 4DQ United Kingdom), thus limiting the possibility of cross contamination: Blank, Lithium Heparin, EDTA. Tubes were inverted (as per manufacturer's guidelines) to ensure complete mixing of sample and anticoagulant and stored on-ice (for a maximum of 30 minutes) prior to the next stage (Chapter 2.2.5).

Samples containing heparin and EDTA were centrifuged at 1000x g for 30 minutes at room temperature. The supernatant (plasma) was transferred to 300µl aliquots and stored at -70°C. The blank samples were permitted to clot at room temperature (20-

22°C) for 60 minutes and then centrifuged at 1000x g for 10 minutes at 4°C. The supernatant (serum) was transferred to 300µl aliquots and stored at -20°C.

Determining Leukocyte Activity

Leukocyte responsiveness to *in vitro* PMA stimulation was assessed for each pair of whole blood capillary samples (Chapter 2.1.2 and Table 2.1 for full description of LCC protocol), at each of the three specified time points.

ELISA Technique

Using standard ELISA (Chapter 2.3), the change in concentration of each of the nine selected mediators between 45 minutes pre- and immediately post-test was assessed (Figure 5.1). Each assay required the use of either heparin or EDTA as the anticoagulant, exceptions included assay of E-selectin and L-selectin where serum was required (clotted sample) (Figure 5.1).

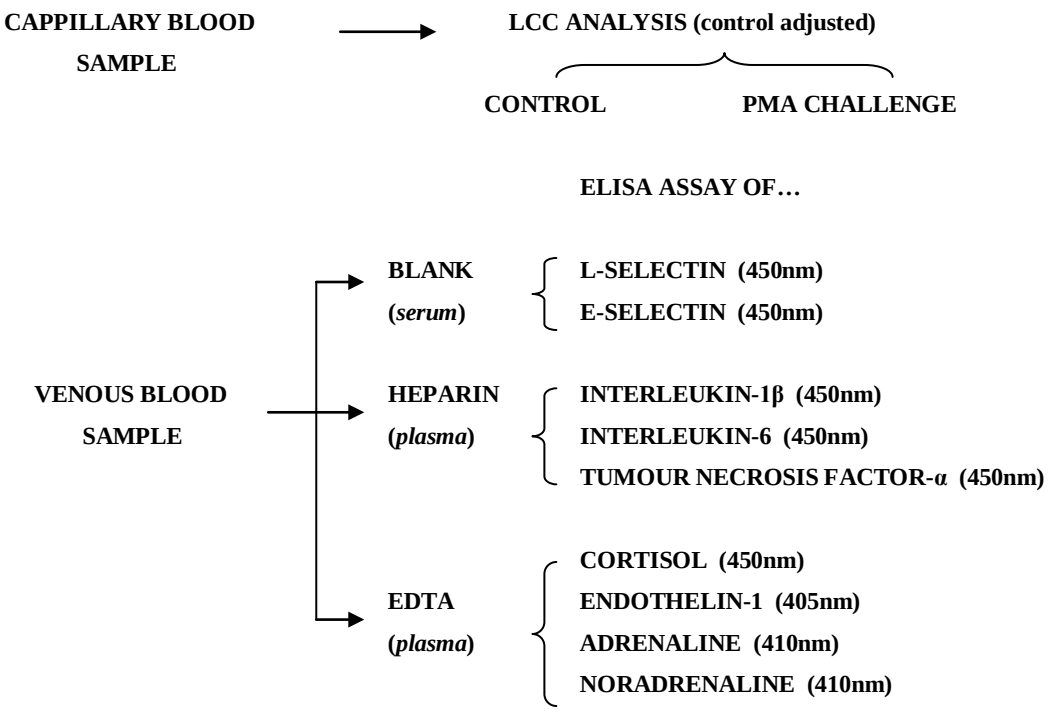
Each assay was conducted following the suppliers instructions, where all blanks, standards, samples and controls were tested in duplicate and the means calculated.

> Interleukin-1β, Interleukin-6, and Tumour Necrosis Factor-α. (Diaclone 1, Bd A. Fleming, BP 1985, 25020 Besançon cedex. France).
>
> Catcombi Adrenaline and Noradrenaline, Cortisol, E-Selectin and L-Selectin. (IBL Gesellschaft Fûr Immunchemie Und Immunbiologie MBH, Flughafenstrasse 52a, D-22335 Hamburg, Germany).
>
> Endothelin-1 (Cayman Chemical Company, 1180 E, Ellsworth Rd, Ann Arbor, MI 48108)

The absorbance of each microwell was read using a spectro-photometer (Anthos Plate Reader 2001, Anthos Labtec Instruments, using Stingray version 1.1 software, Dazdaq Ltd.) adjusted to the required wavelength (Figure 5.1).

For each assay, standard linear reference curves were produced using Microsoft Excel 2003 (Microsoft Corporation) by plotting the mean absorbance for each standard concentration on the ordinate (produced via serial dilution of standard stock solutions provided within each kit) against the respective mediator concentration on the abscissa. For each sample the concentrations for each mediator were calculated by firstly finding the mean optical density (O.D.) value on the ordinate and extending a horizontal line to the standard curve. At the point of intersection, a vertical line was extended to the abscissa and the corresponding mediator concentration was read.

Figure 5.1 Assays conducted using capillary and venous blood samples



Flow diagram illustrating allocation of blood samples for LCC and ELISA analysis for time points 45 minutes pre- and immediately post-test. Details on anticoagulant specificity and wavelength (nm) required for absorbance measurement using a spectro-photometer are included for each of the mediators (n=9) assayed.

Qualitative Assessment

To compare quantitative psychometric methodologies of assessing mental loading with LCC, subjects completed a written version of the NASA-task load index following completion of the test protocol and after blood samples and all other physical measurements had been completed. (Appendix 2). Each subject's responses were then input into the NASA-tlx software for analysis. The questionnaire comprised two stages: Firstly subjects were requested to rate (on a scale of 1 – Low to 20 – High) how the following criteria were affected during attempts to complete the task: Mental demand, Physical demand, Temporal demand (whether the time allowed to complete the task was sufficient), Performance, Effort, and Frustration. The second stage consisted of 15 paired combinations of the previously listed criteria, where in each case the subject had to decide which criteria in each pair was the most important to allow successful completion of the task. (Appendix 2).

As a second means of quantitatively assessing mental stress, subjects were asked to rate their level of perceived mental stress using a continuous likert scale (where 1 was relaxed and 10 stressed), 45 minutes pre- and immediately post-test.

Data Analysis

Standard statistical methodology (Chapter 2.5) was applied to investigate and compare the effect of the psychological stressor on leukocyte activity (for both treatment groups n=15 for each) (Chapter 2.5.1) and also heart rate, systolic and diastolic blood pressure and core body temperature (Chapter 2.5.2). For both treatment groups each subject's leukocyte activity was adjusted to compensate for baseline activity via subtraction of Sample A (control) from Sample B (stimulated sample containing PMA) (Chapter 2.1.2). The difference between 45 minutes pre- and immediately post-test for leukocyte activity and also the other physical measures (e.g. heart rate) were calculated in order to reduce the effect of inter-individual variation.

Post-test changes in leukocyte activity (activity between 45 minutes pre-and immediately post-test) were compared between treatment groups using single factor analysis of variance, in order to explore whether the process of venous sampling was acting as a secondary stressor. Each of the five attributes of leukocyte activity (e.g. Hmax-RLU_{adj}) was used in-turn as the dependent variable, with treatment as the fixed factor.

The relationships between post-stressor changes in leukocyte activity and heart rate, systolic and diastolic blood pressure and core body temperature, were analysed using Bivariate correlation (Chapter 2.5.3).

Perceived psychological stress was qualitatively assessed using likert scales (using a continuous scale) pre- and immediately post-stressor. To test whether the observed post-test changes were significantly different between treatment groups, post-test differences were calculated and applied to a single factor ANOVA model. With post-test difference in perceived psychological stress acting as the dependent variable and treatment group as the fixed factor. Bivariate correlation was used to test whether the observed post-test change in perceived stress was related to post-test changes in leukocyte activity. The truncated product method (Zaykin *et al*. 2002) was used to correct for multiple comparisons (Chapter 2.5.4).

Treatment group B (n=15) provided venous blood samples 45 minutes pre- and immediately post-stressor in order to examine the change in concentration of bio-mediators believed to be associated with leukocyte activation. Mean concentrations of each bio-mediator ± S.E.M. were recoded for both sampling points, and the differences between pre- and post-test were applied as the dependent variable to a single factor ANOVA model, with time acting as the fixed factor.

Bivariate correlation was used to investigate the relationship between post-test changes (difference between 45 minutes pre- and immediately post-stressor) in leukocyte activity (responses 1-5) and post-test changes in concentration of each of the nine bio-mediators tested. The truncated product method (Zaykin *et al*. 2002) was used to correct for multiple comparisons.

The NASA-task load index was used as a means of quantitatively assessing mental loading. This gives a total mental workload value for each subject and also for each treatment group (n=15 for each). Single factor ANOVA, where total mental workload acted as the dependent variable with treatment as the fixed factor, was used to investigate whether the addition of venous sampling affected total mental workload. Bivariate correlation was used to investigate the significance of the relationship between total mental workload (end product of NASA-tlx calculation) and post-test change in leukocyte activity. Each of the 5 responses of the leukocyte activity luminescence profile (e.g. Hmax-RLU_{adj}) for 45 minutes pre- and immediately post-test were compared, in turn, with total mental workload, correcting the overall *P*-values for the effect of multiple comparisons via the Truncated Product Method.

5.4 RESULTS

Leukocyte Activity

The LCC profiles displayed in Figure 5.2a and b show that for samples taken at each of the three time points, PMA challenge stimulated a rapid increase in the release of ROS, with the maximum rate of release occurring at T=15 minutes, after which, activity steadily decreased. Following the test the mean LCC response for each treatment group showed significant decreases in leukocyte activity from baseline at T=10 and 15 minutes, and at maximum leukocyte activity (Hmax-RLU_{adj}), with the most pronounced post-test change ($P = 0.001$ Tukey's *post hoc* procedure) occurring at T=15 minutes (RLU_{adj}) (adjusted response in leukocyte activity recorded 15 minutes into the 45 minute activity profile) (Table 5.1). The differences between treatment groups were not statistically significant, showing a similar pattern of depression of response in both cases (Table 5.1).

Perceived Stress

Changes in self-assessed post-test mental workload, measured using the likert scale, demonstrated significant post-test increases (group A 1.2 ± 0.31 Units; group B 2.0 ±

0.41 Units ($F_{1,59}$ = 18.45, P < 0.001). No significant difference was reported for self-assessed post-test change in mental workload between treatment groups ($F_{1,59}$ = 0.13, P = 0.72). Post-test changes in leukocyte activity and mental workload were not significantly correlated (Hmax-RLU_{adj}) bivariate correlation P = 0.59 (Pearson correlation = -0.1).

Mental Workload: NASA-Task Load Index

Assessment of mental workload using NASA-tlx showed that a significant difference in total mental workload existed between treatment groups ($F_{1,29}$ = 5.6, P = 0.03) (Table 5.2). For each treatment group, mean rating and calculated weight for each of the six criteria (mental, physical and temporal demand, performance, effort, and frustration) are presented. A significant difference in mental rating was shown between treatment groups (Figure 5.3).

Core Body Temperature

No significant changes in core body temperature occurred between 45 minutes pre- and immediately post-test, or between treatment groups (Table 5.3).

Heart Rate, Blood Pressure

Significant post-test increases in both heart rate (P = 0.05 Tukey's *post hoc* procedure) and systolic blood pressure (P = 0.02 Tukey's *post hoc* procedure) were observed between 45 minutes pre- and immediately post-stressor. No significant difference was shown between treatment groups for both parameters (Heart rate $F_{1,29}$ = 2.28, P = 0.11; systolic BP $F_{1,29}$ = 0.24, P = 0.79) (Table 5.3).

Heart rate was significantly correlated with adjusted leukocyte activity 10 minutes into the 45 minute activity profile (T=10 minutes (RLU_{adj}) (bivariate correlation P = 0.01 (Pearson correlation = 0.44) (Table 5.4).

No significant change in diastolic blood pressure occurred between 45 minutes pre- and immediately post-test, or between treatment groups (Table 5.3).

Bio mediator Analysis

Standard ELISA of blood samples from treatment group B demonstrated a significant increase in the post-test plasma concentration of Adrenaline (0.18 ± 0.03 ng/ml ($F_{1,29}$ = 22.23, $P < 0.001$), and which approached significance for E-Selectin (4.25 ± 6.87 ng/ml ($F_{1,29}$ = 0.78, $P = 0.08$) (Table 5.5). The post-test increase in adrenaline was not significantly correlated with the observed post-test increase in leukocyte activity, whereas a weak relationship was shown with E-selectin (Table 5.6).

FIGURE 5.2a and b. Luminescence profiles showing mean leukocyte coping capacity (RLU_{adj}) ± S.E.M. for Groups A (capillary sampling only) and B (capillary and venous sampling) (n=15 for both). * Significant changes ($P < 0.05$) in leukocyte activity between 45 minutes pre- and immediately post-test occur at T=10 and 15 minutes, as well as for Hmax (maximum leukocyte activity).

Figure 5.2a – Group A

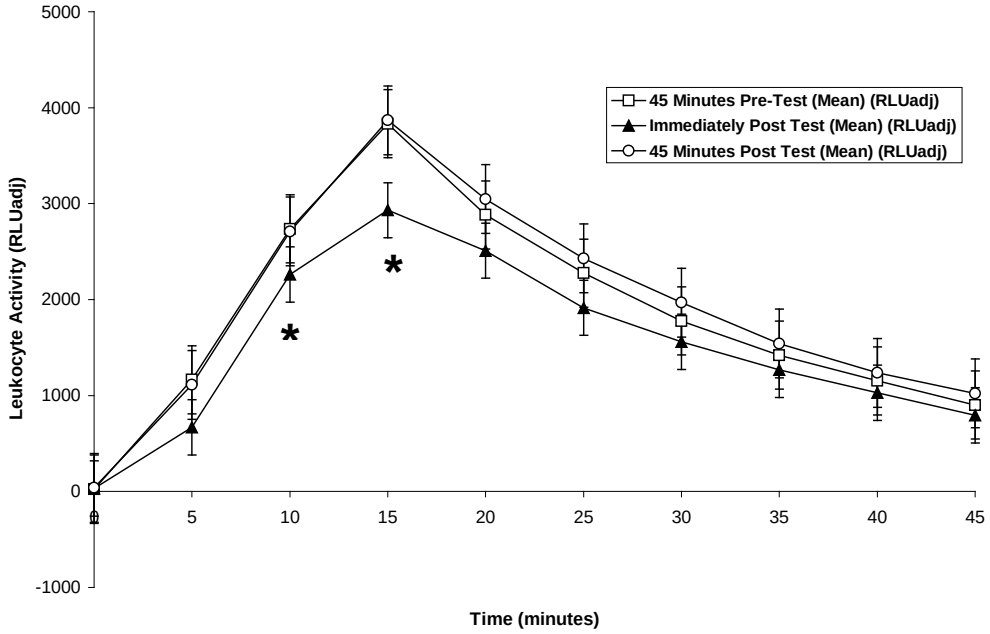


Figure 5.2b – Group B

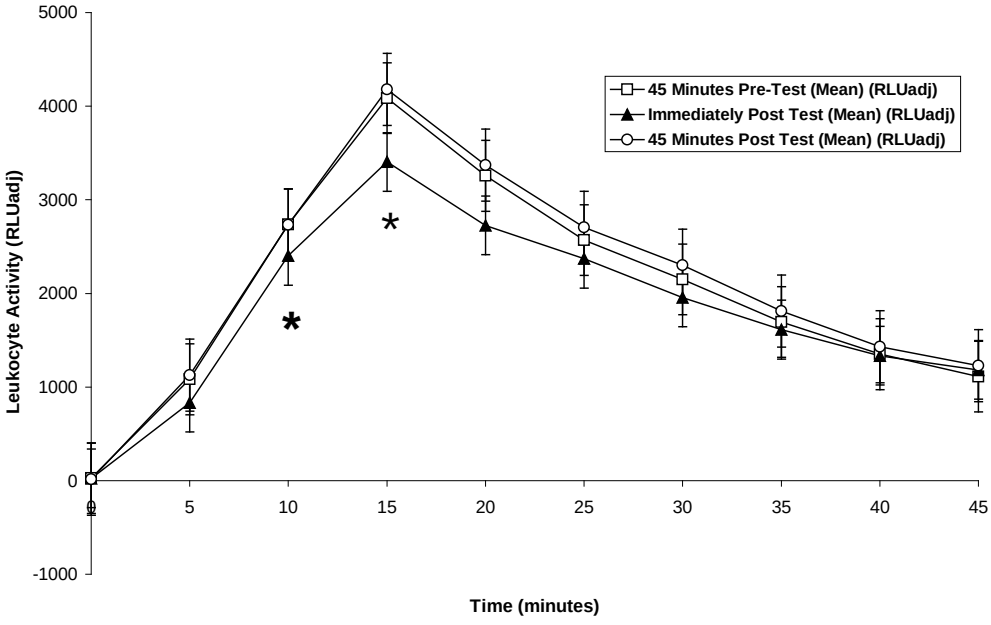


TABLE 5.1 Effect of stressor on leukocyte activity

	GROUP A	GROUP B	*P* (F)
Δ Hmax (RLUadj)	-671.0 ± 179.0 •	-1018.0 ± 229.0 •	0.47 (0.52)
Δ T-max (minutes)	0.0 ± 0.77	0.0 ± 0.45	0.65 (0.21)
Δ t=5 minutes (RLUadj)	-249.0 ± 111.0	-495.0 ± 408.0	0.67 (0.18)
Δ t=10 minutes (RLUadj)	-333.0 ± 203.0 •	-475.0 ± 325.0 •	0.63 (0.23)
Δ t=15 minutes (RLUadj)	-682.0 ± 170.0 •	-901.0 ± 212.0 •	0.26 (1.28)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) for all of the specific attributes of the leukocyte luminescence profiles (with the exception of T-max), for both treatment groups (n = 15 for each) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}). Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}) For the difference in the time taken to reach maximum adjusted leukocyte activity (T-max) (minutes) between 45 minutes pre- and immediately post-test samples, the median is presented along with S.E.M. Single factor ANOVA was used to test the effect of treatment on leukocyte activity (*P*) (*d.f.* = 29). In addition, for each group the effect of treatment on changes in leukocyte activity between 45 minutes pre- and immediately post-test was investigated using single factor ANOVA and Tukey's *post hoc* procedure.

• Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

* Difference between Group A and Group B ($P < 0.05$)

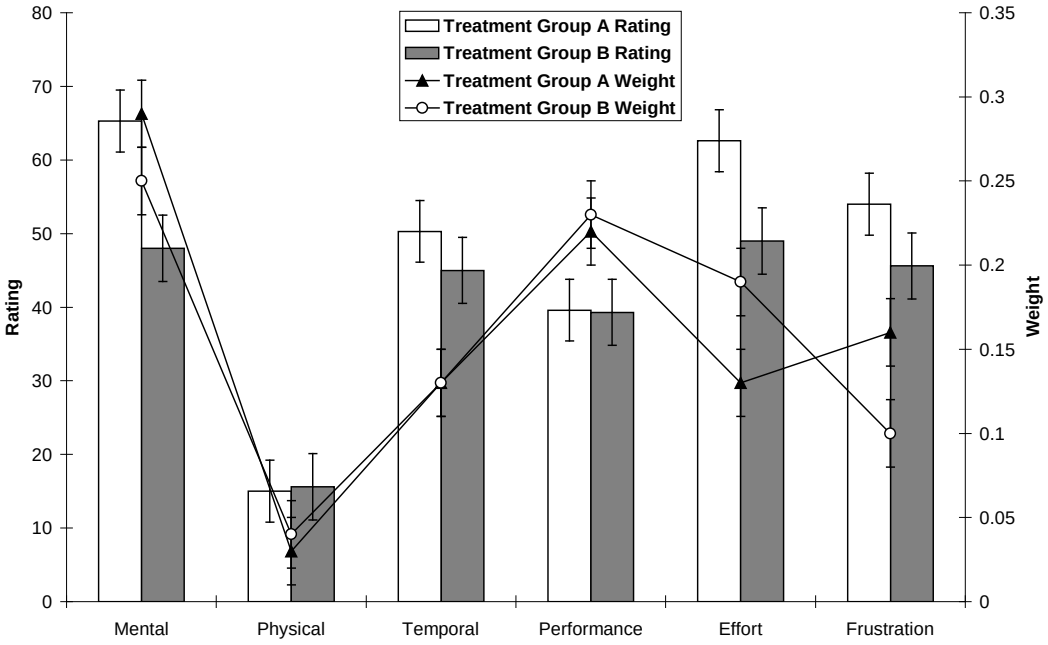
TABLE 5.2 NASA task load index

	GROUP A	GROUP B	*P*
Mental Demand	48.0 ± 5.16 (0.26 ± 0.02)	65.33 ± 4.15 (0.29 ± 0.02)	0.01* (6.84) 0.2 (1.68)
Physical Demand	15.67 ± 3.51 (0.05 ± 0.015)	15.0 ± 2.34 (0.03 ± 0.02)	0.87 (0.02) 0.5 (0.34)
Temporal Demand	45.0 ± 3.0 (0.14 ± 0.019)	50.33 ± 2.9 (0.14 ± 0.02)	0.21 (1.64) 0.98 (0.001)
Performance	39.33 ± 4.97 (0.24 ± 0.017)	39.67 ± 5.89 (0.22 ± 0.02)	0.96 (0.002) 0.49 (0.5)
Effort	49.0 ± 5.61 (0.2 ± 0.02)	62.67 ± 4.8 (0.14 ± 0.02)	0.07 (3.45) 0.04* (4.54)
Frustration	45.67 ± 5.09 (0.1 ± 0.03)	54.0 ± 5.24 (0.16 ± 0.02)	0.26 (1.3) 0.14 (2.29)
TOTAL WORKLOAD	**45.7 ± 3.25**	**56.64 ± 3.26**	**0.03* (5.65)**

For each treatment group (n=15 for each) mean and standard error of the mean (S.E.M.) are presented for the total workload score in addition to the rating and average score (in brackets) of each of the six criteria associated with successful completion of the set task, used during the NASA-tlx evaluation process. Single factor ANOVA was used to investigate the effect of treatment group on total workload and each of the 6 stated parameters in turn (*P*) (*d.f.* = 29).

* $P < 0.05$ Statistically Significant

Figure 5.3 NASA-task load index mean ratings and weights



Combined histogram and line chart illustrating mean ratings ± S.E.M. (histogram) and average weighting ± S.E.M. (line chart) of each of the six NASA-tlx criteria for each treatment group (n=15 for each).

TABLE 5.3 Effect of stressor on heart rate, core body temperature and blood pressure.

	GROUP A	GROUP B	*P* (F)
Δ Heart rate (bpm)	2.0 ± 1.0 •	3.27 ± 1.41 •	0.11 (2.28)
Δ Core Body Temperature (°C)	0.0 ± 0.0	0.0 ± 0.0	0.68 (0.17)
Δ Systolic Blood Pressure (mmHg)	0.0 ± 2.0	4.0 ± 1.0 •	0.79 (0.24)
Δ Diastolic Blood Pressure (mmHg)	3.0 ± 2.0	3.0 ± 2.0	0.88 (0.13)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in heart rate, core body temperature and blood pressure (difference between 45 minutes pre- and immediately post-test samples) for each treatment group (n = 15 for each). The significance of the effect of treatment was tested using single factor ANOVA (*P*) (*d.f.* = 29). In addition, for each group the effect of treatment on changes in each of the stated parameters between 45 minutes pre- and immediately post-test was investigated using single factor ANOVA and Tukey's *post hoc* procedure.

• Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

* Difference between Group A and Group B ($P < 0.05$)

TABLE 5.4 Relationship between change in leukocyte activity and changes in heart rate, core body temperature and blood pressure.

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes
Δ HR	-0.03 (0.89)	-0.34 (0.07)	0.22 (0.24)	**0.44** (0.01*)	-0.17 (0.37)
Δ Core Body Temperature	-0.002 (0.99)	0.31 (0.09)	0.06 (0.74)	-0.03 (0.89)	-0.07 (0.72)
Δ Systolic BP	-0.02 (0.91)	0.12 (0.54)	0.09 (0.64)	-0.23 (0.23)	-0.05 (0.79)
Δ Diastolic BP	0.15 (0.43)	-0.06 (0.76)	0.02 (0.91)	-0.23 (0.23)	0.19 (0.33)

Combined data from both treatment groups (n=30) was tested using bivariate correlation to investigate the significance of the relationship between post-test changes (Δ) in leukocyte activity and heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test) (Pearson correlation with *P*-values in brackets). The Truncated Product Method (Zaykin *et al.* 2002) was used to correct for the use of multiple comparisons (*p*). Only the relationship between leukocyte activity and HR proved to be significant using the truncated product method ($p \leq 0.02$) (n = 30).

* $P < 0.05$ Statistically Significant

TABLE 5.5 Effect of stressor on bio-mediator concentration.

Mediator	45 Minutes Pre-Test	Immediately Post-Test	Post-Test Difference	*P* (F)
Interleukin-6	2.72 ± 0.14 pg/ml	2.64 ± 0.19 pg/ml	0.08 ± 0.25 pg/ml	0.7 (0.15)
Interleukin-1β	28.73 ± 2.13 pg/ml	28.17 ± 1.73 pg/ml	0.56 ± 3.36 pg/ml	0.83 (0.05)
Tumour Necrosis Factor-α	10.6 ± 1.33 pg/ml	10.93 ± 1.46 pg/ml	0.28 ± 2.25 pg/ml	0.88 (0.02)
Endothelin-1	3.59 ± 0.77 pg/ml	3.59 ± 0.74 pg/ml	0.01 ± 1.26 pg/ml	0.92 (1.6)
E-Selectin	72.55 ± 8.54 ng/ml	76.8 ± 9.98 ng/ml	4.25 ± 6.87 ng/ml	**0.08 (0.78)**
L-Selectin	2751.5 ± 91.9 ng/ml	2532.0 ± 122.0 ng/ml	219.0 ± 88.9 ng/ml	0.16 (2.05)
Cortisol	130.8 ± 12.6 ng/ml	151.0 ± 12.0 ng/ml	20.2 ± 11.9 ng/ml	0.26 (1.32)
Adrenaline	0.34 ± 0.02 ng/ml	0.52 ± 0.02 ng/ml	0.18 ± 0.03 ng/ml	**<0.001* (22.23)**
Noradrenaline	0.49 ± 0.04 ng/ml	0.39± 0.05 ng/ml	0.09 ± 0.03 ng/ml	0.22 (1.6)

Mean and standard error of the mean are presented for the change in concentration of each of the nine bio-mediators between 45 minutes pre- and immediately post stressor. The significance of time on mediator concentration (*P*), are reported based on a single factor ANOVA model (*d.f.* = 29).

* Difference between Pre- and Post-Test Bio-Mediator Concentration ($P < 0.05$)

TABLE 5.6 Relationship between post-test changes in leukocyte activity and changes in bio-mediator concentration.

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes
Δ Interleukin-6	0.25 (0.38)	0.48 (0.07)	-0.26 (0.36)	0.38 (0.16)	-0.19 (0.5)
Δ Interleukin-1β	0.38 (0.16)	0.28 (0.32)	-0.22 (0.43)	0.39 (0.15)	0.34 (0.22)
Δ Tumour Necrosis Factor-α	0.42 (0.12)	-0.17 (0.54)	0.15 (0.6)	0.17 (0.55)	0.36 (0.19)
Δ Endothelin-1	0.07 (0.8)	0. 4 (0.14)	-0.4 (0.17)	0.23 (0.42)	-0.01 (0.96)
Δ E-Selectin	-0.3 (0.28)	0.36 (0.19)	-0.003 (0.99)	**-0.55*** (**0.03**)	-0.31 (0.26)
Δ L-Selectin	-0.19 (0.49)	-0.14 (0.61)	0.19 (0.51)	-0.44 (0.11)	-0.17 (0.55)
Δ Cortisol	-0.12 (0.67)	-0.29 (0.3)	-0.13 (0.65)	-0.17 (0.55)	-0.14 (0.62)
Δ Adrenaline	0.17 (0.54)	0.2 (0.42)	-0.11 (0.69)	0.44 (0.1)	0.15 (0.6)
Δ Noradrenaline	0.2 (0.47)	0.5 (0.06)	-0.18 (0.53)	0.14 (0.61)	0.19 (0.5)

The significance of the relationship between post-test changes (Δ) in leukocyte activity and bio-mediator concentration (difference between 45 minutes pre- and immediately post-test), using data from treatment group B only, was investigated using bivariate correlation (Pearson correlation with *P*-values in brackets). The Truncated Product Method (Zaykin *et al*. 2002) was used to correct for the use of multiple comparisons (*p*). Only the relationship between leukocyte activity and E-selectin proved to be significant using the truncated product method ($p \leq 0.05$) (n = 15).

* $P < 0.05$ Statistically Significant

5.5 DISCUSSION

The results show that passive observation and identification of potentially hazardous situations on a computer screen (in this case, those which may be encountered during driving), resulted in psycho-physiological arousal (specifically increased leukocyte activity) of a magnitude comparable to that observed during exposure to events that could potentially elicit actual physiological harm, such as performing a basic driving manoeuvre in an unfamiliar motor vehicle (Chapter 3). This suggests that the mechanism of psychological induced leukocyte activation is incapable of differentiating between stressors which pose a real physical threat, from those that are simply a result of an emotional response. This observation adds further weight to the findings of Mian *et al*. (2003), who observed increases in both number and activation state of circulating leukocytes following exposure to a fictitious stressful event (observation of an 83 minute horror film).

The results provide further evidence for the link between psychological stress and immune responsiveness (Altemus *et al*. 2006, Slominski 2007), demonstrating the diagnostic benefits of leukocyte responsiveness in quantitative assessment of psychological stress. Viswanathan *et al*. (2005) reported that acute psychological stressors (lasting a period of minutes to hours) induced significant immune-enhancement, promoting immune-protection in the case of wounding or infection. LCC analysis supports this observation, whereby the acute stressor promoted leukocyte activation, *in vivo*, which resulted in a significant reduction in ROS release / concentration, following *in vitro* PMA stimulation.

In addition to statistically significant post-stressor changes in heart rate and systolic blood pressure, changes that were in line with published data (Mian *et al*. 2003). Both treatment groups showed significant post-test increases in perceived mental workload (using likert scales). As with leukocyte activity, all parameters showed no significant post-stressor differences between treatment groups, confirming that the informed consent process made subject recruitment self selecting, and that the process of venous sampling was not perceived, both psychologically and physically, as being stressful. Within this study the LCC protocol was used as a means of confirming the effectiveness of the test protocol for stimulating increased mental and physical stress

of a sufficient magnitude to provide the best possible chance for identifying post-stressor concentration changes in the selected bio-mediators. Therefore as long as the post-test stress increase was of sufficient magnitude, whether the process of venous sampling did or did not act as a secondary stressor was not seen to be detrimental within the design of this study.

A significant difference in total mental workload between treatment groups was returned for NASA-tlx analysis. The data suggests that subjects who underwent venous blood sampling in addition to capillary sampling and exposure to the primary psychological stressor (group B) found the experience more psychologically and physically stressful than the participants of group A. This observation contradicts the findings of the other tested parameters, particularly LCC, where no significant difference in the post-test change in leukocyte activity was shown between groups.

The original study design aimed to use the identification of developing driving hazards within randomly selected scenarios as the potential mental stressor. However, as only a single stressor was utilised, this study did not lend itself as being an ideal design for comparing altered leukocyte activity with a subjective, quantitative mental workload assessment tool, such as the NASA-task load index. As NASA-tlx is intended for post-test assessment only during comparison of multiple stressors (e.g. differing design configurations of a motor vehicle interface), the technique was better suited, in the case of this study, for comparing the effect of venous sampling between treatment groups and not for assessing the effect of the intended primary stressor. In future, NASA-tlx assessment could be included within a study where multiple stressors were investigated, such as during the comparison of two different motor vehicle touch screen interfaces (Chapter 6).

A number of bio-mediators were measured and a significant post-stressor increase in plasma adrenaline concentration was recorded which follows accepted trends. Vale (2005) reported that psychological stress triggered the sympathetic nervous system (SNS) to release catecholamines and initiate a number of pro-inflammatory molecular cascades, which promote nuclear transcription of several inflammatory cytokines, including Tumour Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6). In respect to adrenaline, this study supports these conclusions. However no

significant changes were recorded for IL-1β, IL-6 or TNF-α. This could be due, firstly, to the intensity and duration of the stressor; examples cited by Vale (2005) included exposure to events such as earthquake or periods of extreme anger, secondly, the timescale of mediator release following stressor exposure. During the acute phase of the inflammatory response, HPA axis activation results in the release of catecholamines (adrenaline) (Chapter 1.1.3) (Heinrich *et al*. 2003). As part of this response activated leukocytes (primarily monocytes and macrophages) release both IL-1β and TNF-α (Heinrich *et al*. 2003). IL-6 has been shown to negatively regulate TNF-α secretion possibly via HPA axis activation (Lyson *et al*. 1991, Naitho *et al*. 1988, Chida *et al*. 2004), however, this observation was noted following exposure to a psychological stressor lasting a minimum duration of 30 minutes (LeMay *et al*. 1990). Although acute, the emotional intensity and duration of exposure of the stressors utilised within these examples was much greater than could be expected as a result of the stressor utilised within this chapter. Consequently insufficient stimulation was present to elicit full activation of the molecular cascades required for leukocyte activation following *in vitro* PMA stimulation, therefore only the mediators involved within the preliminary stages of the acute inflammatory response and leukocyte activation exhibited quantifiable post-test changes in concentration.

Adrenaline plasma concentration returned the most significant post-test change, providing evidence that stimulation of the acute inflammatory stress response did occur as a reaction to the stressor. Adrenaline secretion occurs via stimulation of the HPA axis (Chapter 1.1.3) and influences a diverse array of physiological systems (e.g. cardiovascular and musculoskeletal systems) with the aim of preparing the body for rapid exertion (as part of the "fight-flight" response (Chapter 1.1.1). The immune response has also been shown to undergo modification during periods of acute stress (Dhabhar *et al*. 2000, Dhabhar and McEwen 2001, Saul *et al*. 2005) (Chapter 1.1.1). Mian *et al*. (2003) reported significant increases in both number and activation of peripheral circulating leukocytes following the observation of an 83-minute fictitious psychologically stressful event (a horror film). While Walsh and Whitham (2006) demonstrated similar leukocyte response trends during exercise within extremes of environmental temperature. It is therefore suggested that psychological stress induced leukocyte activation is mediated, in-part, as a result of β-adrenergic stimulation.

The presence of a post-test increase in E-selectin plasma concentration in the absence of a post-test concentration change in TNF-α (a primary mediator for leukocyte recruitment and activation), suggests that E-selectin up-regulation can result following stimulation from other mediators. This, when combined with the observation that no significant increase in L-selectin occurred post-stressor, suggests that *in vivo*, neutrophils can be activated without the requirement for undergoing the process of migration and emigration from sheer flow. This would result in the release of toxic metabolites including ROS, directly into the systemic circulation, a process which may be part of many disease states that have associations with high incidences of psychological stress.

A limitation with the design of this study was associated with treatment groups. Ideally two additional treatment groups should have existed to act as controls for both the primary and secondary stressors. Both groups would have sat quietly for the 6 minute test-duration instead of completing the hazard perception protocol, one group would have undergone venous and capillary sampling, the other just capillary. The reasons why this design was not followed were, firstly financial. In order to test twice the number of blood samples, in duplicate as instructed by the manufacturer, twice the number of ELISA test kits would have been required. This would have been prohibitively expensive. Secondly, and most importantly, the number of subjects available for testing was severely limited. Due to the process of informed consent, subjects were notified regarding the use of venepuncture. From the established pool of 40 volunteers, only the 30 subjects tested consented to the procedure, and of these, half had to be allocated to the capillary sampling only control group (group A).

In conclusion this study has demonstrated that with similar response trends being exhibited for subjective quantitative mental workload (NASA-tlx and likert ratings) and post-test changes in leukocyte activity, the LCC protocol has been shown to possess the potential to act as an objective, quantitative physiological measure of altered psychological stress. Also, circulating neutrophils appear to possess the ability to react to direct increases in plasma adrenaline concentration, possessing the ability to become activated via an alternate mechanism which negates the prerequisite of neutrophil endothelial interaction.

6.0 Quantifying Changes in Leukocyte Responsiveness Following Primary and Repeated Exposure to Two Different Motor Vehicle Touch Screen Interfaces.

6.1 RATIONALE

Chapters 3, 4 and 5 provide evidence that the LCC protocol is able to differentiate between the changes in leukocyte reactive oxygen species production and release resulting from exposure to different short-term stressors. The aims of this chapter were to first investigate the diagnostic capability of the LCC protocol within a commercial environment (in this case the automotive industry) by applying its use as a means of assessing the ergonomic impact of two different human machine interface (HMI) designs. The second aim was to explore how repeated exposure to the same stressor affects leukocyte reactivity.

6.2 INTRODUCTION

Motor vehicle design now incorporate numerous technologies aimed to improve both the comfort (e.g. electronically controlled air conditioning and in-car entertainment systems) and safety of the driver (e.g. satellite navigation and hands-free mobile telephone). The volume of systems deemed necessary has raised issues regarding the ergonomic arrangement of each systems controls, so as to ensure that they are easily located and safe to use whilst driving (a process which already imposes high levels of mental loading). In order to facilitate this interaction, all of the leading manufacturers employ a form of Human Machine Interface (HMI) to integrate many of the key systems into a single control interface. However, there is still much debate as to which format is best in terms of ease of use and, more importantly, the safest to use whilst driving. Many of these systems employ menu and sub-menu orientated formats. Although this form of “deep” HMI is beneficial in terms of ergonomics and aesthetics, it can lead to potentially hazardous driving practice for those who are not used to the system (first-time driver) or for those who are not particularly confident

with the use of computer-based technology, when compared with a more traditional control layout, with dedicated dash-mounted controls for each function.
The process of driving is inherently dangerous, due to the high mental demand required to cope with the vast number of associated risk factors, including environmental conditions (e.g. adverse weather) as well as other road users. The introduction of a secondary task that competes for driver attention serves to increase both mental loading and the potential for unsafe driving practice. Driver distraction can result from a wide range of situations which may or may not be associated with the task of driving, including interaction with non-essential in-car systems (e.g. satellite navigation and in-car entertainment). Concern about this has prompted a change in British legislation. The death by careless driving act, which came into force in August 2008, allows the prosecution of motorists who caused injury or death to others whilst being "avoidably distracted" by activities such as adjusting a car stereo or eating or drinking at the wheel. Of particular interest in recent years is the use of mobile telephones whilst driving, which until the introduction of the death by dangerous driving act only carried a maximum fixed penalty of £60 and 3 penalty points on the driver's licence (Directgov 2008). Hendrick and Switzer (2007) showed that vehicle braking reaction time was severely compromised as a result of conducting both hand-held and hands-free mobile telephone conversations. McEvoy *et al*. (2005) suggested that the danger associated with mobile phone use is not simply isolated to the physical act of making a call (hands-free or otherwise), but the distraction and altered mental loading can persist for minutes after the conversation has ended. Of the population tested it was suggested that such use led to a four-fold increase in the likelihood of being involved in a hazardous driving situation. It, therefore, follows that any activity that affects driver attention can potentially result in hazardous driving practice. The design of any HMI must therefore take into account its imposed demands on driver mental loading.

Quantifying psychological stress resulting from environmental challenges or social interactions is generally accomplished by one of two approaches. First, qualitative measurement of perceived mental workload through subjective self assessment (including the NASA task load index inventory and subjective workload assessment techniques (SWAT) (Hart and Staveland 1988, Reid and Nygren 1988) (Chapter 1.3.1). Second, quantitative analysis monitoring characteristics of the

cardiopulmonary system and assay of specific stress hormones including salivary cortisol (Moon and Cho 2001, Hodgson *et al*. 2004, Powers *et al*. 2006) and catecholamines (Brown *et al*. 2003) (Chapter 1.3.2).

The techniques currently used to assess altered mental workload, resulting from the ergonomic interaction with existing and experimental technologies within the automotive industry, are highly subjective due to their reliance upon qualitative data. There are a number of quantitative techniques in general use, including the use of eye-tracking and response times. Both are effective, however each relies on the measurement of parameters that are indirectly associated with altered mental work load. Both the eye tracking and response time techniques were designed on the premise that an individual's attention capacity is finite. Therefore, when an individual who is already subjected to a mentally challenging task, such as driving, is subjected to a secondary stressor (e.g. the use of a new in-car control interface), their ability to successfully deal with the secondary stressor would require attention to be redirected away from the primary task of driving. Increased mental loading is proportional to, in the case of eye-tracking, the time driver gaze is directed away from the road ahead. In the case of response time, the time required for the driver to recognise and react (usual protocol involves applying the brake) to the introduction of a simulated or genuine hazard. In both cases altered mental workload is indirectly assessed through the modification of a secondary response. Although an accurate interpretation of increased mental demand can be established, extensive set-up and calibration, as well as interpretation of results must be undertaken. The advantages of using the LCC protocol are in the fact that no calibration is required, as each subject acts as their own control (comparison of pre- versus post-stressor leukocyte activity). Also, results are provided within 10-20 minutes post-stressor (maximum leukocyte activity (Hmax-RLU_{adj}) reached by T=15 minutes following PMA challenge for all subjects within this study and which followed the same trend for all other studies), with the requirement of minimal interpretation. This study aimed to investigate the capability of the LCC protocol to differentiate between the changes in leukocyte reactivity following exposure to two HMIs of differing ergonomic configuration.

Many of the latest HMIs are menu based and as such require a degree of familiarity in the use of computer-based technology in order for the user to feel comfortable. The

possibility therefore exists that a greater degree of mental loading would be exhibited by an individual who may not feel confident in the use of such technology, compared to an individual who does possess the necessary confidence. Such a relationship could lead to a greater incidence of hazardous driving practices within the less technically confident proportion of the general population. In order to explore the existence of such a relationship, a basic questionnaire, designed to gauge each volunteer's confidence in the use of computer-based technology, was used prior to testing (Chapter 6.3 and Appendix 3). Each subject was placed into one of two categories, depending upon their score, individuals with a score equal or greater than 5 were classed as having the confidence to use computer-based technology, whereas those who scored less than 5 were rated, due to their decreased familiarity of computer-based technology, as being less confident in the use of such systems.

Repeated exposure to the same stressor has been shown to cause habituation of the cardiovascular system (Veit *et al*. 1997, Bhatnagar *et al*. 2006, Barnum *et al*. 2007). The mechanism of cardiovascular regulation relies in part on the HPA axis (Chapter 1.1.3), which provides a neural and biochemical link between the emotional stimulus and physiological response. The HPA axis has also been shown to regulate aspects of the immune system. It, therefore, follows that the immune response may also demonstrate habituation. In animal models, repeated social defeat has been used as a mental stressor and has been shown to evoke a reduction in the degree of immune response with repeated stress (Beitia *et al*. 2005), as well as alter leukocyte trafficking and distribution patterns (Engler *et al*. 2004). In humans, chronic psychological stress has been shown to modify the immune response. Altemus *et al*. (2006) described how, following acute stress, cell mediated immune function is enhanced in individuals who have been diagnosed with post traumatic stress disorder (a condition that imposes chronic physiologic and mental stress on sufferers), and depressed in healthy individuals. However, no evidence that directly tests the effect and magnitude of repeated exposure to acute psychological stressors on the immune response, in otherwise healthy individuals, could be identified in published literature. This study aimed to investigate if such a relationship exists in humans, by observing leukocyte responsiveness following repeated exposure to two mental stressors.

Two multi-modal touch screen interfaces, a new model and one of its antecedents, from the same car manufacturer were used to explore firstly, the concept of leukocyte habituation and secondly, whether progressive interface design has led to a reduction in total mental workload (reported via a decreased post-stressor change in leukocyte responsiveness to PMA challenge).

6.3 METHODS

The Subjects

Local ethical committee approval from Coventry University Ethics Committee, and informed consent, was obtained before commencement of the study, in accordance with the declaration of Helsinki (World Medical Association 2004).

Subjects were 15 (7 male and 8 female) moderately fit and healthy individuals, aged between 26 and 56 years. Potential subjects were excluded on the following criteria: suffering from psychiatric illness; suffering from respiratory or cardiovascular disease; smokers; had taken prescription medicine within the previous month, and if they possessed any prior knowledge or experience of the test equipment.

Perceived Technical Ability and Psychological Stress

Prior to testing, each subject's perceived confidence in the use of unfamiliar computer-based technology was qualitatively assessed via response to 10 technology based questions (e.g. do you own an MP3 player? to can you program a video recorder?) (Appendix 3). Each was placed into one of two categories dependent upon their score, those with a score equal or greater than 5 were rated as being relatively confident in the use of computer-based technology. Individuals with a score of less than 5 were rated as lacking the confidence to embrace such technologies. Post-test changes in leukocyte activity (used as a means of assessing altered mental loading) were then compared between these two groups in an attempt to establish if confidence in the use of such technology affects overall mental loading.

Perceived psychological stress was established pre- and immediately post-test for each stressor (Interfaces A and B) by means of likert scales (using a continuous scale with 1 being relaxed and 10 stressed).

Design

The experimental protocols were rigorously standardised, and testing was confined to between 10am and 2pm. Subjects were required to avoid any strenuous activity for at least 2 hours prior to testing (e.g. they were instructed to take the lift to the laboratory, rather than climb the stairs).

Prior to obtaining resting heart rate, BP, and core body temperature following the standardised procedure (Chapter 2.4), subjects sat quietly and were instructed to breathe orthonasally for 15 minutes. The first pair of capillary blood samples was then taken 45 minutes before exposure to the test apparatus (45 minutes pre-test) (Chapter 2.2.3 for sampling methodology). During the 45 minute pre-test period subjects were asked to sit quietly or read (reading material was of a non-stimulating content).

Two minutes prior to testing, the subject was directed to an isolated, previously unseen area of the laboratory, where he or she was instructed to sit in front of one of two touch screen interfaces. Interface test order was assigned using a counter balanced design, where each subject was assigned an identification number (1 to 15), the first 8 numbers that were randomly selected were to complete the protocol using Interface A (earlier design) first, followed by Interface B (newer design incorporating a greater depth of menu orientated controls and superseding Interface A). The remaining 7 volunteers were instructed to use Interface B first, then Interface A. Although both interfaces employed touch screen technology, Interface A (Figure 6.1) incorporated dedicated buttons (located to the left and right of the touch screen) allowing selection of the specific operational screens (e.g. climate control, or satellite navigation menu). Once the required menu was selected all other commands were via a series of menu screens displayed and accessed via the touch screen. With Interface B (Figure 6.2) the main menu was displayed and accessed via the touch screen, thus eliminating the need for dedicated physical buttons. The system employed a display

with greater resolution and improved graphics, with a design that closely resembled the menu, sub-menu format of a personal computer.

Ensuring the subject was comfortable, and could access all the interface controls, the examiner explained the test protocol.

Figure 6.1
Interface A
Pre-existing HMI design format

Figure 6.2
Interface B
Latest HMI design, intended to supersede Interface A

All subjects completed the following tasks within the allotted 6 minute test period:

1. Select a specific radio station - Radio 1 (97.9 FM) and to increase the volume.

2. Program a specific destination into the Satellite Navigation System and initiate guidance (19 Rugby Rd, Barby, Warwickshire).

3. Re-tune the Radio to a different station (BBC Coventry and Warwickshire (94.8 FM).

It was explained that once the test had begun no further verbal communication was allowed, and that assistance would only be permitted after 2 minutes of attempting to

complete each task. Immediately upon completion of the task, and again 45 minutes afterwards, heart rate, BP and core body temperature were recorded and further blood samples taken.

After sitting quietly or reading (reading material was the local daily free newspaper) for 45 minutes (allowing base line leukocyte activity to re-establish) subjects were instructed to perform the same experimental protocol using the second touch screen interface. Sampling protocols were identical.

Repeat Testing

Veit *et al*. (1997) demonstrated how cardiovascular reactivity decreased following repeated exposure to a mild psychological stressor (arithmetic stress). To investigate if leukocyte activity is affected in a similar manner, 4 of the original 15 subjects (2 male and 2 female) were randomly selected. 1 male and 1 female were selected from each test order group and their respective test order remained the same throughout repeat testing. Each subject returned weekly for re-testing on both interfaces (experimental protocols were identical to original testing) on 2 further occasions (including original testing, all repeat volunteers were tested using both interfaces on 3 separate occasions).

Determining Leukocyte Activity

Two 10µl blood samples were taken using a finger lancing device (Roche Products Ltd. Hertfordshire, AL7 1TW United Kingdom) at each of the time periods specified (Chapter 2.2.3 for sampling methodology).

Leukocyte responsiveness to *in vitro* PMA stimulation was assessed for each pair of whole blood samples (Chapter 2.1.2 and Table 2.1 for full description of LCC protocol), at each of the specific time points.

Data Analysis

Standard statistical methodology was applied to investigate and compare the effect of each interface (n=15 for each treatment group) on leukocyte activity (Chapter 2.5.1) and also heart rate, systolic and diastolic blood pressure and core body temperature (Chapter 2.5.2). The relationship between post-stressor changes in leukocyte activity and heart rate, systolic and diastolic blood pressure and core body temperature, was analysed using Bivariate correlation (Chapter 2.5.3).

Test order was determined using a counterbalanced design (using the method described earlier in this chapter). Repeated measures single factor analysis of variance was used to investigate whether the change in activity exhibited for each interface differed significantly according to test order. Each of the 5 attributes of leukocyte activity acted in-turn as the dependent variable with test order acting as the fixed factor. Test order was shown to have no significant impact upon leukocyte activity for each interface ($F_{1,29} = 0.28$, $P = 0.65$).

Perceived psychological stress was qualitatively assessed using likert scales (using a continuous scale) pre- and immediately post-stressor. To test whether the observed post-test changes were significant between treatment groups, post-test differences were calculated and applied to a repeated measures single factor ANOVA model. With post-test difference in perceived psychological stress acting as the dependent variable and treatment group as the fixed factor. Bivariate correlation was used to test whether the observed post-test change in perceived stress was related to post-test changes in leukocyte activity. The truncated product method (Zaykin *et al*. 2002) was used to correct for multiple comparisons (Chapter 2.5.4).

Perceived technical confidence was assessed via response to 10 technology based questions (ranging from do you use an MP3 player? to can you program a video recorder?) (Appendix 3). Dependent on score, each subject was placed into one of two categories – those who were confident in using unfamiliar computer-based technology (score equal to or greater than 5), and those who were less confident in using unfamiliar technology (score less than 5). Using the 5 responses of leukocyte

activity in-turn as the dependent variable and perceived technical confidence category as the fixed factor, repeated measures single factor ANOVA was used to assess if perceived technical confidence influenced post-test changes in leukocyte activity.

To investigate the changes in leukocyte reactivity, and also heart rate, BP and core body temperature in response to repeated exposure to the putative stressors, 4 of the original test group were re-tested using each interface on a weekly basis on two further occasions. Each of the five attributes of leukocyte activity (differences between leukocyte activity at 45 minutes pre- and immediately post-test e.g. Hmax-RLU_{adj}), also heart rate, BP and core body temperature were applied in-turn as the dependent variable to a repeated measures 2-way ANOVA model, with treatment and test week as fixed factors. The interaction between treatment and test week was also investigated. Tukey's honestly significant difference test for multiple comparisons was used *post hoc* for test week.

6.4 RESULTS

Primary Testing

Leukocyte Activity

The LCC profiles displayed in Figure 6.3a and b show that following PMA challenge, maximum leukocyte activity (ROS release) for samples taken 45 minutes pre- and immediately post-stressor occurred at T=15 minutes, after which time, both profiles showed a steady decrease in activity. Pre-stressor activity for both treatment groups was of a similar magnitude, whereas immediately post-stressor, treatment group A showed a greater decrease in activity compared to treatment group B. Post-test changes in activity for all attributes of each luminescence profile, except for the time taken to reach maximum activity (T-max) are expressed as mean differences between leukocyte activity 45 minutes pre-and immediately post-stressor (RLU_{adj} ± standard error of mean (S.E.M.) are given in Table 6.1. Because the data for T-max is discontinuous, the median differences ± S.E.M. are presented. The results show that following the use of Interface A leukocyte activity significantly decreased from

baseline for all 5 attributes of the luminescence profile (except T-max and T=5 minutes), with the most pronounced change occurring at T=10 minutes (adjusted leukocyte activity 10 minutes into the 45 minute sampling period) (P = 0.03 Tukey's *post hoc* procedure), unlike Interface B where although a decrease in post-test activity was recorded, the magnitude of change proved non-significant. A significant difference in post-test leukocyte activity (difference between pre- and immediately post-test activity) between treatment groups, occurred at Hmax-RLU_{adj} and 5 minutes into the 45 minute luminescence profile (T=5 minutes). Both attributes show the use of Interface A led to a decrease in leukocyte activity that was significantly greater in magnitude compared to Interface B (Table 6.1). All subjects (n=15) reached maximum leukocyte activity (Hmax-RLU_{adj}) by T=15 minutes (15 minutes after PMA challenge) (Figure 6.3a and b).

Trends in post-test mental workload measured using likert scales paralleled those in LCC scores, with both treatment groups showing post-test increases. Although the use of Interface A led to an increase of greater magnitude than Interface B (Interface A 0.8 ± 0.3 Units, Interface B 0.1 ± 0.3 Units), the difference was not statistically significant ($F_{1,59}$ = 0.89, P = 0.34).

Core Body Temperature

Although both treatment groups followed a similar trend, demonstrating post-test increases in core body temperature, no significant differences were observed between pre- and post-test, or between treatment groups (n=15 for each) during primary testing (Table 6.2).

Heart Rate, Blood Pressure

As with core body temperature, no significant differences in heart rate, systolic blood pressure or diastolic blood pressure were observed between pre- and post-test or between treatment groups (n=15 for each) during primary testing (Table 6.2).

Perceived Technical Confidence

A basic measure of each subject's (n=15) technical confidence was evaluated pre-test, via their response to 10 technology based questions (e.g. did the subject own an MP3 player, and did the subject favour the use of pen or keyboard?). 10 subjects were classed as being confident in the use of unfamiliar computer-based technology (score of greater than or equal to 5) returning a modal score of 7 ± 0 (scores ranged from 7 to 10). The remaining 5 subjects achieved a modal score of 5 ± 0 (scores ranged from 3 to 4), suggesting that they lacked confidence in using unfamiliar computer-based technology. A significant difference between the two technical confidence groups and post-test difference in leukocyte activity was shown for maximum adjusted leukocyte activity (Hmax-RLU_{adj}) ($F_{1,29} = 6.55$, $P = 0.02$) and T=5 minutes ($F_{1,29} = 8.4$, $P = 0.008$). With those who were more confident in the use of unfamiliar computer-based technology demonstrating a post-test decrease in leukocyte activity that was significantly smaller in magnitude compared to individuals who were rated as lacking the confidence to use unfamiliar technology (Table 6.6).

Secondary Testing

Leukocyte Activity

4 subjects underwent repeat testing on 2 further occasions. Data for these 4 subjects only is presented in Table 6.4. LCC profiles for the 4 subjects (Figure 6.4a and b) showed the same response trends as observed for primary testing, with maximum leukocyte activity (Hmax-RLU_{adj}) occurring at T=15 minutes for both interfaces, and the magnitude of the post-test response proving to be significantly different between treatment group ($F_{1,23} = 13.55$, $P = 0.003$). In week 1 (primary testing) these 4 subjects showed that the use of Interface A resulted in a significant decrease in leukocyte activity of 9% from pre-test values ($P = 0.002$ Tukey's *post hoc* procedure), whereas following the use of Interface B, leukocyte activity showed a 1.8% decrease which proved to be non-significant ($P = 0.29$ Tukey's *post hoc* procedure).

Repeated use of both interfaces resulted in post-test decreases in leukocyte activity which progressively decreased in magnitude (Figure 6.4a and b). For Interface A post-test differences proved significant between week 1 (primary testing) and week 2 (P = 0.03 Tukey's *post hoc* procedure), whereas the magnitude of change was non-significant (P = 0.36 Tukey's *post hoc* procedure) for Interface B. For both Interfaces A and B post-test differences proved non-significant for tests in week 3 (Interface A P = 0.52 and Interface B P = 0.74 Tukey's *post hoc* procedure). The observed decrease in LCC response with repeated exposure to the same stressor suggests that leukocyte reactivity does exhibit habituation as familiarity to a situation increases (mental loading decreases).

Core Body Temperature

Although all 4 repeat test subjects showed a similar trend for core body temperature with post-test values being higher compared to pre-test, the differences were not significant from baseline or between treatment groups for all test weeks. However for both treatment groups the magnitude of the response significantly decreased following successive test weeks ($F_{1,23}$ = 4.69, P = 0.03) (Table 6.5).

Heart Rate, Blood Pressure

As with core body temperature, for the 4 repeat test subjects, post-test changes in heart rate, and systolic and diastolic blood pressure proved non-significant from baseline and between treatment groups for all test weeks. However, post-test differences in heart rate and diastolic blood pressure showed significant decreases in magnitude following repeated exposure to each interface (Table 6.5).

FIGURE 6.3a and b. Mean control adjusted Leukocyte Coping Capacity (RLU_{adj}) ± S.E.M. for use of Interfaces A and B (n=15 for each) for **primary test phase**.
* indicates significant difference in activity between 45 minutes pre- and immediately post-test ($P < 0.05$).

Figure 6.3a Interface A (n=15)

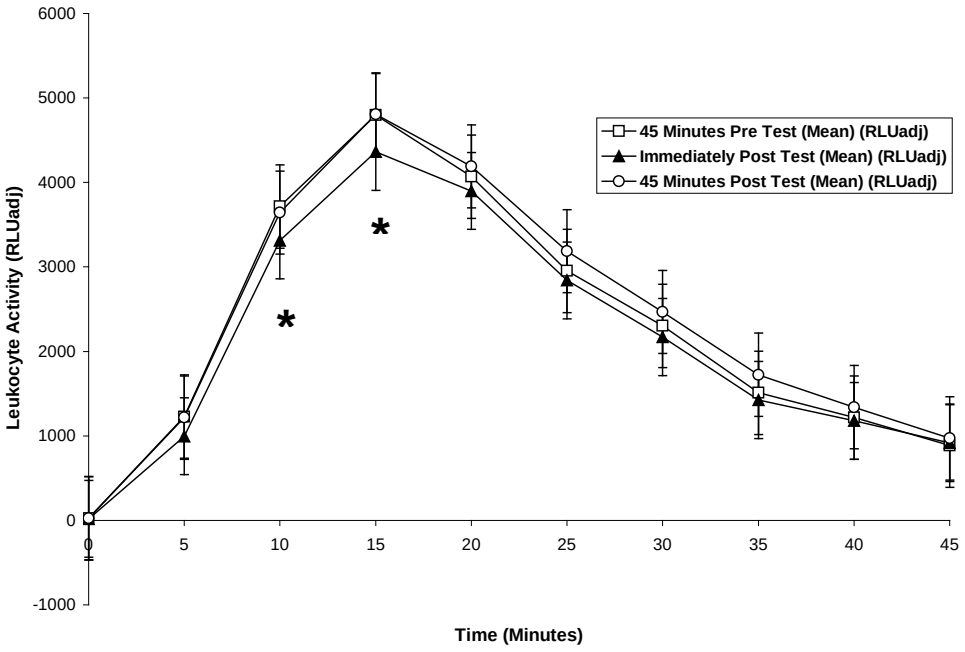


Figure 6.3b Interface B (n=15)

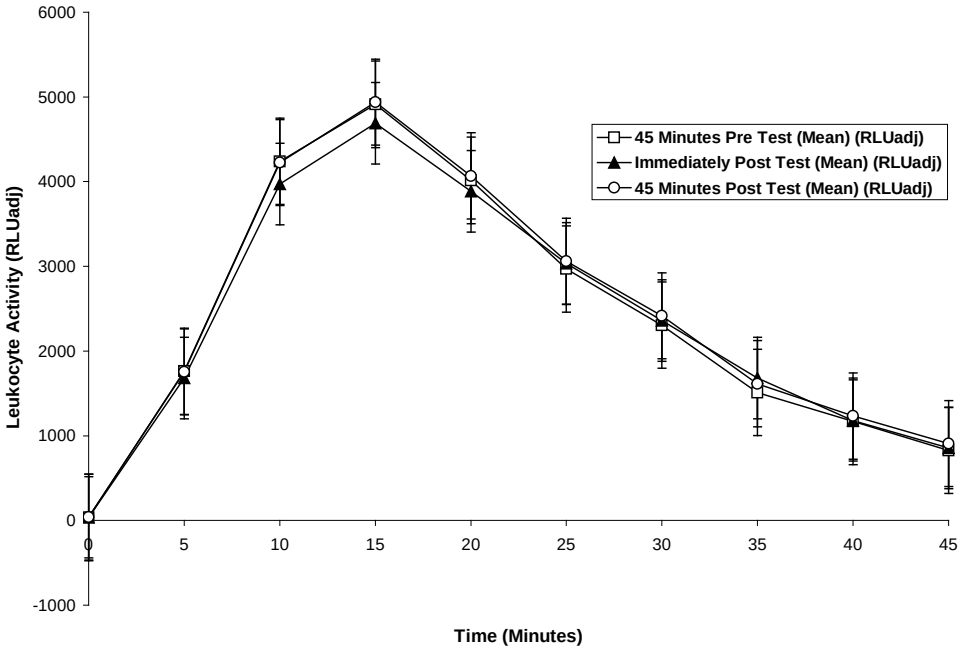


TABLE 6.1 Effect of stressor on leukocyte activity – Primary Test Phase

	INTERFACE A	INTERFACE B	*P* (F)
Δ Hmax (RLUadj)	-429.6 ± 177.38 •	-86.93 ± 91.91	0.04* (2.98)
Δ T-max (minutes)	0.0 ± 0.44	0.0 ± 0.53	0.62 (0.26)
Δ T=5 minutes (RLUadj)	-231.46 ± 64.92	-78.53 ± 70.83	0.05* (2.87)
Δ T=10 minutes (RLUadj)	-403.33 ± 78.29 •	-469.66 ± 247.72	0.79 (0.07)
Δ T=15 minutes (RLUadj)	-435.0 ± 162.13 •	-222.06 ± 201.37	0.12 (2.55)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) for each of the four specific attributes of the leukocyte luminescence profiles for both treatment groups (n = 15 for each) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}). Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}). For the difference in the time taken to reach maximum adjusted leukocyte activity (T-max) (minutes) between 45 minutes pre- and immediately post-test samples, the median is presented along with S.E.M. Repeated measures single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (*P*) (*d.f.* = 29).

• Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$) (Tukey's *post hoc* procedure)

* Difference between Interface A and Interface B ($P < 0.05$)

TABLE 6.2 Effect of stressor on heart rate, core body temperature and blood pressure – Primary Test Phase

	INTERFACE A	INTERFACE B	*P* (F)
Δ Heart rate (bpm)	0.0 ± 0.0	0.0 ± 0.0	0.09 (3.07)
Δ Core Body Temperature (°C)	2.0 ± 0.4	1.6 ± 0.2	0.08 (3.39)
Δ Systolic Blood Pressure (mmHg)	1.0 ± 1.0	1.0 ± 1.0	0.87 (0.03)
Δ Diastolic Blood Pressure (mmHg)	1.0 ± 0.0	0.0 ± 0.0	0.38 (0.8)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in heart rate, core body temperature and blood pressure (difference between 45 minutes pre- and immediately post-test samples) for each treatment group (n = 15 for each). Repeated measures single factor ANOVA was used to investigate the effect of treatment on each of the stated parameters (*P*) (*d.f.* = 29). For all parameters, no significant differences between pre- and post-test and also between treatment groups were observed.

TABLE 6.3 Relationship between post-test changes in leukocyte activity and changes in heart rate, core body temperature and blood pressure – Primary Test Phase

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes
Δ HR	-0.46 (0.01)	0.0 (1.0)	-0.26 (0.17)	-0.27 (0.14)	-0.39 (0.03)
Δ Core Body Temperature	-0.47 (0.009*)	0.14 (0.47)	-0.5 (0.004*)	-0.33 (0.08)	-0.52 (0.003)
Δ Systolic BP	-0.01 (0.95)	-0.12 (0.54)	0.2 (0.29)	0.07 (0.72)	-0.01 (0.94)
Δ Diastolic BP	0.04 (0.84)	-0.24 (0.2)	0.23 (0.23)	0.03 (0.89)	0.15 (0.42)

Combined data from both treatment groups (n=30) was tested using bivariate correlation to investigate the significance of the relationship between post-test changes (Δ) in leukocyte activity and in heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test) (Pearson correlation with *P*-values in brackets). The Truncated Product Method (Zaykin *et al*. 2002) was used to correct for the use of multiple comparisons (*p*). The only significant truncated product was for core body temperature ($p \leq 0.0003$), therefore the relationships between core body temperature and maximum leukocyte activity (Hmax-RLU_{adj}) and activity at 5 minutes into the luminescence activity profile (T=5 minutes) could be classed as significant.

* $P < 0.05$ Statistically Significant

TABLE 6.4 Effect of repeat testing on leukocyte activity

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes
INTERFACE A					
Week 1	-346.0 ± 91.6 • †	0.0 ± 0.0	-90.5 ± 146.54	-322.0 ± 138.62 • †	-311.3 ± 111.8 • †
Week 2	-52.25 ± 30.68	0.0 ± 0.0	-77.0 ± 62.71	-96.75 ± 52.32	-60.25 ± 25.04
Week 3	* 34.75 ± 10.4	0.0 ± 0.0	-25.25 ± 25.98	* 61.5 ± 43.22	* 24.75 ± 13.76
INTERFACE B					
Week 1	-45.25 ± 112.64	-1.25 ± 1.25	-62.0 ± 57.71	-102.5 ± 82.09	-64.5 ± 113.02
Week 2	-24.0 ± 16.27	0.0 ± 0.0	-4.25 ± 3.68	-19.25 ± 14.71	-10.0 ± 18.41
Week 3	1.25 ± 10.96	0.0 ± 0.0	-12.75 ± 11.75	1.25 ± 7.31	17.0 ± 14.61

4 subjects carried out repeat testing, 2 from test order Interface A then B, and 2 from test order Interface B then A. Combined data for all 4 subjects is presented here as mean and standard error of the mean (S.E.M.) for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}) (for T-max the difference in median values is presented). The difference between the values for Interfaces A and B and values between test weeks (1-3), along with the interaction between Interface and test week are presented.

- Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.01$) (Tukey's *post hoc* procedure)

* Difference between Interface A and Interface B ($P < 0.05$)

† Difference between Test Week 1 and 2 ($P < 0.05$) (Tukey's *post hoc* procedure)

Figure 6.4a and b. Effect of repeat testing on leukocyte activity (RLU_{adj})

Figure 6.4a Interface A

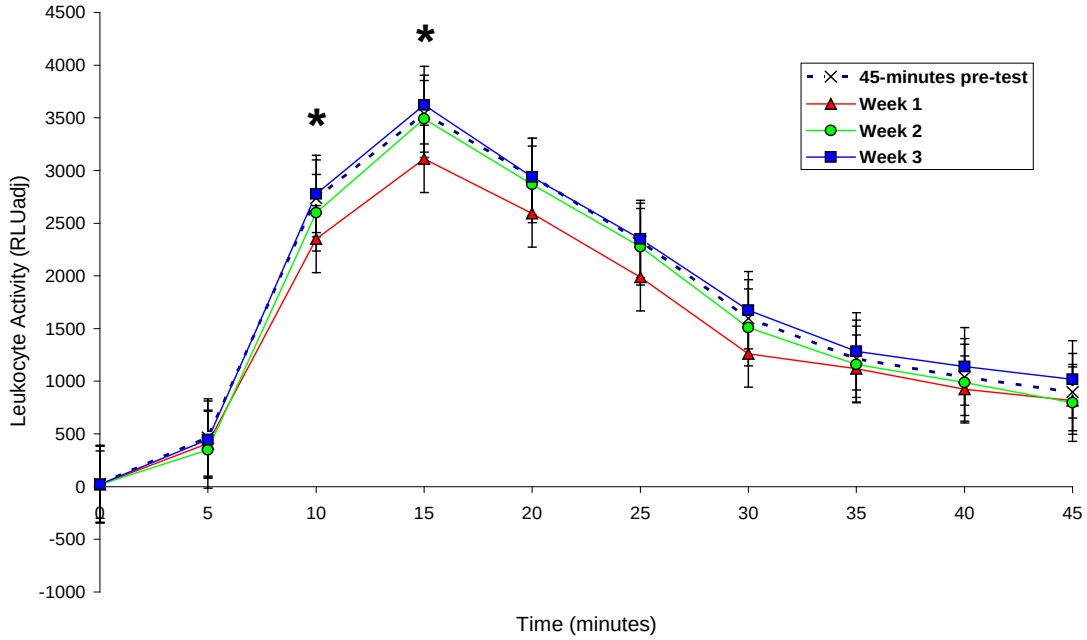
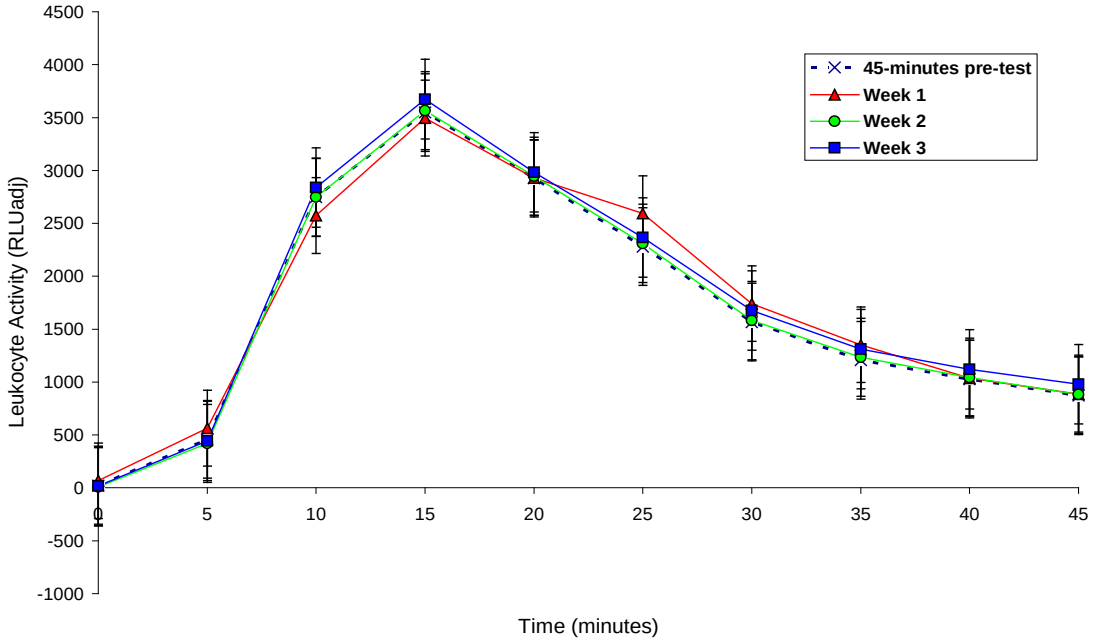


Figure 6.4b Interface B



Line charts showing the effect of weekly repeat testing for 3 weeks using both interfaces on mean immediately post-test leukocyte activity (RLU_{adj}) ± S.E.M. for repeat test subjects (n=4), (week 1 red line, week 2 green line, week 3 blue line). In each case mean pre-test leukocyte activity is also included (Crosses with bold dashed line). * Difference between week 1 and week 2 ($P < 0.05$).

TABLE 6.5 Effect of repeat testing on heart rate, core body temperature and blood pressure

	Δ Core Body Temperature (°C)	Δ Heart Rate (bpm)	Δ Systolic Blood Pressure (mmHg)	Δ Diastolic Blood Pressure (mmHg)
INTERFACE A				
Week 1	0.18 ± 0.03 *	3.25 ± 0.75 *	2.5 ± 1.65	1.75 ± 0.47 *
Week 2	0.13 ± 0.05	1.75 ± 0.47	0.25 ± 0.62	-0.25 ± 0.62
Week 3	0.08 ± 0.05	0.5 ± 0.28	0.75 ± 0.47	-0.25 ± 0.25
INTERFACE B				
Week 1	0.15 ± 0.06 *	2.5 ± 0.29 *	2.0 ± 1.41	1.25 ± 0.75 *
Week 2	0.08 ± 0.05	1.5 ± 0.5	0.25 ± 0.25	0.75 ± 0.85
Week 3	0.03 ± 0.03	-0.75 ± 0.25	0.75 ± 0.85	-0.75 ± 0.47

4 subjects carried out repeat testing, 2 from test order Interface A then B, and 2 from test order Interface B then A. Combined data for all 4 subjects is presented here as mean and standard error of the mean (S.E.M.) for the change (Δ) in core body temperature, heart rate and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test samples). No significant difference between pre- and post-test values and between treatment groups were shown for each of the listed parameters.

* Difference between Test Week 1 and 2 ($P < 0.05$) (Tukey's *post hoc* procedure)

Table 6.6 Effect of technical confidence on leukocyte activity

	CONFIDENT	NON-CONFIDENT	*P* (F)
Δ Hmax (RLUadj)	-123.1 ± 75.9	-528.7 ± 257.7	0.02* (6.55)
Δ T-max (minutes)	1.0 ± 0.46	0.5 ± 0.5	0.5 (0.89)
Δ T=5 minutes (RLUadj)	-134.9 ± 46.9	-654.7 ± 232.2	0.008* (8.4)
Δ T=10 minutes (RLUadj)	-417.0 ± 162.0	-475.4 ± 216.1	0.73 (0.52)
Δ T=15 minutes (RLUadj)	-138.9 ± 54.4	-707.8 ± 351.1	0.6 (0.67)

The effect of perceived technical confidence (rated according to the responses to 10 questions designed to gage an individuals confidence in using unfamiliar computer-based technology (subjects were classed as being either confident in the use of such technology (score equal to or greater than 5) or lacking in confidence (score of less than 5)) on post-test changes (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test) using combined data from both treatment groups (n=15 for each) was assessed for each of the five specific attributes of the leukocyte luminescence profiles (Hmax (RLU_{adj}) - the maximum adjusted response exhibited during the 45 minute sampling period, T-max – the time taken to reach maximum leukocyte activity, and T=5, 10 and 15 minutes - the adjusted response in leukocyte activity recorded at 5, 10 and 15 minutes into the 45 minute activity profile (RLU_{adj}) using a repeated measures single factor ANOVA model. Where attributes of the leukocyte luminescence profiles were used as the dependent variable and perceived technical confidence rating as the fixed factor.

* Difference between technically confident and non-confident groups ($P < 0.05$).

6.5 DISCUSSION

Following exposure to mild psychological stressors, immune activity responds in a rapid and reversible manner, demonstrating similar response trends to those observed within the cardiovascular system (Veit *et al.* 1997). Initial exposure to the two stressors, resulted in significantly different post-test decreases in the magnitude of leukocyte response exhibited for maximum adjusted leukocyte activity (Hmax-RLU_{adj}) and for leukocyte activity 5 minutes into the 45 minute luminescence profile (T=5 minutes) (Table 6.1). These differences demonstrate the capability of the LCC technique to quantify the differential responses in altered leukocyte activity that resulted from performing the same series of basic in-car related tasks using two different touch screen interface designs.

As the use of Interface A (antecedent version) resulted in a post-test decrease in leukocyte activity that was of greater magnitude compared to the use of Interface B (a newer version designed to facilitate ease of use, therefore decreasing total mental workload) (Table 6.1), it could be concluded that the degree of mental loading associated with the use of Interface A was significantly greater than with Interface B. These findings suggest that the LCC technique could be used as an effective measure of altered mental loading during ergonomic evaluation.

It was previously known that psychological familiarity to specific situations promotes habituation of heart rate, blood pressure and core body temperature responses (Veit *et al*. 1997, Bhatnagar *et al*. 2006, Barnum *et al*. 2007). However, this trend has been generally reported following exposure to large stressors, including periods of acute physical exertion (Lockwood and Frost 2007). The current study confirms the existence of this relationship (Table 6.5) and suggests that a similar trend also occurs for leukocyte responsiveness following exposure of low-level stressors. Hmax-RLU_{adj} and leukocyte activity at 10 and 15 minutes into the 45 minute luminescence profile (T=10 and 15 minutes) ceased to produce a significant decrease in activity by the second stressor exposure (Table 6.4).

Subjects (n=15) were categorised according to their responses to 10 questions designed to provide a crude assessment of their perceived confidence in the use of

unfamiliar technologies. 10 were found to be comfortable with the use of new computer-based technology and 5 found the use of computer technology more daunting. A significant difference in the magnitude of post-test change in leukocyte activity was shown between these 2 groups (Table 6.6). This suggests that subjects classed as being confident with computer-based technology were "pre-habituated" to the use of such technologies and therefore demonstrated a reduction in post-test leukocyte activity that was significantly lower in magnitude compared to those individuals who were rated as lacking confidence. Both groups did show habituation to the stressors following repeat exposure. These findings suggest the possible existence of a physical link between an aspect of the immune response and the degree of self-imposed mental loading that occurs in anticipation of an impending, possibly stressful, event.

The results from this study suggest that habituation to specific experiences can occur as rapidly as by the second exposure. This rapid adaptation to novel experiences, especially when attempting to evaluate the ergonomics of new technologies, means that in order for results to be significant, it must be ensured that prior experience of the test apparatus is minimised. The current study proposes that customers rapidly become habituated to the use of their vehicles' in-car systems, suggesting that after only a few encounters, drivers are able to interact with their HMI whilst maintaining safe driving practice. However, it is also the case that due to cost, many of these vehicles are favoured by the short-term car hire market. In this instance, the driver may not have sufficient time to become familiar with the vehicle and as a consequence, may exhibit unsafe driving practice. It is therefore important that the motor industry is able to further improve the ergonomics of such systems, and possess the ability to objectively quantify such improvements.

During primary testing, no significant post-test differences in heart rate, systolic and diastolic blood pressure or core body temperature were shown. A basic subjective measure of perceived stress, employing likert scales (with a continuous scale where 1 represented relaxed and 10 stressed) was also used pre- and immediately post-stressor. Although subjects reported increased perceived mental stress following exposure to both stressors, with the greatest magnitude following the use of Interface A, the observed differences were not significant. Therefore, none of the tested parameters

were able to statistically differentiate between the use of Interfaces A and B. In contrast, the magnitudes of the post-test changes observed for leukocyte activity were significantly different between stressors, providing evidence of the ability of LCC to discriminate between two similar acute mental stressors.

In conclusion, this study has shown that the LCC technique is capable of differentiating between the magnitudes of leukocyte responsiveness occurring as a result of using two Interfaces with only subtle ergonomic differences. Also, that as with the cardiovascular system, leukocyte responsiveness displays habituation as familiarity to a specific mental stressor increases.

7.0 Quantifying Changes in Leukocyte Activity in Response to the Use of Different Automotive Interface Technologies, Whist Simultaneously Maintaining Lane Discipline within a Computer Simulated Environment.

7.1 RATIONALE

In chapter 6 leukocyte activity was used to assess alterations in mental loading resulting from interaction with two different human machine interface (HMI) designs, under static laboratory conditions. Interaction with a vehicle's HMI usually occurs while driving, a task which in itself demands considerable mental loading. It is therefore a design requisite that the impact on driver attention, of such systems is minimised. Each motor manufacturer has its own opinion as to which sensory modality (audio, visual or a combination of the two (multimodal) is the most efficient for both presenting and facilitating HMI interaction, especially while the vehicle is in motion. This study aimed to investigate how the use of 3 such interface systems, each employing a different sensory modality, impacts mental loading whilst simultaneously driving. For safety and consistency a computer simulated test track environment was selected, in preference to test track or public highway.

7.2 INTRODUCTION

The ability to quantify psychological stress poses an intriguing problem with profound practical implications. The most common tradition measures, including heart rate, blood pressure, core body temperature and the plasma concentration of stress hormones (cortisol and adrenaline) (Okutsu *et al*. 2005) have been discussed (Chapter 1.3.2) and their effectiveness tested and compared with the LCC protocol throughout this experimental programme. Within the automotive industry, ergonomic assessment of new and developing in-car technologies employs many of the aforementioned techniques. However the requirement to accurately assess driver attention capacity and situational awareness whilst using such technologies, which could literally mean life or death for the end user, has resulted in the development of

methodologies which assess attention focus (eye-tracking) and reaction time (Chapter 1.5). Both of which can be related to the degree of mental loading exhibited by the test subject. Despite the ability of both technologies to provide a quantifiable indication of mental loading, the equipment, especially in the case of eye-tracking, requires extensive set-up and calibration, prior to testing each volunteer. The advantages of using a technique such as LCC are in the speed of detection (results are calculated in 15-20 minutes), the fact that no lengthy initial set-up and calibration is required (each subject acts as their own control). Most importantly, leukocyte activity has been shown to be directly affected by HPA and SAM activity (Chapter 5), which allows the inference that changes in leukocyte activity are directly related to an individual's mental state, and can therefore be used as an indicator of mental loading.

In general any stimuli which distract the driver from the road ahead will affect both mental work load and driving performance, severely affecting the ability to drive safely (Funke *et al*. 2007, Ma and Kaber 2007). Horberry *et al*. (2006) report how driver performance and response to roadway hazards in a simulated environment is affected as a result of being distracted by a hands-free mobile phone conversation and whilst attempting to operate a vehicle entertainment system. Both tasks degraded responses to hazards and increased subjective workload. The user-friendliness of any novel in-car system is an essential consideration during the design process. The requirement to understand the driver's needs and limitations associated with effective use and assimilation of such systems has caused ergonomic research to explore how the format for presenting information affects overall mental loading and attention capacity. The addition of secondary tasks (such as the use of a novel HMI) during driving, places increased mental demand upon the driver, resulting in a considerable reduction in the driver's situational awareness (SA) of the external environment. The hypothesis was formed that as interface complexity is increased, the ability of the driver to follow a pre-established route would also be affected, due to the increased need to shift attention away from the road in order to identify the correct control. For the current study, a computer simulated test track comprising three lanes was used. Subjects were required to remain in the centre lane throughout the test. The frequency of deviation was recorded by the examiner and used as a basic means for assessing "eyes-off-the road" time and situational awareness.

For drivers, information displayed visually has both advantages and disadvantages (Wickens and Liu 1988, Dingus and Hulse 1993). Using navigation systems as an example, drivers prefer a format that visually informs them of their current location (Streeter *et al*. 1985). Auditory formats can be superior to visual displays in presenting navigation and warning information in that there is a reduced need for the driver to shift attention away from the road (Liu 2001), resulting in faster response times compared to visual stimuli (Simpson *et al*. 1985, Sorkin 1987). Labiale (1990) found that workload was reduced when navigation information was presented audibly, rather than visually, with drivers expressing a preference for audible information. A simulator experiment, conducted by Walker *et al*. (1991), evaluated seven in-vehicle navigation devices that varied in complexity and mode of presentation. The conclusion was that drivers using auditory navigation devices of low, medium or high complexity made significantly fewer navigation-related errors, compared to those using visual mode devices. In terms of complexity, the participants using the complex devices drove more slowly than those using the simpler devices, and high-complexity displays were the least preferable. In addition to making fewer driving errors, drivers using an audible device also reduced travel distance and travel time (Streeter *et al*. 1985, Parkes and Coleman 1990).

Many HMI systems utilise a combination of auditory and visual formats (a multimodal interface). The advantage of such systems is in their ability to present and receive key information using the sensory format that least competes for driver attention (auditory), while still allowing the presentation and interaction with a more detailed and complex version of the information for clarification of commands. Lui (2001) proposed that for safe driving, short auditory information combined with a visual display would optimize perceptual and cognitive performance. McKnight *et al.* (1992) reported that a multimodality display where an audible beep was provided prior to visual direction demonstrated better route guidance results. In theory a multimodality display (visual plus auditory) should allow drivers to perceive more information without significantly increasing their workload.

The reviewed studies employed techniques that were, in general, highly subjective in nature (Chapter 1.5). Using PMA-induced leukocyte reactive oxygen production (LCC) this study aimed to provide objective data elucidating which of the three

interface formats (visual, auditory and multimodal) resulted in the greatest change in leukocyte reactivity following completion of a series of interface related tasks whilst simultaneously driving within a computer simulated environment. Mental workload would already be high as a result of maintaining road position and speed. The addition of a secondary task which involved the subject having to shift attention from the external environment to the vehicles control system should serve to increase workload. It was believed that the extent of this extra workload would vary according to the interface format and the sensory modality which it targets.

7.3 METHODS

The Subjects

Local ethical committee approval from Coventry University Ethics Committee and written informed consent was obtained before commencing the study, in accordance with the declaration of Helsinki (World Medical Association 2004).

Subjects were 15 (7 male and 8 female) moderately fit and healthy individuals, aged between 26 and 55 years. Potential subjects were excluded on the following criteria: suffering from psychiatric illness; suffering from cardiovascular or respiratory disease; smokers; had taken prescription medicine within the previous month, and if they had prior knowledge or have owned a motor vehicle fitted with one of the interfaces to be investigated, or any other sort of computer based HMI (Chapter 2.2.2).

Design

At the time of testing no single vehicle was available which contained a HMI that utilised all three interface modalities. Instead, two test vehicles including a BMW 535D, incorporating the iDrive multimodal control interface (Interface A) and Jaguar S-Type R fitted with a touch screen interface (Interface B) and also voice control (Interface C) were individually interfaced with a computer driving simulator (Low

Cost Simulator™). The simulation software allowed the responses of the vehicles basic control systems to be displayed, in real-time, and used to guide the user around a computer simulated test track viewed from the driver's perspective that was projected onto a 2 metre square screen in front of the test vehicle. Steering inputs were received by means of a sensor placed under each of the front wheels, while acceleration and braking commands, from the respective pedals, were taken by direct feedback from the vehicles engine management system. Only these 3 key controls were required to successfully navigate the virtual test track. As their placement is generic throughout all makes of car, the fact that two different unfamiliar vehicles were used was not seen as a possible source of secondary stress.

Two days prior to testing, subjects received information outlining basic operating instructions for each interface, including how specific voice commands should be structured (Appendix 4). The content was similar to that included in the basic tutorial which accompanies the purchase of either vehicle.

The experimental protocols were rigorously standardised, and testing was confined to between 10am and 2pm. Subjects were required to avoid strenuous activity for at least 2 hours prior to testing.

Prior to obtaining resting heart rate, BP, and core body temperature, following the standardised procedure outlined in Chapter 2.4, subjects sat quietly and were instructed to breathe orthonasally for 15 minutes. Each subject's pre-test perceived psychological stress rating was established by means of likert scales (using a continuous scale with 1 being relaxed and 10 stressed). A resting capillary blood sample was then taken (Chapter 2.2.3 for sampling methodology) 45 minutes before exposure to the test apparatus (45 minutes Pre-Test).

During the 45 minute Pre-Test period subjects were taken from the laboratory (Coventry University) to Jaguar Cars Ltd. research and development centre at Whitley, Coventry, United Kingdom – a 5 minute car journey.

Upon entering the first test vehicle (selected using a counterbalanced crossover design) the subject adjusted the seat and other driving controls to the correct driving

position. As these actions themselves had the potential to cause increased mental stress and subsequent changes to leukocyte activity, the examiner helped with these tasks. To eliminate all other external stimuli, all windows except the windscreen were covered. The subject then proceeded to familiarise themselves with the responsiveness of the steering and also acceleration and braking by driving around the virtual test track for 2 minutes (e.g. as engine was not running, power steering was not available which made trying to turn the steering wheel much harder than during normal operation) . With the vehicle stationary within the simulated environment the examiner explained the test protocol.

The test lasted a maximum of 15 minutes. Subjects were requested to complete the following tasks using the selected interface modality, whist simultaneously driving within the centre lane of the virtual test track at a constant speed of 60mph.

1. Whilst the vehicle was stationary within the simulated environment, program the destination – Euston Road, London NW1 - into the satellite navigation system, and initiate guidance.

2. With the vehicle in motion within the simulated environment, adjust the climate control to a temperature of 18°C with a moderate fan speed.

3. Tune the radio to a specific station (100.7 FM).

4. Turn both the radio and climate control off.

For each interface, the frequencies of lane deviation to the left and right of centre were recorded, by the researcher, as means of assessing situational awareness (SA) of the external environment whilst attempting to complete the interface related tasks.

Each of the selected interfaces targets either a single sensory modality or a combination of the two. With the touch screen (Interface B), information is both provided and acted upon using a visual format via a series of menus and command screens. For voice control (Interface C) all commands are provided using specific voice commands and phases (all subjects received an information sheet detailing the

commands necessary for successful completion of the task, described earlier). The system is primed for a command via depression of a button located left of the steering wheel, at the end of the indicator control stalk. To acknowledge the system is primed an audible beep is initiated, after which each command can be clearly spoken. The final system, Interface A (present within the BMW), employed a multimodal system incorporating both visual and audible commands that could be accessed through the use of a multifunction control wheel, located next to the gear stick, which allowed the user to navigate and select various commands displayed in menu format on an LCD screen mounted within the centre stack of the dash board.

It was explained that once the test had begun no further verbal communication was allowed, and that assistance would only be offered after 4 minutes of attempting to complete the task. Immediately upon completing the tasks the subject was asked to come to a controlled stop in the centre of the virtual test track, whereupon a blood sample, BP, heart rate and core body temperature measurements, as well as perceived psychological stress (using a continuous likert scale) were taken (Immediately post-test).

On two further occasions (ensuring a minimum interval of 2 hours existed between tests for baseline leukocyte activity to re-establish) each subject was tested using the remaining two interfaces using identical protocol (test order was decided using a counterbalanced design).

Determining Leukocyte Activity

Two 10µl blood samples were taken using a finger lancing device (Roche Products Ltd. Hertfordshire, AL7 1TW United Kingdom) at each of the time periods specified (Chapter 2.2.3 for sampling methodology).

Leukocyte responsiveness to *in vitro* PMA stimulation was assessed for each pair of whole blood samples (Chapter 2.1.2 and Table 2.1 for full description of LCC protocol), at each of the specific time points.

Data Analysis

Standard statistical methodology (Chapter 2.5) was applied to investigate and compare the effect of each of the 3 interfaces (n=15 subjects for each treatment group) on leukocyte activity (Chapter 2.5.1) and also heart rate, systolic and diastolic blood pressure and core body temperature (Chapter 2.5.2). The relationship between post-stressor changes in leukocyte activity and heart rate, systolic and diastolic blood pressure and core body temperature, was analysed using Bivariate correlation (Chapter 2.5.3).

Perceived psychological stress was assessed using likert scales (using a continuous scale with 1 representing relaxed and 10 stressed) pre- and immediately post-stressor. To test whether the observed post-test changes were significant between treatment groups, post-test differences were calculated and applied to a repeated measures single factor ANOVA model. With post-test difference in perceived psychological stress acting as the dependent variable and treatment group as the fixed factor. Bivariate correlation was used to test whether the observed post-test change in perceived stress was related to post-test changes in leukocyte activity. The truncated product method (Zaykin *et al*. 2002) was used to correct for multiple comparisons (Chapter 2.5.4).

Test order was determined using a counterbalanced design. Repeated measures single factor analysis of variance was used to investigate if the change in activity exhibited for each interface differed significantly according to test order. Each of the 5 attributes of leukocyte activity acted in-turn as the dependent variable with test order (3 combinations) acting as the fixed factor. Tukey's honestly significant difference test for multiple comparisons was used *post hoc* for test order.

The mean ± S.E.M frequency of lane deviations to the left and right of the centre lane were calculated for each of the 3 treatment groups. Repeated measures 2-way analysis of variance was used to investigate the difference between lane deviation direction and frequency for each interface, using deviation frequency as the dependent variable and treatment group and deviation direction as fixed factors. Tukey's

honestly significant difference test for multiple comparisons were used as *post hoc* tests when applicable. Bivariate correlation was used to investigate the relationship between the post-test change in leukocyte activity (difference between 45 minutes pre- and immediately post-test) and lane deviation frequency. *P*-values were corrected for the use of multiple comparisons using the Truncated Product Method.

7.4 RESULTS

Leukocyte Activity

The LCC profiles displayed in Figure 7.1a, b and c show that following PMA challenge, maximum leukocyte activity (ROS release) for samples taken at each of the 3 sampling points 45 minutes pre-, immediately post- and 45 minutes post-stressor occurred between T=15 and 20 minutes following exposure to Interfaces B (Figure 7.1b) and C (Figure 7.1c) and between T=20 and 25 minutes for Interface A (Figure 7.1a), after which time, all profiles showed a steady decrease in activity. Changes in leukocyte activity were both rapid and reversible with pre-stressor activity and activity 45 minutes post-stressor for all treatment groups being of a similar magnitude. Post-test changes in activity for all attributes of each luminescence profile, except for the time taken to reach maximum activity (T-max) are expressed as mean differences between leukocyte activity 45 minutes pre-and immediately post-stressor (RLU_{adj} ± standard error of mean (S.E.M.) are given in Table 7.1. With the data for T-max being discontinuous, the median differences ± S.E.M. are presented. The results show that a significant difference in post-test leukocyte activity, between treatment groups, occurred 10 minutes into the 45 minute luminescence profile (T=10 minutes) (Table 7.1) In general, LCC scores following the use of Interface A showed the greatest decrease in leukocyte activity with Interface C exhibiting the least (Figure 7.1a, b and c). Table 7.1 shows how the magnitude of the post-test change in activity following the use of Interface A was significantly greater compared to the use of Interface B ($P = 0.01$ Tukey's *post hoc* procedure) and also Interface C ($P \leq 0.001$ Tukey's *post hoc* procedure). Whereas the use of both Interfaces B and C resulted in

post-test changes in leukocyte activity of similar magnitude (P = 0.47 Tukey's *post hoc* procedure).

Test Order

Subjects were assigned, using a counterbalanced crossover design, to one of three test order combinations:

1) Interface A, B then C
2) Interface B, C then A
3) Interface C, B then A

A significant difference was found to exist between test order and adjusted post-test change in leukocyte activity for Hmax-RLU_{adj} ($F_{1,134}$ = 10.83, P < 0.001) and T=15 minutes ($F_{1,134}$ = 11.02, P < 0.001). In both cases the significant difference occurred between test order combination 1 and 3 (in each case P = 0.02 Tukey's *post hoc* procedure). Indicating that individuals who were first tested using Interface A found the subsequent use of Interface C significantly less mentally demanding. Conversely those who first used Interface C found Interface A significantly more demanding.

Subjective Mental Loading

Overall perceived mental stress was significantly different between treatment groups ($F_{1,89}$ = 7.73, P = 0.001). The use of Interface A resulted in an increase in perceived stress (2.0 ± 0.22 units) that was significantly greater in magnitude compared to the use of Interface B (1.13 ± 0.13 units) ($P \leq 0.001$ Tukey's *post hoc* procedure) (rating based on a continuous arbitrary scale where 1 represented relaxed and 10 stressed). The use of Interface C to complete the required tasks resulted in a post-stressor increase of 0.06 ± 0.18 units, which was of a similar magnitude to that observed following the use of Interface B (P = 0.19 Tukey's *post hoc* procedure). Perceived mental loading was not found to be significantly correlated with the observed post-test decreases in leukocyte activity.

Core Body Temperature

Core body temperature increased significantly from baseline following the use of Interfaces A and B (Table 7.2). The magnitude of change between the use of Interface A and B was similar (P = 0.92 Tukey's *post hoc* procedure), whereas the use of Interface C resulted in a post-test increase that was significantly lower in magnitude compared to the use of Interface A (P = 0.04 Tukey's *post hoc* procedure).

Heart Rate, Blood Pressure

Treatment groups A and B demonstrated significant increases in heart rate compared to baseline (45 minutes pre-test), whereas the use of Interface C resulted in no significant change (Table 7.2). No significant difference in the magnitude of change between treatment groups was observed.

As with heart rate, only treatment groups A and B showed significant increases in systolic blood pressure compared to baseline (Table 7.2). The magnitude of the post-test increase was significant between treatment groups ($F_{1,44}$ = 7.87, P = 0.001). The use of Interfaces A and B resulted in a similar magnitude of change (P = 0.81 Tukey's *post hoc* procedure), whereas using Interface C resulted in a post-test increase in systolic BP that was significantly lower in magnitude compared to Interface A (P = 0.002 Tukey's *post hoc* procedure) and Interface B (P = 0.01 Tukey's *post hoc* procedure) (Table 7.2). No significant change in diastolic BP was observed compared to baseline or between treatment groups.

Although the differences in post-test change in heart rate between treatment groups only approached significance ($F_{1,44}$ = 2.9, P = 0.07), change in heart rate was significantly correlated with the change in time taken to reach maximum adjusted leukocyte activity (T-max) and adjusted leukocyte activity at 5 minutes into the 45 minute profile (T=5 minutes) (Table 7.3).

Lane Deviation

Subjects were requested to remain within the centre of 3 lanes throughout the test. Any deviation was used as an indication of increased mental workload and decreased situational awareness of both external and internal driving environments. Lane deviation was significantly different between treatment groups ($F_{1,89} = 40.47$, $P \leq 0.001$). Completion of the test using Interface A resulted in a number of deviations that was significantly greater compared to the use of both Interface B ($P = 0.005$ Tukey's *post hoc* procedure) and Interface C ($P \leq 0.001$ Tukey's *post hoc* procedure) (Table 7.4). For each interface the frequency of deviation to the left was greater than to the right (In each case $P \leq 0.001$ Tukey's *post hoc* procedure), with the use Interface A leading to the greatest number of deviations and Interface C the least (Table 7.4). No significant relationship between post-test change in adjusted leukocyte activity and total lane deviation frequency was found.

FIGURE 7.1a b and c Mean adjusted Leukocyte Coping Capacity (RLU_{adj}) ± S.E.M. for Interfaces A, B and C (n=15 for each). * indicates significant difference in activity between 45 minutes pre- and immediately post-test ($P < 0.05$).

Figure 7.1a Interface A (n=15)

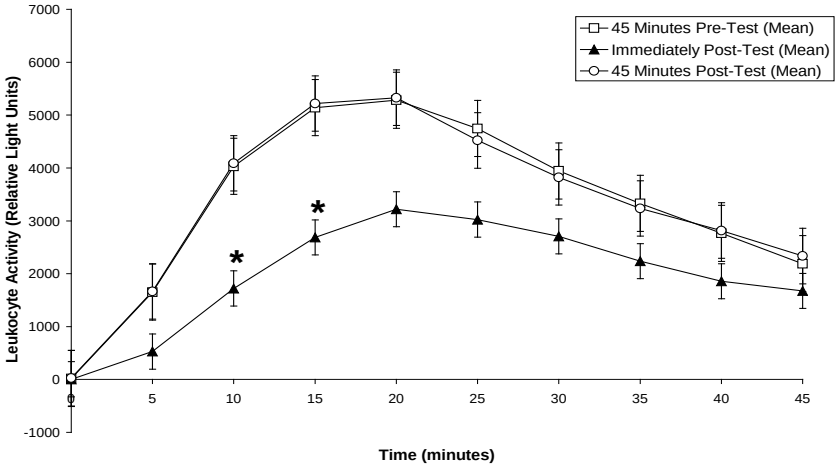


Figure 7.1b Interface B (n=15)

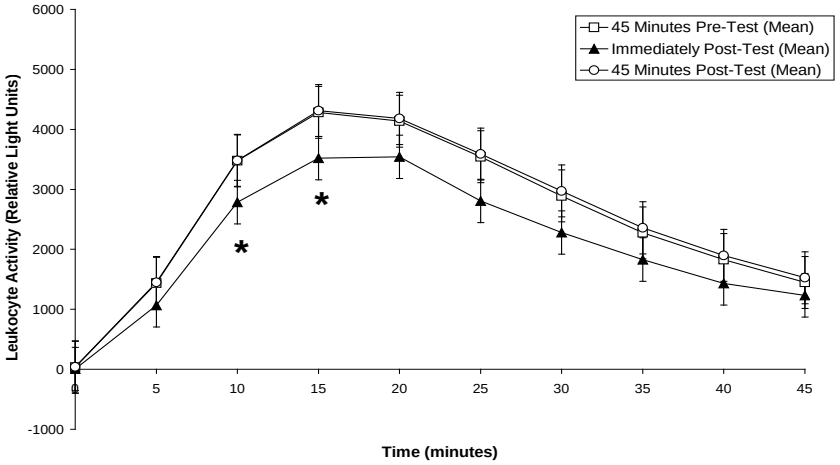


Figure 7.1c Interface C (n=15)

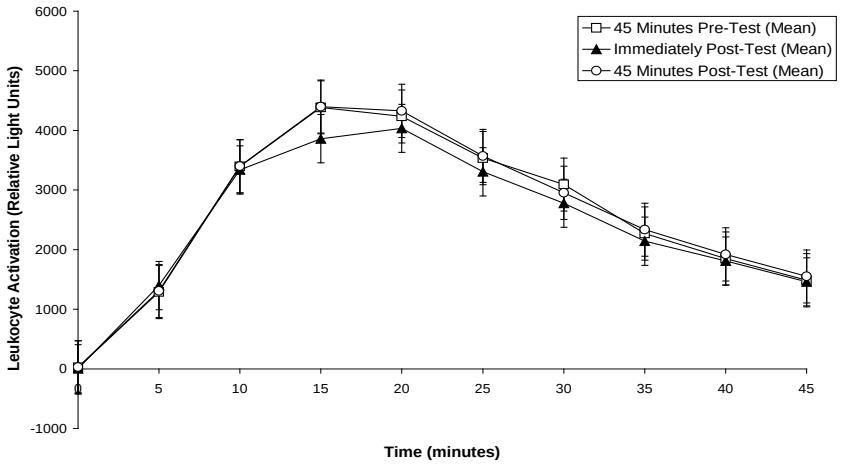


TABLE 7.1 Effect of stressor on leukocyte activity

	INTERFACE A	INTERFACE B	INTERFACE C	*P* (F)
Δ Hmax (RLUadj)	-2418.06 ± 714.24 •	-775.8 ± 229.43	-518.26 ± 219.44	0.07 (2.82)
Δ T-max (minutes)	-5.0 ± 2.01 •	0.0 ± 0.48	0.0 ± 0.48	0.15 (2.01)
Δ T=5 minutes (RLUadj)	-1124.0 ± 348.71 •	-369.06 ± 121.88	-107.0 ± 169.8	0.09 (2.65)
Δ T=10 minutes (RLUadj)	-308.73 ± 689.16 •	-688.8 ± 182.55 •	-57.4 ± 120.15	0.04* (3.46)
Δ T=15 minutes (RLUadj)	-2453.93 ± 705.31 •	-763.33 ± 313.34 •	-525.13 ± 411.97	0.12 (2.26)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in leukocyte activity (difference between 45 minutes pre- and immediately post-test samples) (Leukocyte Activity - Adjusted Relative Light Units – RLU_{adj}) (for T-max the difference in median values is presented) for each treatment group (n = 15 for each). Repeated measures single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (*P*) (*d.f.* = 44). For T=10 minutes a significant difference was observed between all treatment groups.

▲ Difference between treatment groups ($P < 0.05$) (Tukey's *post hoc* procedure)

• Difference between Pre- and Post-Test Leukocyte Activity ($P < 0.05$)

* $P < 0.05$ Statistically Significant

TABLE 7.2 Effect of stressor on heart rate, core body temperature and blood pressure.

	INTERFACE A	INTERFACE B	INTERFACE C	*P* (F)
Δ Heart rate (bpm)	3.0 ± 1.0 •	3.0 ± 1.0 •	1.0 ± 0.0	0.07 (2.9)
Δ Core Body Temperature (°C)	0.3 ± 0.1 •	0.2 ± 0.1 •	0.1 ± 0.1	0.03* (3.85)
Δ Systolic Blood Pressure (mmHg)	2.0 ± 1.0 •	1.0 ± 1.0 •	0.0 ± 0.0	0.001* (7.87)
Δ Diastolic Blood Pressure (mmHg)	0.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.72 (0.33)

Mean and standard error of the mean (S.E.M.) are presented for the change (Δ) in heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test samples) for each treatment group (n = 15 for each). Repeated measures single factor ANOVA was used to investigate the effect of treatment on each of the stated parameters (*P*) (*d.f.* = 44).

▲Difference between treatment groups ($P < 0.05$) (Tukey's *post hoc* procedure).

• Difference between Pre- and Post-Test ($P < 0.05$)

* $P < 0.05$ Statistically Significant

TABLE 7.3 Relationship between post-test changes in leukocyte activity and changes in heart rate, core body temperature and blood pressure

	Δ Hmax (RLU_{adj})	Δ T-max (minutes)	Δ T=5 minutes	Δ T=10 minutes	Δ T=15 minutes	*P*
Δ Heart Rate (bpm)	0.08 (-0.26)	0.03* (-0.33)	0.05* (0.29)	0.2 (0.19)	0.35 (0.14)	0.02*
Δ Core Body Temperature (°C)	0.71 (-0.05)	0.65 (0.07)	0.49 (0.1)	0.62 (0.07)	0.67 (0.06)	0.8
Δ Systolic BP (mmHg)	0.000* (0.65)	0.6 (-0.08)	0.16 (0.21)	0.03* (0.33)	0.01* (0.37)	1.0
Δ Diastolic BP (mmHg)	0.001* (-0.49)	0.46 (-0.114)	0.95 (-0.01)	0.66 (-0.068)	0.68 (-0.062)	1.0

The significance of the relationship between post-test changes (Δ) in leukocyte activity and in heart rate, core body temperature and systolic and diastolic blood pressure (difference between 45 minutes pre- and immediately post-test) was investigated by means of bivariate correlation (Pearson correlation values included). To correct the overall *P*-values for the use of multiple comparisons the Truncated Product Method was applied (Zaykin *et al.* 2002) (*P*) (n = 45).

* $P < 0.05$ Statistically Significant

Table 7.4 Frequency of Lane Deviation

	DEVIATION LEFT	DEVIATION RIGHT	TOTAL NUMBER OF DEVIATIONS
Interface A	11.0 ± 2.0	5.0 ± 1.0	8.0 ± 1.0 ▲
Interface B	6.0 ± 1.0	3.0 ± 1.0	5.0 ± 1.0 ▲
Interface C	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

As a means of assessing driver attention, the frequency of lane deviations to the left and right of the centre of the virtual test track were recorded. The mean number of deviations to the left and right of centre, and also the mean total deviation frequency ± standard error of the mean (S.E.M.) are displayed for each treatment group (n = 15 for each).

▲ Difference between treatment groups ($P < 0.05$ Tukey's *post hoc* procedure)

7.5 DISCUSSION

Immune responsiveness is rapidly affected by an individual's psychological state, and can be used a means for objective assessment of an individual's degree of mental loading. A previous study that analysed mental workload and situational awareness during the use of high feedback vehicles (employing the latest electronic driver aids designed to provide detailed information of all aspects of the internal and external driving environment), as opposed to low feedback vehicles (which provide only the bare minimum level of feedback required for safe driving practice), concluded that situational awareness was improved and was coupled with lower perceived workload (Walker *et al*. 2001). In contrast, the current study suggests that although psychologically a person may perceive high specification driver aids as beneficial, physiologically the act of performing the simplest task (e.g. selecting a radio station) leads to short-term increased stress, which could detrimentally affect attention. The findings of this study also suggest that complex multimodal systems such as Interface A, designed to facilitate interaction with everything from climate control to satellite navigation, evoked greater increases in stress, compared to simpler systems such as Interfaces B and C (which necessitated less hunting through layers of menus to find the correct control, thus reducing additional mental load). Interface C, being voice control, almost completely avoided the need for the driver to shift attention from the external environment, which meant that mental loading was not significantly affected (no significant post-test change in leukocyte activity compared to pre-test values was observed (Figure 7.1c). Chapter 7.4 showed how subjective assessment of perceived mental loading followed a similar pattern as was observed for leukocyte activity, with subjects rating the use of Interface A as being significantly more stressful compared to the use of Interface C.

In chapter 6 (Table 6.4) it was shown that repeated exposure to the same stressor, over several weeks, resulted in a significant progressive decrease in the magnitude of the observed post-test change in leukocyte activity. Within the current study a similar relationship was observed where subjects who were first tested using the more complex interface (Interface A – multimodal HMI), demonstrated decreased post-test leukocyte activity that was significantly greater in magnitude compared to subjects who were tested using the other interfaces before Interface A. In addition to this

learning effect being attributed to the differing complexity of each interface and the mental demand placed upon the subject, it may also be due to the rate at which the subject becomes familiar with the test protocol. It could be the case that test order number 1 (Interface A then B then C) may provoke a more rapid rate of habituation to the protocol, compared to subjects within test order 3 (Interface C then B, finishing with A). If this trend is indeed device non-specific, it can be suggested that repeated use and increased familiarity to a specific task results in a decrease in associated stress and mental demand, resulting in increased situational awareness, which when associated with the use of motor vehicles would result in safer driving practice.

Young and Stanton (2007) describe how vehicle automation systems can reduce driver mental workload and suggest that attention capacity and mental workload are directly related. Within the automotive industry, direct monitoring of eye movement and direction of focus is used as means of assessing situational awareness during periods of increased mental loading (Chapter 1.5). The primary task associated with safe driving practice is ensuring that attention remains fixed on the external environment, therefore eye gaze should be in the direction of the road ahead. With the introduction of secondary tasks, such as interaction with the vehicles HMI, the driver's direction of focus is shifted from the external to the internal environment. The extent of "eyes off the road time" can be related to the complexity of the secondary task. As task complexity and therefore mental workload increases, the amount of time where attention is shifted from the road ahead is also increased. Unfortunately the sponsor's eye tracking hardware was unavailable during this study. Instead a basic measure of situational awareness, where the frequency and direction of deviations from the centre lane of the computer simulated test track were counted. The hypothesis was formed that as interface complexity increased, the ability of the driver to follow a pre-established route would be affected, due to the increased need to shift attention away from the road in order to identify the correct control. With the more challenging and ergonomically complex interfaces (e.g. Interface A which utilised a complex menu system displayed on an LCD that was accessed using a control device located on a different area of the dashboard), mental workload was increased which was accompanied by a higher incidence of lane deviation (particularly to left), as driver attention was redirected from maintaining lane discipline in order to focus on the interface display. In contrast, the use of Interface C

(voice control) resulted in far fewer lane deviations, as there was no need for visual attention to be directed away from the road ahead. The system was primed using a button located at the end of the indicator stalk and the required tasks were accomplished using voice commands. It can therefore be inferred that this system contributed the least to total mental workload.

The format by which information is both received and supplied by the driver can significantly alter response time, accuracy and performance whilst undertaking basic button pushing and navigational tasks. Liu (2001) demonstrated how navigational commands supplied in a multimodal (both visual and auditory commands) or audible only formats led to improved response times and lowered subjective workload ratings, compared to visual only displays. LCC analysis offers support to these findings on a psycho-physiological level. The use of Interface C (voice control), where commands were both given and received audibly, resulted in the smallest change in leukocyte reactivity (Table 7.1). When information was supplied visually, as with both Interfaces A and B, there was a significantly greater decrease in post-test leukocyte activity (Table 7.1).

In conclusion, this study has shown that overall mental workload maybe significantly affected according to the sensory modality used during interaction with a vehicles' HMI, in order to achieve specific basic tasks whilst simultaneously attempting to drive around a computer simulated test track. It was found that the subsequent post-test decrease in leukocyte activity was significantly smaller in magnitude when utilising voice control technology (Interface C) compared to visual devices that rely upon the use of either touch screen technology (Interface B) or a separate multimodal control (Interface A).

8.0 GENERAL DISCUSSION

8.1 DISCUSSION OF RESULTS

This chapter presents an evaluation and critique of the research undertaken. First, looking at the original aims of the project and how the five studies address them, key findings will be emphasized and problems and limitations will be discussed. Second, using these findings a hypothetical model for short-term low-level psychological stress-induced leukocyte activation will be presented. Third, future work will be discussed, with suggestions for assessment and further expansion of the model.

This research has demonstrated that leukocyte activation occurs in a rapid and reversible manner in response to subtle changes in low-level psychological and physical stress. Prior to this research, the leukocyte coping capacity (LCC) protocol has been predominantly applied in the assessment of psychological stress in animal models, primarily the trapping and transport of wild badgers (*Meles meles*) (McLaren *et al*. 2003, Montes *et al*. 2003, 2004). Ellard (2003) provided early evidence highlighting the potential benefits for the use of LCC, in humans, as a means of quantifying the psycho-physiological effects of a high intensity acute psychological stressor (anticipation of impending coronary bypass surgery). The main aim of the pilot study within this research was to establish whether similar response trends occur following exposure to particularly short-term, low intensity psychological and physical stressors. Also to establish whether the technique could form the basis for an objective assay for quantifying the degree of stress exhibited as a result of exposure to low-level short-term psychological and physiological stressors encountered as part of daily life.

Many of the studies within the literature describe how the immune system is affected by exposure to severe, chronic stress, however considerably less is known about the effect of short-term low-level stressors, that are encountered as part of our daily lives. Throughout this research a range of different short-term, real life, stressors associated with the ergonomics of driving were utilised. All were of a sufficient magnitude and

duration of exposure to provoke significant post-test changes in leukocyte reactivity, quantified using the leukocyte coping capacity (LCC) protocol. The stressor, within chapter 3 involved performing a number of basic tasks in order to prepare a motor vehicle for a new driver, so as to meet British motoring standards (including adjustment of the driver's seat to the correct height and distance from the pedals, as well as adjusting the wing and rear view mirrors). The second stage was to perform a simple driving manoeuvre that involved moving away from, and returning to, a designated parking space. Subjects were tested within two different cars on two separate occasions. The magnitude of the post-test response was significantly different between treatment groups (treatment group A – Car A, treatment group B – Car B) (Chapter 3.3). This suggests that leukocyte activity could be used as a means of objectively differentiating between the degrees of stress resulting from two similar short-term stressors.

The duration of exposure to each stressor within chapter 3 lasted between 15 and 20 minutes depending upon the proficiency of the test subject. The aim of the second study (Chapter 4) was to establish whether leukocytes produced a similar response trend following exposure to a psychological stressor that was of a much lower magnitude than was used during the pilot (chapter 3), and which had an exposure time lasting seconds rather than minutes. The stressor involved adjustment of a car electric window to four designated positions using the standard rocker switch control. Within the control group the standard window mechanism was used. However, within the treatment group the polarity of the mechanism was covertly reversed after each designated window position. This meant that when the subject gave the command for the window to raise its response was to lower, and visa versa. As with the previous study (Chapter 3) the ability of leukocytes to release reactive oxygen species (ROS) in response to *in vitro* PMA challenge (see Chapter 2.1.2 for theoretical overview of the challenge technique) following stressor exposure (where the polarity of the window mechanism was altered a total of four times during the test) was significantly reduced, compared to control (polarity remained unchanged). This observation illustrates that the LCC protocol possesses adequate sensitivity to detect and quantify extremely short duration low-level stress.

Exposure to stressors such as the example used in chapter 7 (interaction with different interface designs while simultaneously maintaining lane discipline within a simulated environment) resulted in significant post-test changes in both heart rate and systolic blood pressure (both traditional measures of stress) (Table 7.2) and in leukocyte responsiveness (Table 7.1), this suggests that leukocytes respond to the same stress response mechanisms that have been established for the modification of the cardiovascular system in anticipation of, and during stressor exposure (the fight-flight response) proposed by Canon (1929). Further evidence in support of this hypothesis was provided within chapter 5, where standard ELISA analysis was used to measure the pre- and post-stressor changes in plasma concentration of nine biochemical mediators that were believed to be associated with leukocyte activation. Adrenaline was the only mediator that showed a significant change in plasma concentration, increasing in concentration by 0.18 ± 0.03 ng/ml (Chapter 5.4, Table 5.5) post-stressor (stressor involved identification of potential driving hazards within pre-recorded driving scenarios). There is considerable evidence within the literature which describes how increased catecholamine concentration (adrenaline), via HPA axis activity (Thase and Howland 1995, Seeley *et al*. 2003), promotes modification of specific physiological systems including the cardiovascular system, as part of the fight-flight stress response (Canon 1929) (Chapter 1.1.3). Significant post-stressor increases in heart rate and systolic blood pressure were recorded following exposure to the stressors described within chapter 5 (identification of hazardous situations within pre-recorded driving scenarios) and chapter 7 (comparing the use of 3 different interface designs to successfully complete basic driving related tasks whist simultaneously maintaining lane discipline within a simulated driving environment), observations which support the findings that adrenaline concentration has increased following stressor exposure. As leukocyte activity also increased in response to each stressor, it is possible that leukocytes are able to respond to increases in adrenaline concentration (mechanism proposed within Figure 8.1).

Within chapters 6 and 7 the potential benefits of using leukocyte activity as an objective means of assessing the ergonomic impact of different driving related interface technologies was explored. The stressor used within chapter 6 involved performing basic driving tasks (such as adjustment of cabin temperature and radio station) using two different touch screen interfaces from the same manufacturer.

Interface A was of an earlier design, whereas Interface B superseded Interface A and incorporated a greater depth of menu orientated controls, aimed to facilitate interaction. The magnitudes of the post-stressor changes in leukocyte activity were found to be significantly different between treatment groups (group A – Interface A, group B – Interface B) (Chapter 6.4, Table 6.1), whereas no significant post-test change in heart rate, blood pressure or body temperature was recorded. This suggests that the LCC technique is able to objectively discriminate between stressors that traditional stress measures find indistinguishable. The study described within chapter 7 utilised a stressor design that was fundamentally similar to that used within chapter 6. Subjects were required to complete basic driving related tasks (adjust cabin temperature) using, on 3 separate occasions, 3 different interface designs, each employing either touch screen, voice control or a combination of the two formats (multimodal) whilst simultaneously maintaining lane discipline within a computer simulated test track environment. As observed within chapter 6, the magnitude of the post-test change in leukocyte activity differed significantly between each of the three treatment groups (Chapter 7.4, Table 7.1). These findings add weight to the proposal for the use of the LCC technique as a direct objective measure for quantifying the ergonomic impact of new technologies, within automotive design and other industries.

A review of the literature surrounding the physiology and psychology of stress highlights the fact that the term "stress" often has negative connotations. This may well be true where chronic stressors are involved, however levels of acute psychological and physiological arousal and anxiety have been proven to be an essential prerequisite to normal functioning of an organism's stress response (Dhabhar *et al*. 2000, 2003, Dhabhar and McEwen 2001). Viswanathan *et al*. (2005) reported how acute stress, in the case of skin immunity, resulted in immune-enhancement and protection. Proposing that this characteristic, part of the fight-flight stress response, prepared and protected an individual against potential wounding or infection. As leukocyte activity has been shown to increase in response to low-level short-term stressors lasting between seconds and minutes (Table 4.1), the observations within this thesis can be used to further corroborate the conclusions of Viswanathan *et al*. (2005) and others (Dhabhar *et al*. 2003, Altemus *et al*. 2006), that leukocyte activation occurs as part of the stress response. The blood collection protocols utilised during the current research involved the use of cutaneous finger prick

sampling. As each whole blood sample was not extracted from any of the main central blood pools (as occurs during venepuncture), it can also be suggested that leukocyte activation, in response to low-level stress, is a systemic phenomenon and not localised within a specific region of the body.

Viswanathan *et al*. (2005) proposed that systemic leukocyte activation, in anticipation of physical harm, can function as an important and necessary pre-emptive mechanism. Although such activation would allow a more rapid response in the mobilisation of leukocytes to regions of injury and potential infection, the response mechanism is unable to differentiate between stressors, which have the potential to inflict physical harm, from those that are a result of mental challenge (for example, being face to face with a tiger in the wild, as opposed to observing one in captivity). This characteristic suggests that exposure to potentially stressful, non-threatening situations, encountered as part of daily life has the potential to lead to healthy tissue damage and the development and progression of many common disease states and inflammatory disorders, including asthma, rheumatoid arthritis and psoriasis (Dhabhar *et al*.1996, Richards *et al*. 2005, Viswanathan *et al*. 2005, Boscarino 2008). All studies within this thesis support this observation, in that leukocyte activity was increased following stressor exposure. Only the pilot study (chapter 3), where subjects had to perform the same simple manoeuvre using two different test vehicles, had the potential to inflict actual physical harm. Each of the remaining studies all involved the use of stressors that had the potential to increase the overall mental loading of the individual, either from the need to problem solve (Chapter 6 – use of two unfamiliar touch screen devices), induce confusion (Chapter 4 – covert modification of the control for an electric window) or by the addition of a secondary task during execution of an already mentally demanding primary task (Chapter 7 – use of different interface designs to accomplish basic driving related tasks, whist simultaneously maintaining lane discipline within a computer simulated environment). Although, the undifferentiated nature of the innate immune response can potentially be the cause of numerous disease states, the same characteristics have also allowed its use as a highly sensitive means for objectively quantifying the impact of exposure to low-level short-term everyday mental and physical stressors.

Throughout this research attempts were made to compare the effectiveness of the leukocyte coping capacity protocol with other traditional subjective and objective methodologies for assessing altered mental loading. Traditional measures of assessing changes in mental workload rely heavily upon subjective quantitative techniques that were originally designed for diagnosis of clinical psychological disorders (Lemyre and Tessier 2003). These tests are, by nature highly subjective, due to the reliance of self-assessment and the need for subjects to possess a sufficient understanding of the test. The need for reliable dedicated measurement techniques for assessing psychological stress and mental loading in non-clinical populations led to the development of a number of diagnostic systems (discussed in Chapter 1.3.1). The systems most commonly used to quantify altered mental loading during evaluation of new and developing automotive technologies are the Subjective Workload Assessment Technique (SWAT) (Reid and Nygren 1988) and the NASA task load index (NASA-tlx) (Hart and Staveland 1988). Within chapter 5 comparisons were made between the ability of NASA-tlx and the LCC protocol to quantify changes in total metal loading following stressor exposure. In this instance, although both parameters demonstrated significant differences in mental workload, following stressor exposure (stressor involved identification of roadway hazards within pre-recorded driving scenarios presented on a notebook computer), due to limitations associated with the design of the study (discussed in chapter 5.5 and 8.1), a direct comparison between each was not possible.

A distinct advantage of using LCC to quantify altered mental loading and stress levels rather than methodologies, such as NASA-tlx, became apparent due to one of the limitations associated with the study design within chapter 5. The NASA-tlx questionnaire can only be completed post-stressor. The lack of pre-stressor data makes direct assessment of the effect of a single stressor impossible, the technique only allows for direct comparison between multiple stressors. In addition to having the ability to objectively compare differences in workload resulting from exposure to different stressors (LCC samples are taken pre-and post-stressor), each subject produces their own control (demonstrating baseline activity). This allows the effect of a single stressor to be directly and objectively quantified via calculating the difference between the two values.

All studies within this research demonstrated the trend where, as mental loading increased, for example, as a result of the interaction with a novel motor vehicle (Chapter 3), leukocyte activity, *in vivo*, also increased (characterised by increased systemic release of ROS), which resulted in a decreased ability of leukocytes to response to chemical challenge, *in vitro*.

In addition to testing the more complex questionnaire based tools (discussed in the previous paragraph) more basic techniques were assessed. The simplest of these being, subject self-assessed perceived stress. Each subject was asked to rate, using a continuous scale where 1 represented relaxed and 10 stressed what they perceived to be their own stress level, pre- and immediately post-stressor. Of the 5 studies contained within this research, those involving stressors that were relatively greater in magnitude (possessing a greater potential for eliciting a physical response) including chapter 3 (where the stressor involved performing a basic driving manoeuvre in two different cars) and chapter 7 (which again utilised production vehicles to test different interface designs whilst driving within a computer simulated environment), resulted in significantly increased perceived stress ratings post-test, that proved to be significantly different between treatment groups.

Likert scales can only provide a crude subjective indication as to the effect of exposure to short-term low-level stressors. In order to be effective in allowing differentiation between two or more related stressors, subjects must be able to recognise that stressors, although related, are potentially different. During instances when only subtle differences existed between the stressors, subjects found attempts to differentiate between such stressors, particularly difficult. This became apparent within this research during chapter 4 and 6. The stressor within chapter 6 involved comparing the ability to complete basic driving related tasks (including adjusting climate control and radio frequency) using two different touch screen interfaces. Although different post-test increases in perceived stress were recorded between treatment groups, using likert scales, the magnitude of each subject's reaction to the stressor was insufficient to be able to significantly differentiate between the two stressors. This demonstrates the benefit of using LCC when a comparison between closely related low-level stressors is required.

Within chapter 4, the extremely short duration and low-level nature of the stressor (adjustment of the controls of an electric window mechanism), meant that subjects did not report any significant change in perceived stress level following the test (likert scale). However, as with the stressor in chapter 6, significant changes in post-test leukocyte activity (Figure 4.3a and b), and in the magnitude of response between treatment groups was recorded (Table 4.1). This observation suggests that a physiological response is elicited during exposure to low-level short-term psychological stressors which, when asked, the subject may dismiss as trifling and therefore decide not to report. The fact that significantly different post-test changes in leukocyte activity were shown between treatment groups, in response to such an acute low-level stressor, demonstrates that the detection sensitivity of the LCC assay is significantly greater than one of the most basic subjective measures (Likert scale). Not only can LCC detect changes in leukocyte activity resulting from particularly low-level short-term stressors, the technique can also objectively differentiate between multiple stressors that subjective techniques, including perceived stress rating, may not even be capable of detecting, let alone differentiate between. This observation is of particular importance within industry, where ergonomic evaluation is often reliant upon the test subject's own perception of how stressful interaction with a particular device or specific task is. The use of LCC would allow a definitive objective assessment of the physiological impact of a particular stressor.

In an attempt to further validate the effectiveness of LCC for objectively quantifying the effect of low-level stress, the observed post-test changes in leukocyte activity were compared to other traditional objective methods for assessing mental and physical stress including heart rate, blood pressure and core body temperature. Heart rate, BP and catecholamine and cortisol concentrations are often used as indicators of the quantity of stress received (Hiramatsu *et al*. 1981, Holbrook *et al*. 1984, Jern 1991, Tochikubo *et al*. 1996, Clow *et al*. 1999, Seematter *et al*. 2000, Munakata *et al*. 2002, Clow *et al*. 2006). However, blood pressure, heart rate and sympathetic activity possess feedback systems for stabilisation and tolerance to stress (homeostasis), which can alter their effectiveness as measures of mental stress (Delarue *et al*. 2003). Pre- and post-stressor measurements of heart rate, systolic and diastolic blood pressure, and also core body temperature were made. For heart rate significant post-test differences were measured after exposure to the following stressors: identification

of potential driving hazards viewed on a notebook pc (chapter 5), and after the use of Interfaces A (multimodal) and B (touch screen) to complete basic driving related tasks while simultaneously maintaining lane discipline within a computer simulated environment (chapter 7). None of the examples showed a significant difference in the magnitude of increased heart rate between treatment groups. The stressor used during chapter 3 involved performing basic driving related tasks followed by a simple manoeuvre in two different motor vehicles. This was the only stressor, used during this research, which had the potential to inflict actual physical harm on the subject. It was hypothesised that this stressor should have elicited the most intense psychological and physiological reaction, resulting in significant pre- post-stressor differences, and also differences between treatment groups, in all measured parameters. Table 3.2 shows that this was not the case, the only parameter that demonstrated a significant post-test difference was core body temperature (which increased following the use of car A), all other parameters remained unchanged.

Significant post-stressor changes in core body temperature were also recorded following exposure to the stressors within chapter 7, where completion of the required tasks using Interface A (multimodal control) whist simultaneously maintaining lane discipline within a simulated environment resulted in a significant post-stressor increase in body temperature that was significantly greater in magnitude compared to the use of Interface C (voice control) (Table 7.2).

Following exposure to the stressor within chapter 5 (identification of potential hazards within 6 pre-recorded driving scenarios), post-stressor systolic blood pressure increased significantly. A similar trend was also observed after exposure to the stressors within chapter 7, where a significant increase in systolic BP was recorded post-stressor following the use of Interfaces A (multimodal control) and B (touch screen). The magnitude of change following the use of Interface B (touch screen) and C (voice control) was significantly different compared to the use of Interface A. No significant changes in diastolic BP were recorded following exposure to any of the stressors used during this research.

Repeated exposure to the same stressor has been shown to cause habituation of the cardiovascular system (Veit *et al*. 1997, Bhatnagar *et al*. 2006, Barnum *et al*. 2007).

To explore how familiarity to a task affects physical responsiveness, chapter 6 describes how subjects under went weekly repeat testing, using two different touch screen interfaces to successfully complete 3 interface related tasks (including programming a destination into the satellite navigation system). Despite the absence of any significant post-stressor differences occurring for any to the 4 traditional measures of stress, post-test difference in heart rate, diastolic BP and core body temperature all decreased significantly in magnitude following successive exposure (Table 6.5).

All of the traditional measures of stress (heart rate, blood pressure, and body temperature), used during this research, failed to provide a consistent means of quantifying the physical effects of exposure to each of the psychological stressors used within each of the 5 studies. This suggests that such measures are too crude and lack the sensitivity to allow accurate measurement of, and differentiation between such low intensity short-term stressors. In contrast, significant differences in leukocyte activity between pre- and post-stressor and in the magnitude of response between treatment groups were recorded in response to each of the 5 stressors used during this research. These findings suggest that the LCC technique does appear to possess the required sensitivity to allow the objective detection, quantification and discrimination between such short-term low-level stressors.

Like many other physiological measures, including resting heart rate and blood pressure, Leukocyte activity demonstrates considerable inter-individual variation in baseline levels. For example, pre-test baseline activity observed for subjects (n=39) within the pilot study (Chapter 3.0) ranged between 403 ± 323.8 RLU_{adj} and 9554 ± 323.8 RLU_{adj} (Mean baseline activity 2394 ± 323.8 RLU_{adj}). In order to reduce the effects of this, post-test leukocyte activity must be compared with pre-stressor baseline values in each individual. This means that each person acts as their own 'control'. While this does increase the time taken for the test, and doubles the cost, it means that the method can accurately detect small changes in leukocyte activity.

The literature describes that a direct relationship exists between situational awareness, driver performance and mental loading (Young and Stanton 2007). In fact any stimulus which distracts the driver from the road ahead will affect both mental

workload (within this thesis altered leukocyte responsiveness is used as an indication of increased stress and mental workload) and driving performance (Funke *et al*. 2007, Ma and Kaber 2007) (discussed in Chapter 1.5). The technology now exists that allows monitoring of both eye movement and pupil diameter – both of which are indicators of attention focus and can be related to altered mental loading during completion of unfamiliar secondary driving tasks (Sodhi *et al*. 2002, Strayer *et al*. 2003). Unfortunately, the sponsor's eye tracking hardware was unavailable during the assessment of the effect on leukocyte activity of interaction with a vehicle HMI using either visual, audible or a combination of the two modalities, while simultaneously maintaining lane discipline within a simulated driving environment (Chapter 7). As this was a potentially important area of investigation, a more basic method for assessing direction of focus was developed. The simulated driving environment contained a three lane test track, and volunteers were expected to remain within the centre lane. Utilising the premise that a driver will unintentionally steer in the direction that their attention is focussed upon, during periods where full attention is not placed upon the direction of travel, the direction and frequency of deviations from the central lane were used as a crude means of monitoring situational awareness. Although this was a basic method, it proved particularly effective. Existing methods for assessing direction of focus entail the use of hardware that requires complex calibration before use with each individual, and therefore requires a set-up time which far exceeded the time constraints of this study. The results from these more technologically advanced devices possess a greater objectivity compared with the method used within chapter 7, however, the results from chapter 7 (Table 7.4) show that as task complexity increased the amount of time when attention was shifted away from the road ahead also increased (suggesting situational awareness was decreased and mental workload was increased). Although not significantly correlated, the magnitude of change in leukocyte activity post-test followed the same trend as was observed for lane deviation frequency (the use of Interface A resulted in the greatest number of deviations (Table 7.4) and the most pronounced post-test decrease in leukocyte activity, *in vitro* (Table 7.1), whereas the use of Interface C provoked changes in both parameters that were significantly smaller in magnitude). These observations suggest a link between decreased situational awareness, increased mental workload and decreased leukocyte activity, *in vitro*. They also provided further

evidence in support of the use of leukocyte reactivity as a rapid, objective means of quantifying altered mental loading in response psychological and physical stressors.

Other techniques used to quantify changes in stress level involve assay of stress hormone concentration. As part of the study within chapter 5, the plasma concentration of adrenaline and cortisol, in addition to other mediators believed to be associated with the stress response and leukocyte recruitment and activation (discussed in chapters 1.3.2, 1.4 and 5.5) were measured using standard ELISA pre- and immediately post-stressor. The use of changes in plasma catecholamine and cortisol concentration as indicators of mental stress can, as observed for heart rate and BP, report inaccurate measurement. Factors, other than the presence of stress, can influence stress hormone concentration. For example, cortisol concentration is known to possess a diurnal rhythm (with the greatest concentration occurring just after waking, followed by a steady decrease in concentration throughout the day) (Petraglia *et al*. 1983). Many studies report the use of salivary cortisol as a useful indicator of mental stress (Hiramatsu *et al*. 1981, Kuga *et al*. 2002). Thorn *et al*. (2009) demonstrated how cortisol secretory activity during the initial 45 minutes following awakening, termed the Cortisol Awakening Response (CAR), can be used as an effective indication of an individual's psychological and health status. Such a system would prove effective in identifying the presence of chronic stress, however, may lack the ability to identify the physical effects of short-term stressor exposure. Hodgson *et al*. (2004) reported significant increases in cortisol concentration in care home patients who had undergone relocation to a new facility compared to residents who had not yet moved. However, its use can be problematic and may therefore be deemed unsuitable, due to the degree of variation in concentration exhibited diurnally and also between subjects. Hormone analysis may also be deemed unsuitable as many techniques require several hours before results are known. The distinct advantage in using LCC as opposed to stress hormone analysis is that results can be obtained within 15 to 20 minutes post-stressor, rather the after several hours, at a financial cost that is far less than is required for the procurement of ELISA test kits.

The plasma concentration of adrenaline was the only one of the nine mediators tested that showed a significant post-test increase (Table 5.5). It is generally accepted that adrenaline concentration is increased during periods of mental and physical stress

(Ohrui *et al*. 2008), and is involved in the up-regulation of physiological processes responsible for preparing the body for rapid increases in exertion (as described during the fight flight response) (Chapter 1.1.3). A study by Landmann (1992) showed that acute sympathetic activation by adrenaline infusion, short-term exercise, or psychological stress led to a selective increase in lymphocytes that were rich in β-adrenergic receptors. Leukocytes are also known to posses β2-adrenergic receptors, and therefore, as observed with lymphocytes, have the potential to react to changes in plasma adrenaline concentration, which could potentially result in direct systemic leukocyte activation (proposed pathway is described within Figure 8.1). Further evidence in support of this proposed mechanism was the fact that no significant changes in the plasma concentration of mediators known to be involved in the traditional progressive model for leukocyte activation including E-Selectin, L-Selectin and Tumour Necrosis Factor-α (Kunkel and Ley 1996, Simon *et al*. 1998, Chen and Springer 1999, Jung and Ley 1999, Simons and Green 2005), that are involved in the precursory events of capture, rolling, slow-rolling and firm adhesion (described in chapter 1.2.6) were recorded (Table 5.5), yet activation still occurred.

Griffis *et al*. (2007) reported increases in catecholamine concentration following periods of pain, that were correlated with significant increases in the numbers of leukocytes expressing CD11a (lymphocyte function-associated antigen-1 (LFA-1), which plays a central role in leukocyte intercellular adhesion through interactions with its ligands, and is expressed on all leukocytes (Lub *et al*. 1995). It follows that if acute pain can elicit adrenaline secretion, resulting in increased leukocyte recruitment and activation, then a similar response may result following increased mental stress via HPA and SAM pathways.

The literature describes how repeated exposure to the same stressor can result in habituation of the cardiovascular system (Veit *et al*. 1997, Bhatnagar *et al*. 2006, Barnum *et al*. 2007). Chapter 1.1.3 describes how the mechanism responsible for cardiovascular regulation relies, in part, on the HPA axis, which provides a neural and biochemical link between the emotional stimulus and physiological response. As the HPA axis has also been shown to regulate aspects of the immune system (Landmann 1992) it follows that the immune response (specifically leukocyte activity) may also demonstrate habituation. In animal models, repeated social defeat has been used as a

mental stressor and has been shown to elicit a decrease in the magnitude of the immune response with repeated stressor exposure (Beitia *et al*. 2005), as well as alter leukocyte trafficking and distribution patterns (Engler *et al*. 2004). In humans, chronic psychological stress has been shown to modify the immune response. Altemus *et al*. (2006) described how, following acute stress, cell mediated immune function is enhanced in individuals who have been diagnosed with post traumatic stress disorder (a condition that imposes chronic physiologic and mental stress on sufferers), and depressed in healthy individuals. However, no evidence directly testing the effect and magnitude of response of the innate immune system to repeated exposure by short-term psychological stressors, in otherwise healthy individuals could be identified within the published literature.

In chapter 6, subjects were required to complete basic driving related tasks (including selection a specific radio station and adjustment of climate controls) using 2 different touch screen interfaces, a new model and one of its antecedents from the same manufacturer. 4 of the original test group were randomly selected for weekly repeat testing, using both interfaces, on two further occasions. Initial use of Interface A (antecedent) resulted in a significant decrease in leukocyte activity, compared to pre-test values. Unlike Interface B (new version), where although activity decreased post-test, the magnitude of change was not significant (Chapter 6.4). Repeated use of both interfaces resulted in post-test decreases in leukocyte activity that progressively lessened in magnitude (Figure 6.4a and b, Table 6.4). This trend suggests that leukocyte activity does exhibit a habituated response following repeated exposure to the same short-term stressful situation. It also provides evidence suggesting a link between leukocyte activity and the autonomic nervous system, whereby, as familiarity to the stressor increased, the magnitude of the leukocyte response decreased, ultimately resulting in cessation of a quantifiable response. Habituation to the stressor can also be confirmed due to the fact that the magnitude of the post-test response observed for heart rate, blood pressure and also core body temperature decreased following repeated exposure.

A habituated response also imposes implications for the commercial testing of novel equipment (such as an unfamiliar HMI, used as the stressor within chapters 6 and 7). Greater emphasis must be placed, during the recruiting process, to ensure that subjects

have no prior experience or knowledge regarding the specification of the equipment under investigation (chapter 6.5). The findings of chapter 6 (repeated assessment of two different touch screen interfaces) suggest that exposure to short-term low-level stressors, as used within this research can result in a habituated response in leukocyte activity following initial exposure. Within this study leukocyte reactivity was below detection threshold by the third stressor exposure (Table 6.4).

8.2 A THEORETICAL MODEL FOR SHORT-TERM LOW-LEVEL PSYCHOLOGICAL STRESS-INDUCED LEUKOCYTE ACTIVATION

The final aim of this research was to construct a model that explored the possible mechanisms for leukocyte (neutrophil) activation following exposure to acute short-term low-level psychological stress. It has been reported that the causative factor of many disease states, involving the degradation and destruction of healthy tissue, may be attributed to the extra cellular release of toxins by leukocytes, primarily neutrophils (Malech and Gallin 1987). These findings were further elucidated by Weiss (1989), who demonstrated that the release of reactive oxygen species and proteolytic enzymes may be responsible for inducing tissue damage. In 1996, Kang *et al*. presented evidence which suggested that psychological stressors were capable of inducing ROS production, and that as psychological stressor intensity increased so too does leukocyte oxidative activity. A comparison of the extent of the post-test change in leukocyte activity observed following stressor exposure within chapter 3 (performing a basic manoeuvre in two production vehicles from different manufacturers) (Table 3.1) and chapter 7 (completion of basic automotive tasks using HMIs employing either visual, audible or a combination of both as the primary interface format, whist simultaneously maintaining lane discipline within a simulated driving environment) (Table 7.1), provides further evidence in support of the conclusions of Viswanathan *et al*. (2005) who suggested that the innate immune system is unable to differentiate between stressors that have the potential to inflict physical harm, from those that are a result of mental challenge (mounting near identical responses for each). It is

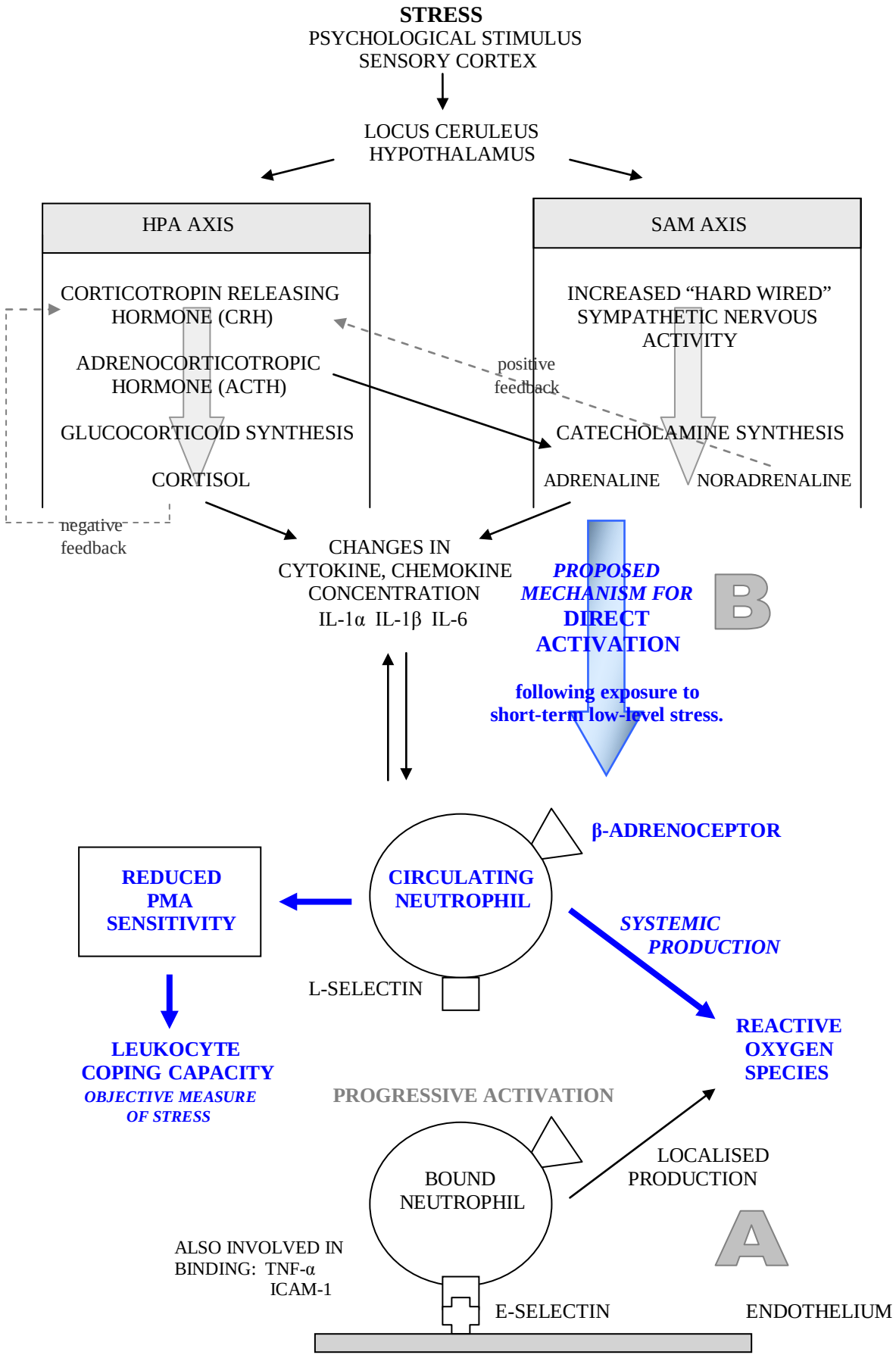
generally accepted that these reactions have evolved as part of the normal physiological response to stress, and must therefore provide some degree of initial protection, whereas prolonged exposure may result in damage (Dhabhar *et al*. 2003, Viswanathan *et al*. 2005, Altemus *et al*. 2006).

In contrast to the belief that exposure to psychological stress is immunosuppressive, studies conducted by Dhabhar and McEwen (1996, 1999) and Dhabhar *et al*. (1996, 2000) have shown that exposure to acute or naturalistic stressors has novel adjuvant-like immune enhancing effects, while chronic stress is immunosuppressive. Viswanathan *et al*. (2005) postulated that this mechanism of short-term enhancement would be beneficial when directed in response to potential wounding and infection in order to facilitate repair (during the fight flight response). However, it would be detrimental if directed against innocuous or self-antigens (e.g. during hypersensitivity or autoimmune diseases). Stress-induced enhancement of immune function (particularly when associated with skin immunity) makes sense when viewed from an evolutionary perspective. An acute stress response is an evolutionarily, adaptive, psycho-physiological survival mechanism (Dhabhar *et al*. 2000). One primary function of the brain is to perceive stressors, warn of danger and promote survival. Stress-responsive neurotransmitters and hormones are the brain's signals to the body. Since stressful natural encounters often result in wounding and infection, it is unlikely that evolution would select for a system exquisitely designed to avoid predation only to render the individual susceptible to the deleterious effects of bacterial infection. It has also been hypothesized that the adaptive value of stress-induced decreased immune-competency is that it may decrease the development of autoimmunity following tissue damage.

It has been shown that stress hormones, such as adrenaline and cortisol serve to reduce immune activity (Altemus *et al*. 2006). Yet it has also been shown that as a consequence of the stress response, innate immune activity is enhanced. It is this paradoxical relationship that has made elucidating the mechanisms of activation particularly complex. One possible explanation, which has formed the basis for the proposed model (Figure 8.1), is that the immune system exhibits differing catecholamine and glucocorticoid sensitivities, which are dependent upon the phase (early versus late) of the immune response, when stressor exposure occurs. At the

beginning of an immune response (following exposure to acute stressors), factors such as leukocyte trafficking, proliferation, cytokine and chemokine function, may become receptive to stress hormone-mediated immune-enhancement, rather than exhibiting the accepted trend of becoming suppressed. This proposed mechanism was developed as a consequence of a similar observation that showed, following antigen exposure the immune response was enhanced or suppressed, dependent upon which stage the immune response was exhibiting when exposure occurred (Viswanathan *et al*. 2005). Such a relationship would account for the observations noted during this thesis, where exposure to acute psychological stress promoted leukocyte activation and ROS release *in vivo*, which resulted in a decreased ability to respond when exposed to PMA challenge *in vitro*.

Figure 8.1 **Proposed model for short-term low-level psychological stress-induced leukocyte activation.**



LCC results from all studies demonstrated that systemic leukocyte activation had occurred in response to short-term low-level mental stressors (lasting between 30 seconds and 20 minutes). This observation and the fact that following exposure to the stressor within chapter 5 (identification of potential hazards within 6 randomly selected pre-recorded driving scenarios) resulted in only a significant increase in the plasma concentration of adrenaline (measured using standard ELISA), and not in the plasma concentration of mediators known to be associated with the progressive model of leukocyte activation (see chapter 1.2.6) including L-selectin, E-selectin, Tumour Necrosis Factor-α, Interleukin-1β, Interleukin-6, Endothelin-1 or Noradrenaline (Table 5.5). It is suggested that exposure to short-term low-level mental stressors (lasting minutes) promotes a systemic, *in vivo*, increase in leukocyte activation and ROS release (characterised by a decreased ability to produce ROS in response to *in vitro* PMA challenge) that is regulated by a mechanism other than the accepted progressive model (Jung *et al*. 1998) (mechanism for progressive model shown as A on Figure 8.1), where adrenaline released via HPA and SAM activity leads to cytokine up-regulation. Similar findings have been shown, by others, to occur during acute, transient (1-2h) administration of catecholamine and glucocorticoid hormones. Both served to enhance delayed-type hypersensitivity reactions (Dhabhar and McEwen 1999) and pro-inflammatory cytokine release (Ackerman *et al*. 1998 Ostrowski *et al*. 1999, Altemus *et al*. 2001).

Landmann (1992) demonstrated the presence of β-adrenoceptors on leukocytes, this evidence, combined with the findings from chapter 5, which show that of the nine mediators tested, the plasma concentration of adrenaline was the only one that showed a significant post-stressor change in plasma concentration (increase of 0.18 ± 0.03 ng/ml), it is proposed that following short-term low-level mental stress, adrenaline acts directly upon leukocytes (primarily neutrophils) in a similar manner as seen with the cardiovascular system (Vlcek *et al*. 2008), utilising β-adrenergic stimulation to promote leukocyte activity (proposed mechanism for direct systemic leukocyte activation shown as B on Figure 8.1 – Highlighted in blue). If this novel pathway does indeed exist, then it could provide an explanation as to how a transient mental stressor can result in systemic leukocyte activation. The existence of such a pathway, and its ability to become activated following exposure to a mental stressor lasting only 30 seconds (as observed within chapter 4), would suggest that leukocyte

activation, in response to short-term low-level stress occurs via the sympathetic pathway.

8.3 LIMITATIONS

Limitations associated with the LCC technique are discussed in Chapter 3.4.

This research has demonstrated the potential benefits of using the Leukocyte Coping Capacity technique as an objective means for detecting and quantifying short-term, low-level psychological and physical stress. However, the process by which the LCC protocol attempts to quantify altered stress levels, utilising the ability of leukocytes to produce ROS, is novel. In order to establish the diagnostic efficacy of the LCC protocol, the technique must undergo comprehensive experimental evaluation with the aim of further elucidating the mechanisms required for leukocyte activation and the time frame in which these pathways operate.

The protocol utilised throughout this research was based on existing studies, which primarily focused upon animal testing (McLaren *et al*. 2003, Montes *et al*. 2003, 2004). Ellard (2003) was the first to investigate the feasibility of using LCC to assess psychological stress in humans, however, the duration and intensity of the stressor utilised in his research, quantifying the difference in the magnitude of stress exhibited following elective or acute coronary bypass surgery, was considerably greater compared to the stressors described in this thesis. Despite the fact that the technique appeared to be effective for use with human models, the relative lack of optimisation data means that the potential sensitivity and diagnostic ability of the technique is still not known. If time had permitted the following factors associated with protocol design would have been investigated.

Throughout this research whole blood samples were harvested immediately post-stressor, using the premise that the optimal level of psychological stimulation had occurred at this point therefore providing the greatest potential for leukocyte

activation. Although it is reasonable to hypothesize that leukocyte activity does indeed follow this trend, further experimental evaluation of the reaction kinetics is required in order to establish the exact point at which maximum activation occurs.

It is accepted that many traditional physical response parameters used to assess psychological stress, such as changes in heart rate (Gelfand *et al*. 2004) and changes in stress hormone concentrations, for example cortisol (Clow *et al*. 2006) vary in magnitude between subjects and between genders, differences which can be attributed to differing physiological resource availability and emotional coping strategy (Burns and Katkin 1993, Hughes 2007) (Chapter 1.1.1 and Chapter 4.5). As leukocyte reactivity follows a similar heterogeneous trend, due to comparable physical and psychological constraints, it can be inferred that the time required to reach maximum activity would also vary between individuals. While it is reasonable to assume that leukocyte activity does indeed follow this trend, experimental verification is still required. If this research was to be repeated with the aim of producing greater sampling consistency, instead of collecting samples immediately post-stressor, where the time point at which sampling occurred differed for each individual, according to how long it took to complete the required stressor paradigm, a more standardised sampling protocol would be followed, with post-test samples being collected at a set time point following initial stressor exposure.

It is also necessary to ascertain whether monitoring leukocyte activity in the luminometer at 5 minute intervals, providing only a 'snap-shot' of the overall reaction kinetics, is sufficient to establish the complete physiological profile associated with a given psychological stressor, or if a more detailed activity profile (e.g. measuring activity every minute) is required.

In addition to investigating the influence of psycho-physiological variation on stress-induced leukocyte activation, the actual methodology associated with the LCC technique must also be tested to ensure an optimised design. As previously mentioned, only a small proportion of the published LCC research, from which the protocol for the research described in this thesis was based, had been conducted using human models. In order to ensure that the technique is optimised to assess human leukocyte function, experiments must be conducted to firstly, ensure that each of the

reagents used (including luminol, heparin, DMSO and PBS) is of a sufficient concentration to ensure that it does not become rate limiting. Secondly, to verify that none of the reagents used influence the course of the reaction. The only factor which should act to limit the rate of the reaction is the leukocyte's capacity for activation. During the LCC protocol, leukocytes were stimulated using 10^{-5}M PMA. To confirm whether this is the optimal concentration to elicit maximum leukocyte activation and also to confirm that the PMA concentration utilised during this research was in excess and therefore not rate limiting, or even cytotoxic, the magnitude of response of a known number of leukocytes should be assessed following exposure to a range of PMA concentrations.

In order to achieve these objectives it would be necessary to utilise a laboratory based stressor paradigm, such as the Trier Social Stress Test (TSST) (Kirschbaum *et al*. 1993), which is capable of providing an effective means of evoking moderate psychological stress, and which also incorporates an effective non-stress control condition (Het *et al*. 2009). The TSST consists of a 10 minute anticipation period immediately followed by a 10 minute test period, where subjects must deliver a free speech and perform mental arithmetic tests in front of an audience. In addition to eliciting significant increases in heart rate, the TSST has been shown to evoke extensive changes in the concentration of cortisol, ACTH and prolactin (Kirschbaum *et al*. 1993), all of which indicate the presence of elevated mental stress, and demonstrate its suitability for use during the LCC validation and evaluation process.

To date, LCC analysis has been used to assess the impact on leukocyte activity to a wide range of mental stressors, including short-term confinement stress in badgers (McLaren *et al*. 2003, Montes *et al*. 2003, 2004), stress associated with impending cardio bypass surgery (Ellard 2003) and now the ergonomic design of unfamiliar in-car technology. Although each provides an indication as to the potential effectiveness of the LCC technique as an objective means of quantifying altered mental stress, until the protocol has undergone validation the direct relationship between changes in psychological stress level and leukocyte activity cannot be confirmed.

As discussed earlier in this chapter, one of the main limitations of this research was associated with the heterogeneous response of the test population to each of the

stressors. The point at which an individual perceives a novel experience as being stressful is dependent upon the availability of resources (both mental and physical) and coping strategies each employs, both of which are unique to each individual (discussed within chapter 1.1). In order to try and limit the effects of inter-individual variability, so as to allow direct comparison of leukocyte activity between individuals, each subject's pre- post-stressor differences were calculated. This process allowed each subject's pre-stressor baseline activity to act as a control. By subtracting baseline activity from post-stressor values, the difference should be generic, and therefore allow direct comparisons between individuals. Ideally, paired data and a cross-over design would have been a better course of action, as it would have allowed comparisons to be made between individuals as well as between treatment groups. This method was used within chapter 6 (comparison of two touch screen interfaces) and also chapter 7 (comparison of the use of three different interface designs to perform basic driving related tasks, whilst simultaneously maintaining lane discipline within a simulated driving environment), however its use was not always possible due, mainly to the fact that all required test equipment was not always available at the same time. All test vehicles were owned by the sponsor and were involved within their own research. Demand was such that it proved logistically impossible to secure more than one vehicle at any give time. This resulted in the inability to design and conduct a cross-over study. In the case of the pilot study (Chapter 3) which involved two test vehicles, each was made available for a two week period with an interval of two months between. Ideally both vehicles would have been made available throughout, allowing subjects to undergo testing on each, with test order being counterbalanced. This would have allowed comparisons to be made between not only treatment groups, but also between the responses measured for each individual. Instead, each volunteer was tested using only one of the two vehicles, again treatment group allocation was randomly decided. This method ensured that each volunteer was exposed to the test protocol and novel testing environment (motor manufacturer design and development facility) on only a single occasion. Although this eliminated the possibility of protocol familiarity and subsequent inhibition of the immune response, it did mean that the experience of a novel situation, such as being allowed access into a research facility not normally accessible to the public, may have led to a degree of anticipation and excitement or apprehension which may have influenced post-test results. In an ideal situation in order to reduce the possibility of this

occurring, instead of taking pre-test samples back at the university laboratory, they would have been taken once the subject had entered the sponsor's research facility and had been allowed time to calm down. However, this was not possible due to the limited time that each volunteer, who did not possess complete site access, was allowed to stay at the test site. If pre-test samples were also taken and measured at the research facility, then each subject would have had to spend an additional 45-60 minutes on site (on top of the 75-90 minutes already required for testing and analysis of post-stressor samples).

Although cross-over studies are usually preferred in science, the transient, low-level nature of the stressors utilised in this research meant that subjects became rapidly habituated to the novel situations, in the case of the stressor paradigm explored in chapter 6 habituation occurred in the majority of subjects after secondary stressor exposure, therefore, in these instances utilising a cross-over study design may not be possible or appropriate.

Within each study it was virtually impossible to eliminate all external stimuli that subjects may have perceived as being stressful (even the sampling protocol, which utilised a finger lancing device that was unfamiliar to many of those tested, could have itself elicited a stress response). This was particularly true for the studies described within chapters 3 and 7, where testing occurred beyond the confines of the laboratory, within a completely novel environment (the sponsor's research and testing facility, Whitley, Coventry).

The original intention of chapter 5, which involved identification of potentially hazardous situations (such as approaching a cyclist wearing dark clothing at dusk) in 6 randomly selected driving scenarios (from a pool of 100) as the stressor, was to attempt to further elucidate the biological pathways that facilitate low-level psychological stress-induced leukocyte activation. In an attempt to explore whether the pathways utilised during the acute inflammatory response are also employed during psychological stress-induced leukocyte activation, standard ELISA was used to assess the changes in plasma concentration of nine specific mediators (known to be involved in leukocyte recruitment and activation during initiation of the acute inflammatory stress response) following exposure to an acute mental stressor of a

similar magnitude to those discussed within the literature, such as mental arithmetic (Uchino *et al*. 1995) and speech tasks (Marsland *et al*. 2002). On this occasion the LCC protocol was used simply to confirm that the selected stressor had resulted in a physiological response, in order to ensure that the subsequent ELISA analysis, that is both expensive and time consuming had the best chance of yielding useful results.

Upon reflection the study design placed too great an emphasis upon whether venous sampling acted as a secondary stressor, devoting half of an already limited pool of volunteers, who were willing to submit to venous sampling to investigate whether this was in fact the case. No significant difference in the magnitude of the post-test leukocyte response was observed between treatment groups (Table 5.1) which suggests that venous sampling did not significantly affect overall results. Even if the reverse were true, and that venous sampling had served to amplify the leukocyte response, this would have been beneficial in that it would have led to an increased chance of identifying any significant post-test changes in mediator concentration. The anticipatory response associated with the stressor, would have also, in itself, served to evoke some degree of mental stress.

In order to look at the effect of anticipation a cross over design could have been adopted, with volunteers being assigned to one of three treatment groups. Group one would be told that there were no hazards and would experience hazard free scenarios (control group), group two would be told to expect hazards, but would again observe hazard free scenarios. Group 3 would be told to expect hazards and observe scenarios containing hazards. All groups would have undergone venous testing. If anticipation had acted as a potent secondary stressor, then the magnitude of leukocyte response exhibited by subjects within group 2 would have been significantly lower compared to group 1 (control). If anticipation was not acting as a stressor then no significant difference in the magnitude of leukocyte activity would be shown between groups 1 and 2.

Within chapter 5 (identification of developing roadway hazards) the intention was to subject volunteers to a laboratory based mental stressor of a similar magnitude to that used within chapter 3 (which involved performing a driving manoeuvre in an unfamiliar vehicle), in order to evaluate changes in bio mediator concentration. The

ideal scenario would have been to use a production motor vehicle as the stressor (as in chapter 3). However, the health and safety requirements of the sponsor dictated that the cabin of such a vehicle, even when stationary, was an unsuitable environment for venous blood collection. Additionally and most importantly, in order to limit loss of mediator bio reactivity, effective and efficient sample preparation and storage could only be achieved within a laboratory environment.

The second aim of chapter 5 was to compare the LCC technique with existing subjective quantitative methods for evaluating mental stress. The most basic technique involved the use of perceived stress ratings using likert scales (using a continuous scale where 1 represented relaxed and 10 stressed). This technique was applied to all studies except chapter 5. In general, post-test changes in perceived stress rating followed a similar trend as was observed for leukocyte activity, where, as perceived stress increased post-stressor, the ability of leukocytes to mount an effective response following PMA-challenge decreased (decreased leukocyte activity *in vitro*, signifies increased mental stress). In chapter 5, a more complex measure of mental loading was tested. The NASA task load index (NASA-tlx) was selected on the basis that it was used by the sponsor during ergonomic evaluation. The results showed that subjects rated mental demand and effort as the most important of the six criteria for successful completion of the task (Table 5.2). Despite the calculated values for total mental workload being significantly different between treatment groups, a direct comparison between total mental workload and post-stressor changes in leukocyte activity was not possible. This was due to the fact that the NASA-tlx test can only be conducted post-stressor, therefore comparisons between pre- and post-stressor are not possible, only comparisons between multiple stressors. This method could have been used within chapter 7, which involved assessment of multiple stressors (3 different interface designs, each targeting a specific sense to facilitate interaction: visual, audio or a combination), in this instance the design format was similar to that utilised by the sponsor during evaluation of specific in-car systems.

Ideally dynamic assessment was to follow static laboratory testing, but was deemed impractical in terms of finance and the time required for subject training. An alternative route was proposed involving the use of production vehicles that were interfaced with computer-based driving simulation technology. This combination of

technologies, used within chapter 7, provided near complete immersion of the driver within a simulated environment, which permitted the inclusion of the dynamic component of maintaining safe road positioning whist simultaneously interacting with the vehicles human machine interface (HMI) system. Within the literature, driving simulation technology has been shown to elicit both psychological and physiological changes. Studies by Yamaguchi *et al*. (2006) and Yamaguchi and Sakakina (2007) demonstrate how salivary amylase activity (considered to be an indicator of sympathetic nervous activity) is affected following the acute psychological effects of driving within a simulated environment. Young and Stanton (2005) used driving simulation technology to assess the psychological impact (represented via altered mental workload) resulting from the use of an adaptive cruise control system. These studies and others (Matthews *et al*. 1998, Lui 2001, Sung *et al*. 2005, De Valck *et al*. 2007) demonstrate that the magnitude of mental response and resultant modification and adaptation of physiological mechanisms that result from interaction with driving simulation technology does not differ significantly, compared to real life driving scenarios, such observations validate the use of driving simulators, as an effective means for provoking a physiological response.

8.4 FUTURE WORK

The research carried out here has opened many doors for future work. Firstly, this study involved testing individuals who did not possess any form of mental health problem. There is a need to identify whether leukocytes respond to acute stressors in a similar manner when the individual has been diagnosed as suffering from chronic stress. It has been reported that individuals with post traumatic stress disorder (PTSD) have disturbance of the major stress response systems, the HPA axis and the sympathoadrenomedullary system (Altemus *et al*. 2006). The results from chapter 5 (ELISA analysis of post-stressor changes in the plasma concentration of 9 mediators believed to be involved in leukocyte regulation) support the findings of others (Viswanathan *et al*. 2005), in suggesting that neutrophil activation occurs via a relationship between glucocorticoid and catecholamine secretion and subsequent

cytokine and chemokine activation and secretion. Chronically stressed individuals exhibit significantly reduced baseline cortisol (urinary and plasma) concentration, which could predispose such individuals to enhanced immune activation, since chronic elevations in glucocorticoids globally suppresses immune system reactivity (McEwen *et al*. 1997). In this instance, exposure to even mild acute mental stressors (as used within this research) could result in a significantly greater leukocyte response. This could, if the individual had failed to disclose their diagnosis of chronic stress, result in an artificially high mental workload value which far exceeded the true rating. If the existence of such a relationship was proved, a series of calibration studies should be conducted, to ensure that accurate interpretation of leukocyte activity can be made in the presence and absence of chronic stress.

Bio mediator analysis (Chapter 5) relied upon comparing venous blood samples taken 45 minutes pre- and immediately post-stressor. Despite harvesting venous blood straight after the stressor, the procedure of single venepuncture meant that by the time blood had actually been harvested at least one minute had passed. In some instances (e.g. catecholamines) this delay may have provided sufficient time to allow altered mediator concentration to return to pre-stressor values. If this study was to be repeated an indwelling catheter would be utilised, permitting immediate blood collection following cessation of the stressor, and also allowing samples to be taken at regular intervals during stressor exposure. Thus, allowing the sequence of mediator stimulation to be investigated.

The proposed model for short duration low-level stress-induced leukocyte activation (pathway B on Figure 8.1) suggests that neutrophils possess the ability to become activated through direct β-adrenergic stimulation, a similar trend was observed during lymphocyte activation by Landmann (1992). In order to test this mechanism, non-stimulated neutrophils could be exposed, *in vitro*, to a range of adrenaline concentrations, established using the pre-, post-stressor concentrations measured using standard ELISA within chapter 5 (range between 0.34 ± 0.02 ng/ml and 0.52 ± 0.02 ng/ml). This method would identify whether leukocyte activation occurred in response to adrenaline stimulation, and also at what concentration range the response is elicited. The technique could also be applied to test the influence of other mediators (such as those used in chapter 5) on leukocyte activity, *in vitro*. This

methodology would allow further elucidation of the mediators and biochemical pathways responsible for stress-induced leukocyte activation. A comparison of leukocytes isolated from both healthy non-stressed and chronically stressed individuals could be used to explore whether the mechanism differs in the present of chronic stress.

Chapters 3 and 7 both used interaction with production motor vehicles as the stressor. By comparing post-stressor changes in leukocyte activity (Tables 3.1 and 7.1) it is suggested that the immune response is unable to discriminate between stressors capable of inflicting physical harm (the stressor within chapter 3 involved performing a basic manoeuvre within an unfamiliar vehicle) from those which serve to increase mental demand (pose no physical threat) (the stressor within chapter 7 involved maintaining lane discipline within a computer simulated driving environment whilst simultaneously interacting with different HMI formats). Despite the subject knowing they could not be physically harmed during the simulator study (chapter 7) the data shows (Table 7.1) that the post-test decrease in leukocyte activity was greater in magnitude compared to the decrease observed following stressor exposure within chapter 3 (Table 3.1) even though, in this instance, there was the possibility that the stressor could inflict physical damage (subject could crash the car). These observations suggest that innate immune activity is, at least partly regulated via sympathetic stimulation. It therefore follows that the coping strategy employed by each gender in the interpretation and response to a non-threatening stressful situation may be different (Burns and Katkin 1993, Hughes 2007). If so, one gender may be more susceptible to such situations and may demonstrate an increased incidence of immune related disorders. In order to investigate this, the effect on leukocyte activity of performing a series of basic interface related tasks (similar to those used within chapters 6 and 7) could be assessed whist the test vehicle is stationary, and also while driving within traffic. Test subjects would be grouped according to gender, whether they were novice or experienced drivers and also whether they possessed prior experience of either the test vehicle or any of the systems contained within.

In conclusion, this research has given some interesting and novel findings that warrant further investigation in a number of different directions. Firstly, to determine exactly how much influence the autonomic nervous system has on the innate immune

response. Secondly, to explore the full potential of this relationship as a means of providing direct, objective, quantitative assessment of altered mental loading. The final area of investigation would be to explore the physiological consequences of repeated exposure to short-term low-level mental stress, and its association with health and disease.

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APPENDIX 1

Standard Medical Health Questionnaire

Overview and Explanation.

Please read the notes below carefully before completing this Medical Form. Unless you are willing to provide a full medical history we cannot allow you to participate in this study.

This form will help to ensure that you are both medically suitable and physically capable of undertaking the required tasks as part of this study. Therefore it is essential you provide accurate, comprehensive and truthful information. You must fully disclose your medical history. We the examiners cannot accept any responsibility whatsoever in the event you do not fully disclose all relevant details. Please note, if you develop any new medical conditions, or experience worsening of existing conditions during subsequent days upon completion of the task, you must inform the examiner.

Full Name……...Age……...……

Height (in metres)…… Weight (in Kg)……...

- Do you Smoke? [ ] Yes [ ] No

  If yes, how many (per day) [ ] < 5
  [ ] 5-10
  [ ] > 10

- Do you regularly drink Alcohol? [ ] Yes [ ] No

  If yes, have you consumed any alcohol in the last [ ] 24 hours
  [ ] 48 hour

- Have you had a Cold in the past 2 Weeks? [ ] Yes [ ] No

- How often do you Exercise (*exercise being anything which makes your heart beat faster than your resting rate for 20 minutes or more*)?

  EVERY DAY. [ ]
  2-3 TIMES A WEEK. [ ]
  ONCE A WEEK. [ ]
  NEVER. [ ]

- What sort of exercise do you do? (*Gym / Sports / Brisk Walk etc*)……

- What activity were you doing before you came to this lab to be tested (*within the last hour*)?.....

- Have you taken any form of medication within the last 48 hours? (Tablet / Lotion / or Potion) [ ] Yes

  [ ] No

  If yes please provide details of what you have taken……...

- Do you take any form of Food Supplement? (e.g. vitamins) [ ] Yes

  [ ] No

  If yes please provide details of what you have taken……...

- Do you take any form of Herbal Medication? [ ] Yes

  [ ] No

  If yes, please name them and state what they are treating.

  Name of Medication…….

  For the Treatment of…….

- Do you use any form of Complementary Medicine (e.g. acupuncture, hypnotism)?

  [ ] Yes

  [ ] No Name of Treatment……...

Do you have a history of any of the following conditions? If so please give details indicating frequency, severity and aggravating factors (what can trigger an occurrence) where necessary, and any treatment you are taking.

1	Raised Blood Pressure	Yes / No	
2	Heart or Circulatory Disease	Yes / No	
3	Rheumatic Fever	Yes / No	
4	Deep Vein Thrombosis (DVT) (if so please give dates & details of treatment)	Yes / No	
5	Asthma Hey Fever	Yes / No Yes / No	
6	Epilepsy	Yes / No	
7	Diabetes	Yes / No	
8	Digestive or Bowl Disorders	Yes / No	
9	Past Injuries (e.g. fractures)	Yes / No	
10	Haematological or Blood Disorders	Yes / No	
11	Cerebral Disease (Stroke, head injuries etc.)	Yes / No	
12	Metabolic or Endocrinological Disorders	Yes / No	
13	Surgical Operations	Yes / No	

14	History of Mental Health Problems	Yes / No	
15	Allergies (Dietary / Drug / Environmental)	Yes / No	
16	Any Hospital Treatment in the Last 3 Years	Yes / No	

If you have any other medical condition not disclosed above, please give details here:

……..
…….
..……

I certify that I have read and understand this medical form, and the attached notes and explanation, and the information I have given is correct.

Signed……. Date…….

APPENDIX 2

NASA Task Load Index (NASA-TLX) Questionnaire

Subject Name. _____

Date. _____

PART 1.

Place a mark on each scale at the point which best indicates your experience of the task.

(For Example: if you found the task mentally demanding you would place a mark at a point greater than 10).

MENTAL DEMAND

1 10 20

LOW HIGH

PHYSICAL DEMAND

1 10 20

LOW HIGH

TEMPORAL DEMAND ***(do you feel as though the time required to complete the task was insufficient (1), about right (10) or excessive (20)?)***

1 10 20

LOW HIGH

PERFORMANCE

1 10 20

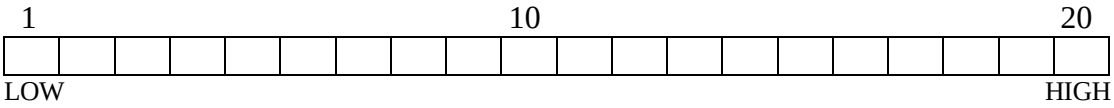
GOOD POOR

EFFORT

1 10 20

LOW HIGH

FRUSTRATION



PART 2.

For each of the following decide which contributor was the most important for you to successfully complete the task.

(Place a tick in the corresponding box)

1	PERFORMANCE		OR	MENTAL DEMAND	
2	EFFORT		OR	PERFORMANCE	
3	PERFORMANCE		OR	TEMPORAL DEMAND	
4	FRUSTRATION		OR	EFFORT	
5	EFFORT		OR	PHYSICAL DEMAND	
6	PERFORMANCE		OR	FRUSTRATION	
7	MENTAL DEMAND		OR	EFFORT	
8	PHYSICAL DEMAND		OR	FRUSTRATION	
9	PHYSICAL DEMAND		OR	PERFORMANCE	
10	TEMPORAL DEMAND		OR	FRUSTRATION	
11	TEMPORAL DEMAND		OR	EFFORT	
12	PHYSICAL DEMAND		OR	TEMPORAL DEMAND	
13	FRUSTRATION		OR	MENTAL DEMAND	
14	MENTAL DEMAND		OR	PHYSICAL DEMAND	
15	TEMPORAL DEMAND		OR	MENTAL DEMAND	

APPENDIX 3

Technical Ability Questionnaire

All the information you provide will be treated in the strictest confidence.

Subject Name……...

Date……..

Date of Birth…….

- Do you prefer [ ] Pen

  [ ] Keyboard

- Do you use a calculator to perform simple calculations or do you do them in your head?

  [ ] Calculator

  [ ] Head

- Do you know how to program your video recorder?

  [ ] Yes

  [ ] No

- Are you confident in using a PC?

  [ ] Yes

  [ ] No

- Do you have internet access at home?

  [ ] Yes Is it [ ] Dial-up

  [ ] Broadband

  [ ] No

- Do you use the internet for online banking or shopping?

  [ ] Yes

  [ ] No

- Do you have to have the latest gadgets?

  [ ] Yes

  [ ] No

- Do you own an MP3 Player?

  [ ] Yes

  [ ] No

- *In your own opinion would you class yourself as being computer literate?*

  [ ] Yes

  [ ] No.

APPENDIX 4

Pre-Test Instruction Leaflet

JAGUAR VOICE CONTROL

Many of the Jaguars operations can be controlled via its voice activation system.

To enter a voice command you must first press the  button located on the steering wheel in order to activate the system. The LCD display on the dashboard now shows that the system is "**LISTENING**".

You must speak the required command clearly in a normal speaking voice.

The system responds to commands using the following sequence….

DEVICE ⟹ FUNCTION ⟹ SETTING

For example, to turn the radio on and select 100.7FM Heart FM the command would be….

RADIO ON ⟹ RADIO TUNE 100.7 FM

You may also require the following commands….

RADIO HELP
RADIO OFF
RADIO TUNE FM

The Climate Control can also be adjusted using voice control. As with the radio the climate control system responds to commands given in the standard Device / Function / Setting sequence.

In order to successfully complete the task you will need to know the following commands…

CLIMATE CONTROL ON / OFF
CLIMATE CONTROL AUTOMATIC
CLIMATE CONTROL TEMPERATURE (17 - 31°C)
CLIMATE CONTROL TEMPERATURE HIGH / LOW

For example, to turn the climate control on and set the cabin temperature to 22°C, the command would be….

Press  button on the steering wheel followed by "**CLIMATE CONTROL ON**"

Press  button then say "**CLIMATE CONTROL TEMPERATURE 22°C**"

The system sounds complex on paper however it is really quite easy to use when you are behind the wheel. If you are completely confused you can also ask the system for assistance by simply saying "**HELP**".

Some of the required tasks may not fall under voice control however during the test you will be required to try to complete that task using voice control first before attempting to use manual controls.

JAGUAR TOUCH SCREEN

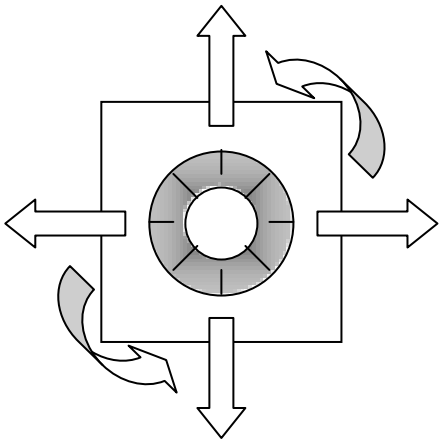
The touch screen fitted in the S-Type R is the same as the one used during the last study. Many of the required tasks can be completed by simply pressing the screen itself and logically working through the menu system. Some of the more frequently used functions also have a dedicated control on the dash board. Either method can be used to successfully complete the task.

For example, to turn the radio on and select 100.7 Heart FM

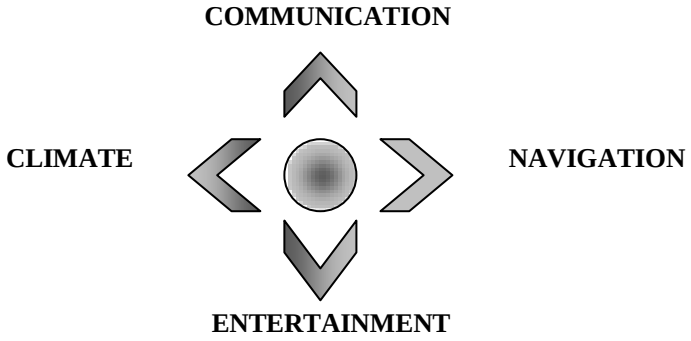
Press AUDIO ⇨ FM ⇨ 100.7 Heart FM

BMW iDRIVE

This system is controlled via a large silver control dial located in the centre console between the two front seats. The control works in a similar manner as a computers mouse. You are able to move the controller backwards and forwards, as well as side to side in order to select the required command on the LCD display.
The controller also spins allowing you to select the required function from the sub-menus. (It will however only spin if such menus exist in order to complete the required task).



Many of the required tasks are accomplished via the iDrive control and are accessed via the **Home Menu Screen**.



For example, to turn the radio on and select 100.7 Heart FM

- From the **Home Menu Screen**
- Using the iDrive Dial – **PULL BACKWARDS** to enter the **Entertainment Menu**
- **Turn** the Dial to highlight the band width (e.g. FM) and select by **PRESSING** the dial
- **Turn** the Dial to highlight the station (e.g. 100.7 Heart FM) and select by **PRESSING** the dial.

Don't worry if all of this sounds extremely confusing, I am sure that once you have each interface in front of you things will start to make a bit more sense. The whole idea of this study is to see how people cope with performing what are usually basic tasks whilst driving. In order to successfully pass the test you do not necessarily need to be able to complete every task, it is more your ability to keep the vehicle on the simulated road whilst try to complete the tasks that is more important.