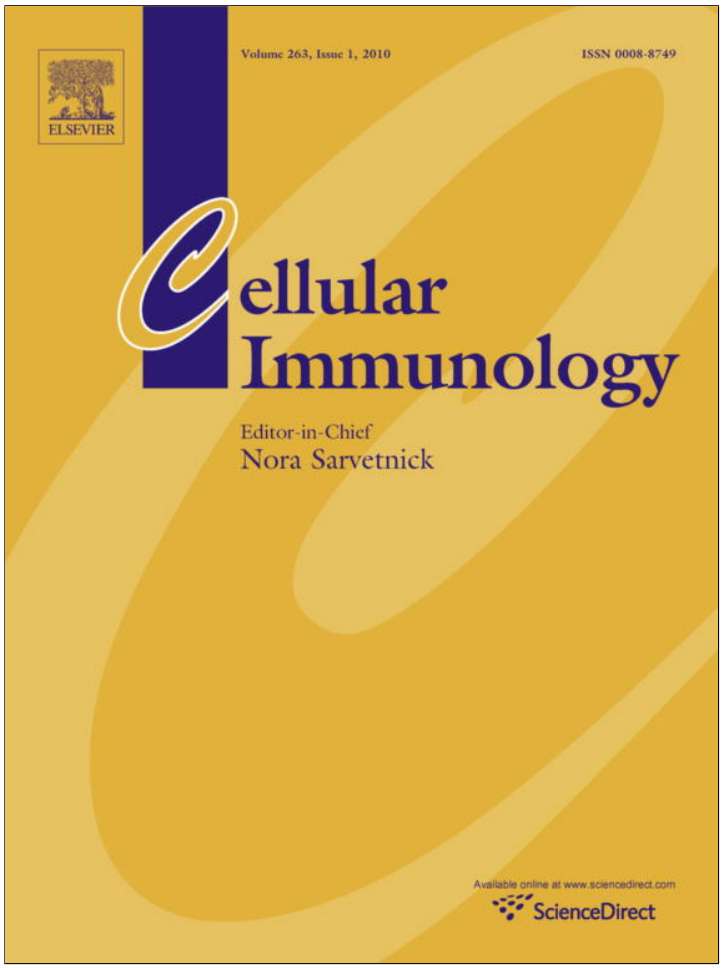

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Leukocyte reactivity as an objective means of quantifying mental loading during ergonomic evaluation

G.K. Shelton-Rayner [a], D.W. Macdonald [b,*], S. Chandler [c], D. Robertson [c], R. Mian [d]

[a] Department of Biomolecular and Sport Sciences, Faculty of Health and Life Sciences, Coventry University, Cox St, Coventry CV1 5FB, UK
[b] Wildlife Conservation Research Unit, Zoology Department, University of Oxford, The Recanati-Kaplan Centre, Tubney House, Abingdon Road, Tubney, Oxfordshire OX13 5QL, UK
[c] Jaguar and Land Rover Research Department, Jaguar Cars Limited, Engineering Centre, Whitley, Coventry CV3 4LF, UK
[d] Oxford MediStress Ltd., Suite 125, 27 Colemore Row, Birmingham B3 2EW, UK

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ABSTRACT

Psychological stress evokes rapid changes to the cardiovascular and neuroendocrine systems, responses that can become habituated following repeated exposure. This study, comprising of two phases, suggests that the immune system follows a similar trend. Phase 1: 15 healthy subjects (aged between 26 and 56 years) provided capillary blood samples before and after completing three basic tasks using, in turn, two automotive touch screen interfaces (Interface 1—antecedent version, Interface 2—improved version). Using a chemiluminescent technique termed leukocyte coping capacity (LCC), the ability of leukocytes to produce reactive oxygen species *in vitro* was assessed. Significant differences in leukocyte activity were shown between treatment groups, where the greatest post-test decrease occurred after using Interface 1. Phase 2: a randomly selected sub-group ($n = 4$) underwent weekly repeat testing using both interfaces. Significant differences in post-test leukocyte reactivity were exhibited between test weeks for each interface—the magnitude of response decreasing with successive exposure.

1. Introduction

Quantification of psychological stress resulting from environmental challenges or social interactions is generally accomplished by one of two approaches. First, evaluation of perceived mental workload using subjective self assessment (including the NASA task load index inventory [1] and subjective workload assessment techniques (SWAT) [2]). Second, monitoring characteristics of the cardiopulmonary system and assay of specific stress hormones including salivary cortisol [3–6] and catecholamines [7]. Brown et al. [8,9] demonstrated how urinary catecholamine excretion can quantitatively indicate the effects of long-term psychological stressors—characterised by the taxonomic classification proposed by Elliot and Eisdorfer [10].

Assessing 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 [11,12] in addition to heart rate, blood pressure and body temperature [5–8]. All are subject to considerable biological variation, introducing uncertainty to comparison between individuals and populations.

The stress response is a complex combination of metabolic, neuroendocrine and behavioural changes. Psychological stress reduces the effectiveness of the immune system, thus leading to an increased risk of infection or disease [13,14]. Even short-term psychological stressors such as academic examinations [15,16] can produce demonstrable physiological changes in the reactivity of specific classes of leukocyte, notably neutrophils [17].

Activated leukocytes release an array of mediators, including reactive oxygen species [18]. Although their function is to attack invading pathogens, the products of leukocytes have the potential to damage healthy tissue and organs [19,20]. A study conducted by Atanackovic et al. demonstrated how exposure to a putatively stressful event resulted in a significant reduction in ROS production, compared to control [21]. More recently, chemiluminescent assay of PMA-induced ROS production by leukocytes has been shown to provide quantitative links between psychological anxiety and immune-competency [22–24]. The leukocyte coping capacity (LCC) technique involves measuring the ability of leukocytes to produce a respiratory burst following chemical challenge, assayed in terms of reactive oxygen species (ROS) and calibrated through the emission of photons via their interaction with Luminol [22].

The LCC test monitors the multifaceted effects of stress using the body's leukocytes (primarily, but not exclusively, neutrophils) as bio-indicators. These cells circulate throughout the body picking up and responding to all of the signals of stress (as indicated in

* Corresponding author. Fax: +44 1865 393101.
E-mail address: david.macdonald@zoo.ox.ac.uk (D.W. Macdonald).

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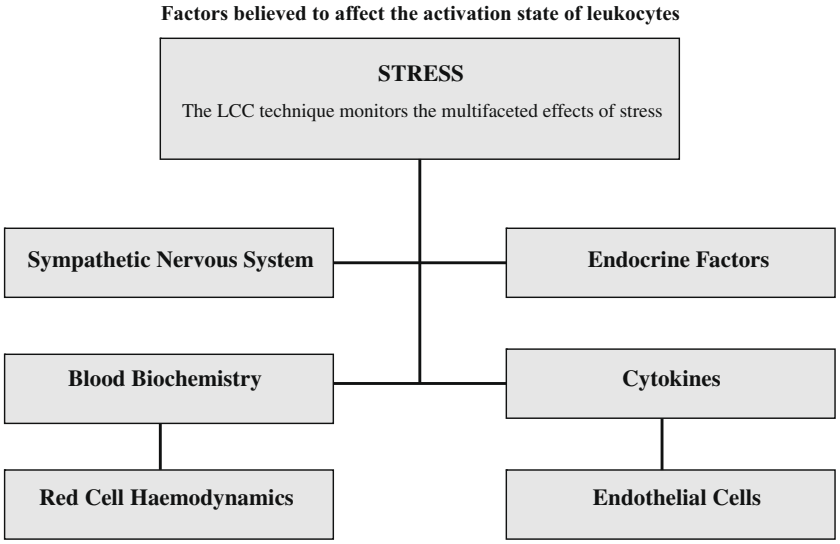

Fig. 1. Flow diagram showing factors believed to affect the activation state of leukocytes.

Fig. 1). Leukocytes (primarily, but not exclusively, neutrophils) have over 150 different receptors [25] which can respond to a diverse range of factors, all of which are sensitive to stress. These include: endocrine factors in the plasma, changes in blood biochemistry, changes in red cell haemodynamics, cytokines and factors released from other cells, both circulating and non-circulating cells such as endothelial cells, and changes in the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system. As stress affects each of these factors, leukocytes make ideal indicators of stress, being constantly exposed to a diverse range of stress stimuli. The coping capacity of leukocytes (LCC), i.e. their ability to respond to an external stimulator and produce reactive oxygen species, will be affected by the immediate external environment in the blood. Leukocytes (mainly neutrophils) which have been exposed to stressors within the body will have a reduced capacity to produce reactive oxygen species in response to an external stimulator (e.g. PMA). This is the underlying technical foundation of the test [25].

The LCC test is a physiologically relevant blood test for objectively assessing the effect of stress. The physiological relevance is convincing since:

- Firstly, the leukocytes are kept in the local environment, i.e. they are suspended in the blood. The suspension of leukocytes in blood allows the cells to dynamically interact with the surrounding red cells and allows cell–cell interaction within and between different leukocyte cohorts. This can dramatically affect the responsiveness of leukocytes. The viscosity and cell–cell interaction with other leukocytes, hormones and cytokines of the surrounding cells can have a dramatic effect on shear stresses and the expression of cell surface receptors. Disruption to cell signalling pathways are minimised, and the responsiveness and integrity of cells is maintained.
- Secondly, the technique avoids centrifugation, a process known to affect cell reactivity, and also avoids 'plating out' cells on glass slides—as used in the NBT test [26]. The cells are stimulated *in vitro* with PMA and the superoxide producing capacity of the cells is measured in real time. As leukocytes release reactive oxygen species in response to stress [22], the stimulation allows us to evaluate the capacity that the cells have to produce further reactive oxygen species. This takes into account the exposure to other stress mediators and makes the test sensitive to true stress; the reactivity of the cells is not altered by deliberate manipulation.

The use of a drop of whole blood is deliberate. Leukocytes are three-dimensional entities, their ability to produce reactive oxygen species is altered by cell signalling pathways of other entities and cells. The aim of this study is to monitor the cellular capacity of leukocytes to produce superoxide radicals in real time. By deliberately leaving the cells in contact with the circulating mediators of stress within blood, the leukocytes are able to actively interact with other cellular components and mediators. Leukocytes (primarily neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress [25]. The use of a 10 μl drop of whole blood that is maintained and which is not spread on glass or preserved, attempts to provide *in vivo* conditions within an *in vitro* environment, thus allowing three-dimensional exposure to, and interaction with hormones (which can alter the reactivity of the cells), other cells such as macrophages, other neutrophils, the haematocrit, and red blood cells (whose viscosity alter during stress).

The objectives of this study were to firstly, investigate the feasibility of using altered leukocyte responsiveness as a means of objectively assessing and discriminating between changes in psychological anxiety/mental workload, elicited as a consequence of interaction with two different touch screen interfaces from the same motor manufacturer (Interface 1—an antecedent model and Interface 2—a new version designed to facilitate ease of use and therefore reduce mental workload). Secondly, we aimed to investigate whether the concept of habituation, as observed within the cardiovascular system [27–29], can also be applied to leukocyte responsiveness, following increased psychological familiarity to a specific situation.

2. Materials and methods

2.1. 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 [30].

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 pre-

vious month, and if they possessed any prior knowledge or experience of the test equipment.

2.2. Perceived technical confidence and psychological stress

Prior to testing, a crude measure of 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?). 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.

Perceived psychological stress was established pre- and immediately post-stressor for each interface (Interfaces 1 and 2) by means of Likert scales (using a continuous scale with 1 representing relaxed and 10 stressed) [31,32].

2.3. Design

For drivers, the primary task of driving involves considerable mental loading. The attempt to accomplish secondary tasks, such as using in-car systems, obviously has the potential to affect this mental challenge. Mental loading can be reduced according to how information is presented—visual, auditory or multi-modal (combination of the two) formats [33]. Two multi-modal touch screen interfaces, a new model (designed to facilitate interaction and ease of use) and one of its antecedents, from the same car manufacturer were used to explore whether progressive interface design has resulted in a reduction in mental workload.

The experimental protocols were rigorously standardised, and testing was confined to between 10 am and 2 pm. Subjects were required to avoid any strenuous activity for at least 2 h 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 outlined below and illustrated in Fig. 2, subjects sat quietly and were instructed to breathe orthonasally for 15 min. The first pair of capillary blood samples was then taken 45 min before exposure to the test apparatus (45 min pre-stressor) (see below).

During the 45 min pre-stressor period subjects were asked to sit quietly or read (reading material was of a non-stimulating content, a local daily newspaper).

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 with subjects being arbitrarily allocated to one of two test order combinations (Interface 1 then 2 or visa versa). Interface 1 was an antecedent version of Interface 2 (a newer and more intuitive design incorporating a greater depth of menu orientated controls designed to facilitate interaction and reduce mental workload). Although both interfaces employed touch screen technology, Interface 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 using the touch screen. With Interface 2, the main menu was displayed and accessed solely by 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.

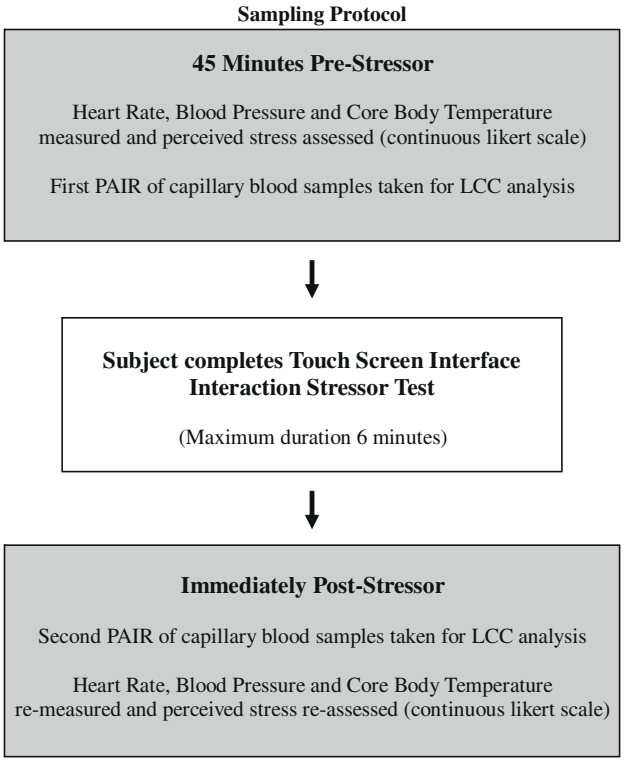

Fig. 2. Flow diagram illustrating the time line for blood sample collection, for leukocyte coping capacity (LCC) analysis, and measurement of other physiological (heart rate, systolic and diastolic blood pressure, and core body temperature) and psychological (perceived stress rating) parameters.

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

Each subject had a maximum of 6 min to complete the following tasks:

(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 min of attempting to complete the task. Immediately upon completion of the task (immediately post-stressor), heart rate, BP and core body temperature were recorded and further blood samples taken (Fig. 2).

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

2.4. Repeat testing

Studies have demonstrated how cardiovascular reactivity decreased following repeated exposure to a mild psychological stressor (arithmetic stress) [29]. 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. One male and one 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 two further occasions (including original testing, all repeat volunteers were tested using both interfaces on three separate occasions).

2.5. Heart rate, blood pressure and core body temperature measurements

At each specified time point (Fig. 2) a heart rate transceiver (Polar 610i™ Heart rate monitor, Polar Electro, Finland) attached directly to the chest, using the belt provided, monitored heart rate. Systemic blood pressure was measured using an oscillometric wrist mounted blood pressure monitor (Omron RX-3, Omron Healthcare, Inc., Illinois 60015, USA). Core body temperature (CBT) was measured using an infra-red ear thermometer (Braun® Thermoscan™, P and G Brooklands, Waybridge AT13 0XP, United Kingdom).

2.6. Blood samples

At each specified time point (Fig. 2) two 10 μl blood samples were taken using a finger lancing device (Accu-Chek® Softclix®, Roche® Ltd., East Sussex, United Kingdom) from the subject's non-contractual hand. Following the procedure illustrated in Fig. 3, one sample was used for the (non-stimulated) control (Sample A) and was placed into 10 μl of murine heparin (concentration 0.1 U) (CP Pharmaceuticals Ltd., Ash Road North, Wrexham LL13 9UF, United Kingdom), 10 μl phosphate-buffered saline (PBS) (Sigma–Aldrich, Dorset SP8 4XT, United Kingdom) and 90 μl of 10^{-4} M Luminol ($C_8H_7N_3O_2$) (Sigma–Aldrich, Dorset SP8 4XT, United Kingdom). The second blood sample (Sample B) was added to the same reagents, except that the 10 μl of PBS was replaced by 10 μl of 10^{-5} M Phorbol 12-Myristate 13-Acetate (PMA) (Sigma–Aldrich, Dorset SP8 4XT, United Kingdom). PMA stimulates leukocytes (primarily neutrophils) causing them to increase their production of oxidative metabolites. This increased production can be measured using Luminol amplified light emission (chemiluminescence) [34].

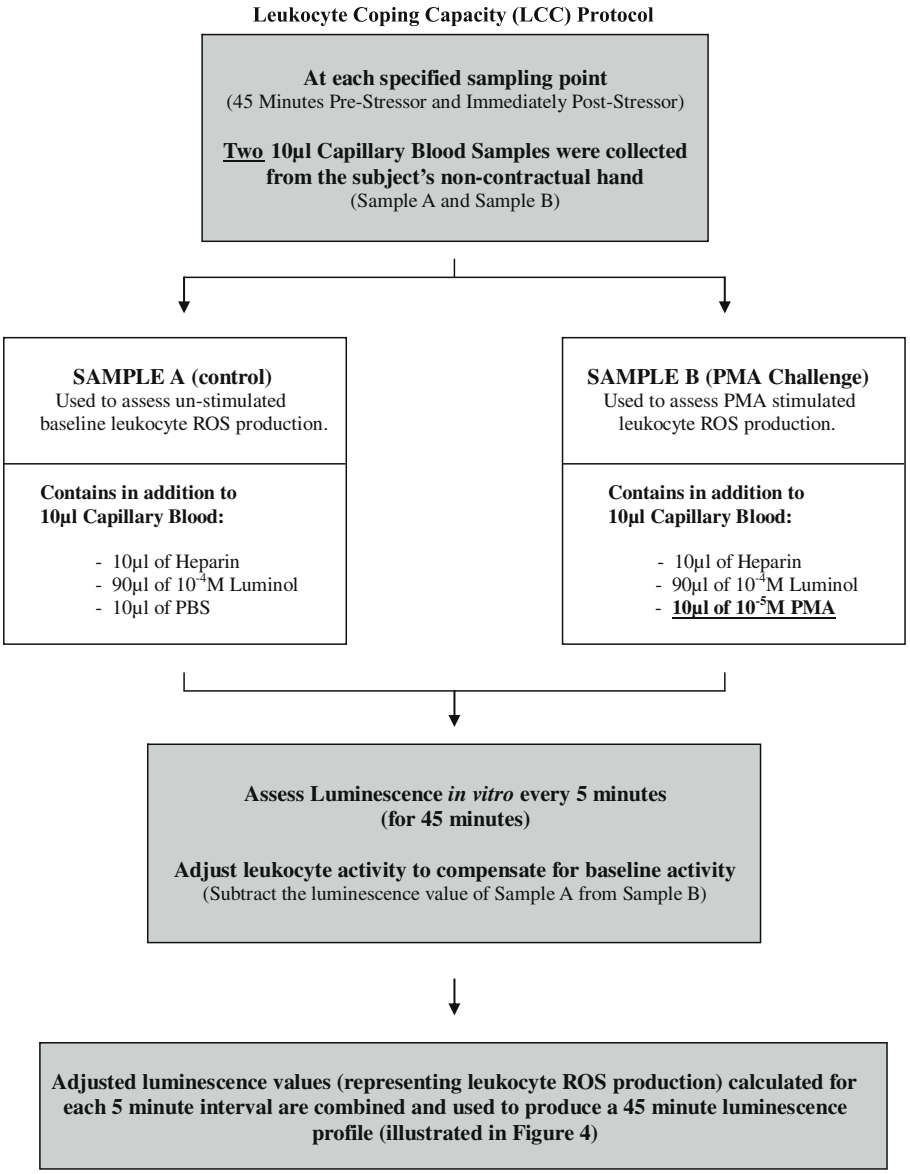

Fig. 3. Leukocyte activity was assessed using whole blood samples taken 45 min pre- and immediately post-stressor, following the protocol illustrated.

2.7. Determining leukocyte activity

Leukocyte coping capacity (LCC) is a measure, made using a Luminometer, of the concentration of reactive oxygen species (ROS) calibrated through the emission of photons as a result of their interaction with Luminol. This is an indicator of the leukocytes' ability to produce a respiratory burst [22]. LCC is defined as the response of leukocytes (mainly neutrophils) to challenge, in this case by PMA or also by fMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine). Subjects whose LCC score is higher have displayed a greater potential to produce a respiratory burst, and are therefore in this respect more able, physiologically, to respond to bacterial challenge (immunologically competent). LCC responsiveness to *in vitro* PMA challenge is inversely related to mental workload.

In this experiment, each pair of blood solutions (Sample A and Sample B) was simultaneously tested every 5 min using a Luminometer (Berthold® Technologies, Junior™ LB9509, Hertfordshire AL3 7LZ, United Kingdom) for a total of 45 min, in order to produce a luminescence profile (Fig. 4). Between chemiluminescence measurements the samples were incubated at 37 °C in a water bath (JB1™ Grant Instruments, Cambridge, United Kingdom). At each 5 min interval an adjusted score, measured in relative light units (RLU_{adj}) was obtained for each subject by subtracting the luminescence score of the control (Sample A—without PMA stimulation) from the PMA challenge sample (Sample B).

2.8. Data analysis

For all measured parameters data are expressed as mean post-test changes ± standard error of mean (SEM). For T_{max} (time taken to reach maximum leukocyte activity), the data were classed as discontinuous as leukocyte activity was measured at 5 min intervals for a total of 45 min, in this case the median ± SEM is presented. Single factor analysis of variance (ANOVA) (SPSS statistical software (release 15.0, Lead Technologies, Inc.) was used to test in turn, the effect of experimental group (Interface 1 and Interface 2) on leukocyte activity, heart rate, BP, core body temperature, perceived psychological stress rating (continuous Likert scale) and perceived technical ability.

A two-way ANOVA model was subsequently used to investigate in turn, the effect of repeat testing on changes to leukocyte reactivity, heart rate, BP and core body temperature. 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.

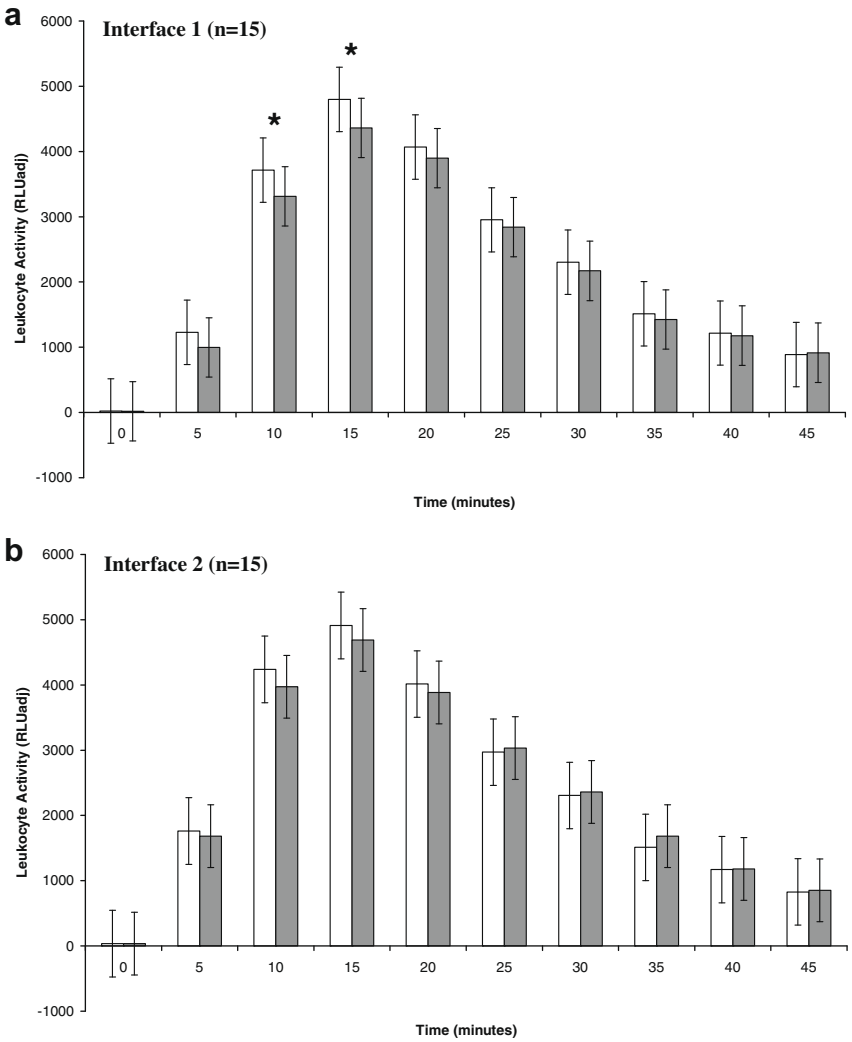

Fig. 4. (a and b) Mean adjusted leukocyte coping capacity (RLU_{adj}) ± SEM for Interfaces 1 and 2 ($n = 15$ for each) for primary test phase. Open bars represents mean adjusted leukocyte activity 45 min pre-stressor and closed bars represents activity immediately post-stressor. * indicates significant difference in activity between 45 min pre- and immediately post-stressor ($P < 0.05$).

3. Results

3.1. Primary testing

3.1.1. Leukocyte activity

Leukocyte activity profiles produced from blood samples taken 45 min pre- and immediately post-stressor for both treatment groups (Interfaces 1 and 2) are displayed graphically (means with SEM bars) in Fig. 4. Pre-stressor activity for both treatment groups was of a similar magnitude, whereas immediately post-stressor, the use of Interface 1 resulted in a greater decrease in activity compared to the use of Interface 2. Post-stressor changes in leukocyte activity are given in Fig. 5. The results show that following the use of Interface 1 leukocyte activity significantly decreased from baseline for all five attributes of the luminescence profile (except T_{max} and $T = 5$ min), with the most pronounced change occurring at $T = 10$ min (adjusted leukocyte activity 10 min into the 45 min sampling period) ($F_{1,14} = 5.14$, $P = 0.03$), unlike Interface 2 where although a decrease in post-stressor activity was recorded, the magnitude of change proved non-significant. A significant difference in post-stressor leukocyte activity (difference between pre- and immediately post-stressor activity) between treatment groups, occurred at H_{max}-RLU_{adj} and 5 min into the 45 min luminescence profile ($T = 5$ min). Both attributes show the use of Interface 1 led to a decrease in leukocyte activity that was significantly greater in magnitude compared to Interface 2 (Fig. 5). All subjects ($n = 15$) reached maximum leukocyte activity (H_{max}-RLU_{adj}) by $T = 15$ min (15 min after PMA challenge) (Fig. 4).

Trends in post-test mental workload measured using Likert scales paralleled those in LCC scores, with both treatment groups showing post-stressor increases. Although the use of Interface 1 led to an increase of greater magnitude than Interface 2 (Interface 1 0.8 ± 0.3 U, Interface 2 0.1 ± 0.3 U), the difference was not statistically significant ($F_{1,59} = 0.89$, $P = 0.34$).

3.1.2. Core body temperature

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

3.1.3. 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-stressor or between treatment groups ($n = 15$ for each) during primary testing.

3.1.4. Perceived technical confidence

A basic measure of each subject's ($n = 15$) technical confidence was evaluated pre-stressor, 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?). Ten 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 five 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-stressor difference in leukocyte activity was shown for maximum adjusted leukocyte activity (H_{max}-RLU_{adj}) ($F_{1,29} = 6.55$, $P = 0.02$) and $T = 5$ min ($F_{1,29} = 8.4$, $P = 0.008$). With those who were more confident in the use of unfamiliar computer-based technology demonstrating a post-stressor decrease in leukocyte activity that was significantly smaller in magnitude compared to individuals who were rated as lacking the confidence to use unfamiliar technology (Fig. 6).

3.2. Secondary testing

3.2.1. Leukocyte activity

Four subjects underwent repeat testing on two further occasions. Data for these four subjects only is presented in Table 1. LCC profiles for the four subjects showed the same response trends as observed for primary testing, with maximum leukocyte activity (H_{max}-RLU_{adj}) occurring at $T = 15$ min for both interfaces, and the

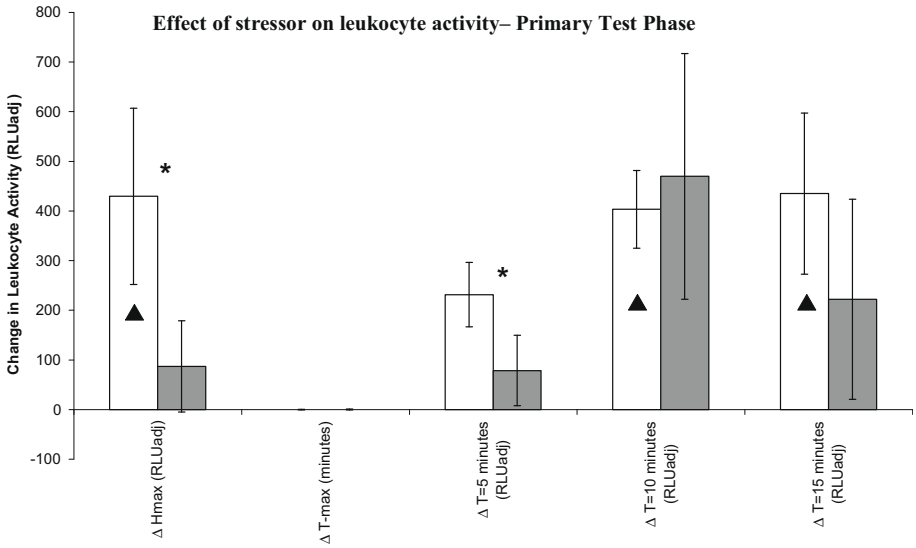

Fig. 5. Mean and standard error of the mean (SEM) are presented for the change (Δ) in leukocyte activity (difference between 45 min pre- and immediately post-stressor samples) for four specific attributes of the leukocyte luminescence profiles for each treatment group ($n = 15$ for each) (leukocyte activity-adjusted relative light units—RLU_{adj}), open bars represent Interface 1 and closed bars Interface 2. H_{max} (RLU_{adj})—the maximum adjusted response exhibited during the 45 min sampling period, $T = 5$, 10 and 15 min—the adjusted response in leukocyte activity recorded at 5, 10 and 15 min into the 45 min activity profile (RLU_{adj}). T_{max} data (difference in the time taken to reach maximum adjusted leukocyte activity between 45 min pre- and immediately post-stressor) is discontinuous; therefore median values with SEM are presented. Repeated measures single factor ANOVA was used to investigate the effect of treatment on leukocyte activity (df = 29). ▲Difference between pre- and post-stressor leukocyte activity ($P < 0.05$). *Difference between Interface 1 and Interface 2 ($P < 0.05$).

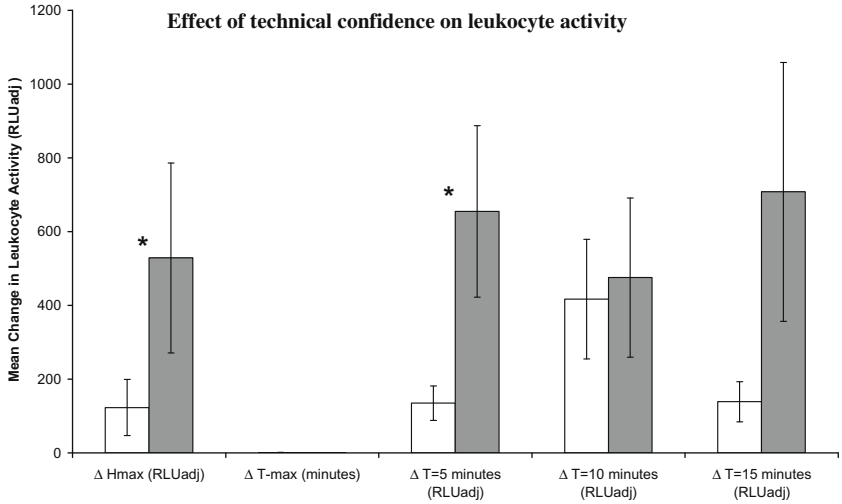

Fig. 6. The effect of perceived technical confidence rated according to the responses to 10 questions designed to gage an individuals confidence in using unfamiliar computer-based technology—subjects were classed as being either confident (open bars) in the use of such technology (score equal to or greater than 5) or lacking in confidence (closed bars) (score of less than 5) on post-stressor changes (Δ) in leukocyte activity (difference between 45 min pre- and immediately post-stressor) using combined data from both treatment groups ($n = 30$) was assessed in turn, for each of the five specific attributes of the leukocyte luminescence profiles H_{max} (RLU_{adj})—the maximum adjusted response exhibited during the 45 min sampling period, T_{max}—the time taken to reach maximum leukocyte activity, and $T = 5$, 10 and 15 min—the adjusted response in leukocyte activity recorded at 5, 10 and 15 min into the 45 min activity profile (RLU_{adj}) using a repeated measures single factor ANOVA model. * Difference between technically confident and non-confident groups ($P < 0.05$).

Table 1
Effect of repeat testing on leukocyte activity.

	ΔH_{max} (RLU_{adj})	ΔT_{max} (min)	$\Delta T = 5$ min	$\Delta T = 10$ min	$\Delta T = 15$ min
Interface 1					
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 2					
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

Four subjects carried out repeat testing, two from test order Interface 1 then 2, and two from test order Interface 2 then 1. Combined data for all four subjects is presented here as mean with SEM for the change (Δ) in leukocyte activity (difference between 45 min pre- and immediately post-stressor samples) (leukocyte activity-adjusted relative light units—RLU_{adj}). The difference between the values for Interfaces 1 and 2 and values between test weeks (1–3), along with the interaction between interface and test week are presented. •Difference between pre- and post-stressor leukocyte activity ($P < 0.01$). * Difference between Interface 1 and Interface 2 ($P < 0.05$). †Difference between test week ($P < 0.05$) (Tukey's *post hoc* procedure).

magnitude of the post-stressor response proving to be significantly different between treatment group ($F_{1,23} = 13.55$, $P = 0.003$). In Week 1 (primary testing) these four subjects showed that the use of Interface 1 resulted in a significant decrease in leukocyte activity of 9% from pre-stressor values ($P = 0.002$, Tukey's *post hoc* procedure), whereas following the use of Interface 2, 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-stressor decreases in leukocyte activity which progressively decreased in magnitude. No significant interaction effect was found between treatment and time, indicating the temporal effect (rate of habituation) was similar for both interfaces. For Interface 1 post-stressor 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 2 (Table 1). For both Interfaces post-stressor differences proved non-significant in Week 3 (Interface 1—$P = 0.52$ and Interface 2—$P = 0.74$, 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).

3.2.2. Core body temperature

Although all four repeat test subjects showed a similar trend for core body temperature with post-stressor values being higher compared to pre-stressor, 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 2).

3.2.3. Heart rate, blood pressure

As with core body temperature, for the four repeat test subjects, post-stressor 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-stressor differences in heart rate and diastolic blood pressure showed significant decreases in magnitude following repeated exposure to each interface (Table 2).

4. Discussion

Following exposure to mild psychological stressors, immune activity responds in a rapid and reversible manner, demonstrating

Table 2
Effect of repeat testing on heart rate, core body temperature and blood pressure.

	ΔCore body temperature (°C)	ΔHeart rate (bpm)	ΔSystolic blood pressure (mm Hg)	ΔDiastolic blood pressure (mm Hg)
Interface 1				
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 2				
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

Four subjects carried out repeat testing, two from test order Interface 1 then 2, and two from test order Interface 2 then 1. Combined data for all four subjects is presented here as mean with SEM for the change (Δ) in core body temperature, heart rate and systolic and diastolic blood pressure (difference between 45 min pre- and immediately post-stressor samples). No significant difference between pre- and post-stressor values and between treatment group were shown for each of the listed parameters.
* Difference between test week ($P < 0.05$) (Tukey's *post hoc* procedure).

similar response trends to those observed within the cardiovascular system [29]. Initial exposure to the two stressors, resulted in significantly different post-stressor decreases in the magnitude of leukocyte response exhibited for maximum adjusted leukocyte activity (H_{max}-RLU_{adj}) and for leukocyte activity 5 min into the 45 min luminescence profile ($T = 5$ min). These differences demonstrate the capability of the LCC technique to objectively quantify, and discriminate between, the differential responses in altered leukocyte activity that resulted from performing the same series of basic in-car related tasks using two touch screen interfaces that differed in ergonomic design and in the graphical format by which information is presented.

Leukocytes (primarily, but not exclusively, neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress. These include: endocrine factors in the plasma, changes in blood biochemistry, changes in red cell haemodynamics, cytokines and factors released from other cells, both circulating and non-circulating cells such as endothelial cells, and changes in the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system [25]. As stress affects each of these factors, leukocytes make ideal indicators of stress, being constantly exposed to a diverse range of stress stimuli. The coping capacity of leukocytes (LCC), i.e. their ability to respond to an external stimulator and produce reactive oxygen species, will be affected by the immediate external environment in the blood. We have previously shown that exposure to a short-term stressor results in a significant change in biochemical mediators [35].

The use of Interface 1 (antecedent version) resulted in a post-stressor decrease in leukocyte activity that was significantly greater in magnitude, compared to the use of Interface 2 (a newer version designed to facilitate ease of use, with information being accessed solely through an enhanced resolution touch screen, unlike the antecedent version which utilised dedicated physical controls in addition to a lower resolution touch screen), whereas no significant differences in subjective perceived stress or in heart rate, BP or core body temperature were observed between treatment groups. These findings demonstrate the diagnostic benefits (increased sensitivity) of using the LCC technique as an objective means of discriminating between the physical effects of two closely related mental stressors, which appear to be below the detection threshold of other traditional physical measures of mental workload.

The architecture and adhesiveness of a cell microenvironment is a critical factor in determining it's responsiveness *in vivo* [36]. The LCC technique maintains the structural integrity and shape of the cell as near to the *in vivo* condition as possible. Deliberately keeping the leukocytes suspended in whole blood, allows them to dynamically interact with the surrounding red cells and permits cell–cell interaction within and between different leukocyte cohorts. This may dramatically affect the responsiveness of leukocytes. The viscosity and cell–cell interaction with other leukocytes, hormones and cytokines, released from surrounding cells, can have a dramatic effect on shear stresses and the expression of cell surface receptors. Disruption to cell signalling pathways are minimised, and the responsiveness and integrity of cells is maintained. Centrifugation a process known to affect cell reactivity, and 'plating out' cells on glass slides (used in the NBT test [26]), both of which affect the reactivity of cells, is deliberately avoided. The superoxide producing capacity of the cells is monitored in real time. As leukocytes release reactive oxygen species in response to stress, the stimulation allows us to see (like a differential equation) the capacity that the cells have to produce further reactive oxygen species. This takes into account the exposure to other stress mediators and makes the test sensitive to true stress and the reactivity of the cells is not altered by manipulation.

It was previously known that psychological familiarity to specific situations promotes habituation of heart rate, blood pressure and core body temperature [27–29]. Within this study, evidence to support such a relationship was shown for heart rate, diastolic blood pressure and core body temperature. A similar trend was also observed for leukocyte responsiveness, with both H_{max}-RLU_{adj} and leukocyte activity 15 min into the 45 min luminescence profile ($T = 15$ min) showing significant post-stressor reductions in the magnitude of response following repeat testing. The results showed that as familiarity to both stressors increased, following repeated exposure, the magnitude of the post-stressor decrease in leukocyte responsiveness significantly reduced. After the third exposure, both treatment groups showed no significant change in post-stressor leukocyte activity. To our knowledge, this study is the first to demonstrate habituation in leukocyte activity. Effective coping (habituation) will mean that the stress response (the multitude of pathways that are triggered) will be activated when needed and terminated afterwards. In a comprehensive review on stress habituation in 'Nature' de Kloet et al. described how repeated exposure to stress can modulate the pathways involved, so that the same stimuli results in reduced activity [37]. This adaptive process is reported to be initiated in the limbic system. Thus the habituation reflected in the reduced LCC reading probably reflects the down regulation of one or more of the pathways involved with stress, which include a down regulation of endocrine factors in the plasma, changes in blood biochemistry, changes in red cell haemodynamics, cytokines and factors released from other cells, both circulating and non-circulating (such as endothelial cells), and changes in the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system (Fig. 1).

In conclusion, this study has revealed the potential benefits over traditional methodologies for the use of LCC analysis as an objective measure of altered mental workload during ergonomic evaluation of new technology. In addition we show how, as with the cardiovascular system, leukocyte responsiveness exhibits habitu-

ated response trends following increased familiarity to a specific mental stressor.

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