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Leukocyte coping capacity: a novel technique for measuring the stress response in vertebrates

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Methods used to quantify the stress response in animals are vital tools in many areas of biology. Here we describe a new method of measuring the stress response, which provides rapid results and can be used in the field or laboratory. After a stressful event, we measure the capacity of circulating leukocytes to produce a respiratory burst *in vitro* in response to challenge by phorbol myristate acetate (PMA). During the respiratory burst leukocytes produce oxygen free radicals, and the level of production can be measured directly as chemiluminescence. When *in vitro* PMA-stimulated whole blood chemiluminescence is measured directly after a stressful event, we define the response as the leukocyte coping capacity (LCC). In an experiment badgers (*Meles meles*), which were caught as part of an on-going population study, were either transported to a central site prior to blood sampling or blood was collected at their site of capture. Transported animals had a significantly lower LCC and showed changes in leukocyte composition that were indicative of stress. We conclude that the stress of transport reduced LCC in badgers and that LCC serves as a quantitative measure of stress. Potential applications of this method are discussed. *Experimental Physiology* (2003) **88.4**, 541–546.

Objective, quantitative and practicable measures of stress are pivotal to studies in many branches of vertebrate biology, including human biology, animal husbandry and wildlife ecology (e.g. Dawkins, 1980; Bateson & Bradshaw, 1997; Palme & Möstl, 1997; Creel, 2001; Goymann *et al.* 2001). The stress response in animals is currently assessed using a variety of techniques, including measurement of cortisol levels (e.g. Beerda *et al.* 1996; Palme & Möstl, 1997; Harper & Austad, 2000) and haematological values (e.g. Millspaugh *et al.* 2000), and observations of behaviour (reviewed by Rushen, 2000). Here we present a new method for quantifying stress, based on direct measures of an immune response. We state the benefits of the technique, and discuss the circumstances under which its use is most valuable.

The method is based on the ability of individuals to mount a challenge-induced immune response after a defined, potentially stressful event. Each individual's capacity to respond to immune challenge is compared with its own baseline level of immune system activity. After the putatively stressful event, we measure the capacity of the individual's leukocytes to produce a quantifiable immune response known as the respiratory burst. During the respiratory burst, oxygen uptake by leukocytes is accelerated in order to produce oxygen free radicals that destroy bacteria (a process reviewed by Halliwell & Gutteridge, 2000). Leukocytes are known to produce oxygen free radicals in response to agonists such as bacterial peptides binding to receptors on their cell membranes (Dietert *et al.* 1996), the activation of protein kinase C with phorbol myristate acetate (PMA; Hu *et al.* 1999) and stress (Ellard *et al.* 2001). It has also been demonstrated that stress affects the respiratory burst: leukocytes isolated from the head kidney of salmon (*Salmo salar*) showed a reduced respiratory burst (40 % reduction in oxygen free radical production) after the fish were subjected to a 2 h period of confinement stress (Thompson *et al.* 1993). Therefore, there is evidence that the respiratory burst activity of leukocytes is affected by stress. In particular we wanted to determine whether a reduction in the respiratory burst of circulating leukocytes (which can be measured in whole blood without using isolation techniques) could be used as a measure of stress. In our *in vitro* challenge–coping approach, after a stressful event, we chemically stimulate a respiratory burst in whole blood *in vitro* using PMA, and measure the capacity of

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Experimental Physiology: Translation and Integration

leukocytes to produce a respiratory burst over a 30 min period.

We define the response of leukocytes to PMA challenge after a stressful event as the individual's leukocyte coping capacity (LCC). Therefore, animals with a higher LCC have a greater potential to produce a respiratory burst, and physiologically, are better able to respond to bacterial challenge after stress. Therefore LCC is an *in vitro* assessment of the animal's current physiological status. We also examine the basal chemiluminescence in samples of blood that have not been stimulated by PMA, and this acts as a baseline with which to compare the individual's LCC. Our hypothesis is that LCC, measured using a challenge–coping approach, will provide a quantitative measure of the stress an individual undergoes during a particular event. This hypothesis predicts that animals subjected to known stressors should have a lower LCC than animals that have not (Sanidas *et al.* 2000; Egger *et al.* 2001; Ostrakhovitch & Afanas'ev, 2001). Here, we tested the prediction that badgers (*Meles meles*) have a lower LCC when they are subjected to trapping and then transport, a known stressor in animals (Blecha, 2000) than when they are trapped but do not experience the additional stress of transport.

METHODS

Trapping and transporting badgers

Badgers were trapped in Wytham Woods, Oxfordshire, UK (for details of the study site and its badger population see Macdonald & Newman, 2002) in cage traps baited with peanuts in August and November 2001. We selected badgers because a measure of stress for this species would be immediately useful in studies of its unusual social system (Macdonald & Newman, 2002), its role in the epidemiology of bovine tuberculosis and because studies, conservation and control of badgers necessitate their capture and handling, so a means of evaluating alternative procedures would be helpful. We selected this population because their individual life histories have been monitored for 14 years, during which our particular trapping and handling procedures have been refined to the highest welfare standards. Badger traps were set adjacent to badger setts between 14.00 and 17.00 h. Traps were checked each morning between 06.30 and 07.00 h and trapped badgers were transferred to individual holding cages, which were then covered.

Animals were then assigned to one of two experimental regimes: sampling at the site of capture, without transport (non-transported, $n = 8$); or sampling immediately after transport (transported, $n = 8$). Transport consisted of a short ride for less than 10 min on a trailer pulled by an all-terrain quad bike. Badgers were anaesthetised, either at their site of capture or after transport, using an intramuscular injection of ketamine hydrochloride at a concentration of 100 mg ml^{-1} (Ketaset, Fort Dodge, USA) and administered to badgers at a dose of 0.2 ml kg^{-1}. Processing consisted of measurements of body weight and length, and recording the sex and condition of the badger. Other measurements were also taken as part of the ongoing badger population study. Blood was removed by needle venepuncture of the jugular vein, collected into a tube containing the anti-coagulant potassium EDTA (BD Vucutainer Systems, Plymouth, UK) and taken immediately for PMA challenge. Differential cell counts were made from blood smears fixed in alcohol and stained with May-Grunwald and Giemsa stains (minimum of 100 cells counted per slide). Mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and haematocrit were calculated using a haematology analyser (Celltac MEK-5108K, Kohden, Japan).

PMA challenge and measurement of leukocyte coping capacity

To measure the unstimulated blood chemiluminescence levels, 10 μl whole blood was transferred into a silicon anti-reflective tube (Lumivial, EG & G Berthold, Germany) to which 90 μl 10^{-4} mol l^{-1} luminol (5-amino-2,3-dihydrophthalzine; Sigma A8511) diluted in phosphate buffer was added. The tube was then shaken gently. To measure the chemiluminescence produced in response to challenge, a further tube was prepared as above, but with the addition of 10 μl phorbol 12-myristate 13-acetate (PMA; Sigma P8139) at a concentration of 10^{-6} mol l^{-1}. The PMA was dissolved in a small amount of dimethyl sulfoxide (DMSO; Sigma D 5879) and then diluted to a concentration of 10^{-6} mol l^{-1} in PBS. For each tube chemiluminescence was measured every 5 min in a portable chemiluminometer (Junior LB 9509, EG & G Berthold) for a total of 30 min. When not in the chemiluminometer, tubes were incubated at 37 °C. Although a range of leukocytes can produce a respiratory burst, neutrophils are responsible for the majority of oxygen free radical production (Ellard *et al.* 2001) and so we also examined LCC per quantity of 10^9 neutrophils l^{-1}; this also provided a method of examining LCC in relation to the potential effects of changes in the number of circulating neutrophils after stress.

Statistical analyses

To compare differences in LCC and other haematological parameters in badgers with and without transport, we used multivariate analysis of variance (MANOVA). For LCC, the chemiluminescence levels at each time interval were the dependent variables. We chose this approach because it allowed us to examine the effect of the treatment at each time interval, giving more detail about the nature of the effects of the treatment, and allowed us to identify the time interval at which the treatment effect was greatest. This procedure was carried out using the software SPSS for Windows release 10.0.5. Non-parametric data were log transformed prior to the analysis to ensure the data met assumptions of multivariate normality (Tabachnick & Fidell, 1996).

Animal welfare considerations

The badger population at Wytham Woods is under long-term scientific investigation, and badgers are trapped and transported regularly throughout their lives. Examining transport stress is part of our continuing refinement of best practice. Work was carried out under English Nature licence 1991537 and UK Home Office licence PPL 30/1826.

RESULTS

LCC and unstimulated blood chemiluminescence levels for transported ($n = 8$) and non-transported ($n = 8$) badgers are shown in Fig. 1. Transport significantly reduced LCC in badgers ($F_{7,8} = 4.5$, $P = 0.03$). The difference in LCC between transported and non-transported individuals was greatest at 15 min ($F_{1,14} = 8.9$, $P = 0.01$; Fig. 1). However, two of the non-transported individuals were caught during a night when ambient temperatures unexpectedly fell below freezing, and these individuals did not show a

typical response for this group, and in both cases their LCC was no greater than their basal response (Wilcoxon Signed Ranks Test, for both cases $Z < -1.0$, $P > 0.1$). Transport had no effect on unstimulated levels of chemiluminescence ($F_{7,8} = 2.5$, $P > 0.05$). However, to ensure that there was no bias in the LCC results because of individual differences in unstimulated levels of chemiluminescence, we subtracted the unstimulated from the PMA-stimulated values for each animal (PMA − unstimulated). Transport had an effect on the PMA − unstimulated values from 10 min ($F_{1,14} = 4.1$, $P = 0.06$), with the difference peaking at 15 min ($F_{1,14} = 9.4$, $P = 0.008$), and thus showed a pattern consistent with the LCC values described above. To examine individual neutrophil activity, we calculated LCC per 10^9 neutrophils l^{-1} (Fig. 1), and found the same pattern as overall LCC, indicating that activity per neutrophil was greater in non-transported individuals.

The effects of transport on the blood parameters we measured are shown in Table 1. Transported individuals had significantly lower numbers of leukocytes and a different leukocyte composition: transported animals had a higher percentage of neutrophils and a correspondingly lower percentage of lymphocytes (Table 1). Haematocrit, neutrophil/lymphocyte ratio, MCHC and MCV were also affected by transport (Table 1).

DISCUSSION

These results indicate that stress dramatically reduces the potential of circulating leukocytes to produce free radicals, and moreover, the results support the hypothesis that LCC is a measure of the stress associated with a specific event. The rather uniform depression of the coping response in all transported individuals suggests that this stressor overrode the sources of individual variation that characterised the coping responses of non-transported individuals. The reduced response of the two individuals caught during a night when temperatures fell below freezing perhaps identifies another stressor worthy of investigation (Glette *et al.* 1982).

Transport also brought about changes in circulating cell composition and number, and this represents the effects of cell trafficking between reservoir sites – the liver, lungs, bone marrow and peripheral blood (Severs *et al.* 1996). In particular, the increases in haematocrit and neutrophil/lymphocyte ratio in the transported animals are indicative of stress (Murata, 1989; Haigh *et al.* 1997; Maes *et al.* 1998). Stress hormones are known to bring about changes in circulating leukocyte composition (McCarthy & Dale, 1988). Immediate leukocytosis is attributed to elevated catecholamine levels, and delayed increases in neutrophils and decreases in lymphocytes are observed due to raised

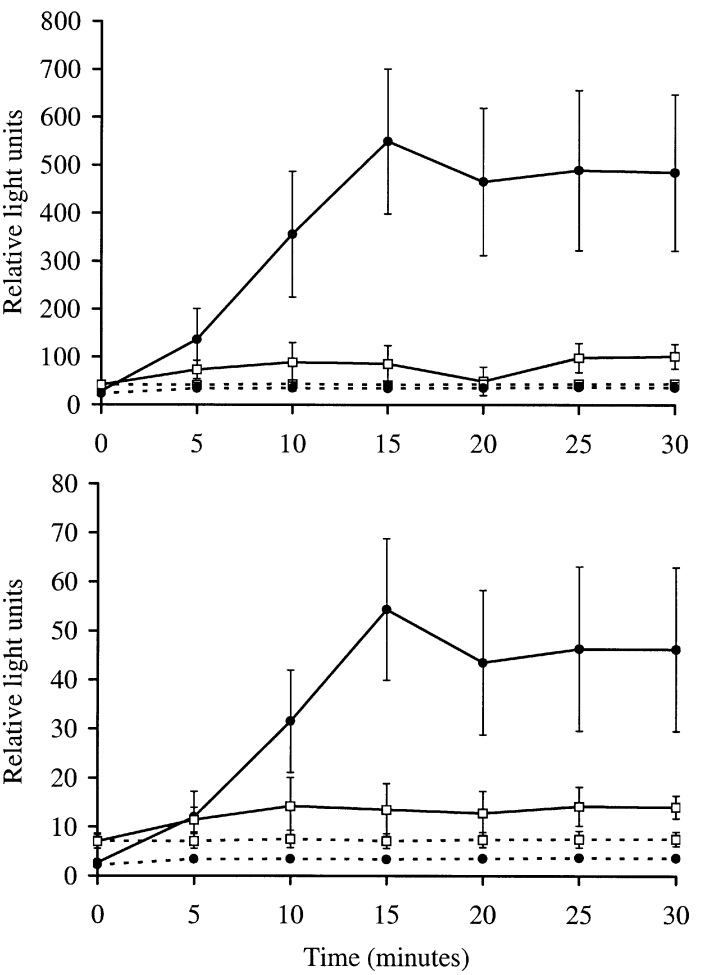

Figure 1

The effect of transport stress on *in vivo* leukocyte coping capacity (LCC). The continuous lines represent mean (± S.E.M.) LCC in badgers after transport (□), and without transport (●). Dashed lines represent unstimulated control samples for transported (□), and non-transported (●) badgers. The lower graph presents LCC per neutrophil, where LCC has been calculated as relative light units divided by the number of neutrophils (10^9 l^{-1}). Lines and symbols are as in upper graph.

Table 1. Blood parameters measured in transported and non-transported animals

	Non-transported		Transported				
	Mean	S.E.M.	Mean	S.E.M.	*t*	d.f.	*P*
Erythrocytes ($\times 10^{12}$ l^{-1})	7.1	0.7	9.8	0.8	−2.5	14	0.025
Haemoglobin (g l^{-1})	113.8	10.1	144.4	11.5	−2.0	14	0.065
Haematocrit (%)	34.0	3.1	44.5	3.3	−2.3	14	0.037
MCV (fl)	48.3	0.6	45.8	0.8	2.6	14	0.020
MCH (pg)	16.2	0.2	14.8	0.2	4.7	14	0.000
MCHC (g l^{-1})	335.4	2.6	324.1	3.0	2.9	14	0.013
Platelets ($\times 10^{9}$ l^{-1})	618.3	87.3	525.5	49.1	0.9	14	0.370
Leukocytes ($\times 10^{9}$ l^{-1})	10.4	0.9	7.0	0.9	2.5	14	0.024
Neutrophils (%)	81.5	2.4	88.5	2.1	−2.2	14	0.047
Lymphocytes (%)	17.4	2.4	10.4	2.2	2.1	14	0.052
Monocytes (%)	1.1	0.5	1.1	0.6	0.0	14	0.999
Eosinophils (%)	0.0	0.0	0.0	0.0	—	—	—
Basophils (%)	0.0	0.0	0.0	0.0	—	—	—
Neutrophils ($\times 10^{9}$ l^{-1})	8.5	0.9	6.3	0.9	1.8	14	0.098
Neutrophil/lymphocyte ratio	5.3	0.7	10.7	1.6	−3.0	14	0.009

Mean and standard error of the mean (S.E.M.) are presented for both treatment groups ($n = 8$ for both), and the significance of the differences between the groups are reported based on a *t* test, for which the degrees of freedom (d.f.) and significance (*P*) are given. MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration.

cortisol levels (Pedersen *et al.* 1994). It is therefore probable that in badgers subjected to transport, increased cortisol levels brought about the changes we observed in the neutrophil/lymphocyte ratio.

Transported badgers had lower numbers of circulating leukocytes. In contrast, short-term stress is also usually associated with a demargination of leukocytes (entry into the circulation): a relationship we have observed in our laboratory (Mian *et al.* 2003). However, this relationship is not simple (Dhabhar *et al.* 1996), and in an elegant series of experiments Oishi *et al.* (2003) demonstrated that immune changes in rats brought about by physical stimulators differed from those induced by visual, olfactory and auditory stimuli. Modification of the receptors on the endothelium and on the leukocytes themselves can dramatically alter the number of adherent (and thus the number of circulating) leukocytes (Ley, 1996). Transport stress could therefore have modified these receptors and caused an increased margination of the leukocytes. Margination is the process by which leukocytes exit the central blood stream, and initiate mechanical contact with the endothelial cells. The margination process is enhanced in vessels of a certain size by the aggregation of erythrocytes, which tend to occupy the centre of microvessels (Firrell & Lipowsky, 1989). The increase in haematocrit observed in this and previous studies (Maes *et al.* 1998) could thus selectively promote margination in some vessels. Previous studies have demonstrated that margination of leukocytes is not a uniform process, and occurs in particular sized vessels within the microcirculation (Mian & Marshall, 1993). The changes in shear stress likely to have been brought about by the increased haematocrit may also serve as a trigger mechanism for leukocyte activation (Schmid-Schonbeim *et al.* 2001). It has been suggested that exposure to hostile conditions, or other psychological stressors, initiates the secretion of several hormones including cortisol, catecholamines, prolactin, oxytocin and renin (Toft *et al.* 1994; Van de Kar & Blair, 1999). Any of these could alter adhesion receptors on circulating leukocytes and thus contribute to an altered leukocyte distribution.

Furthermore, psychological stress alone has been shown to influence the number and distribution of leukocytes in the blood in a rapid and reversible manner (Dhabhar *et al.* 1995, 1996; Kang *et al.* 1996; Goebel & Mills, 2000), and stress has also been found to alter the expression of leukocyte adhesion receptors (Goebel & Mills, 2000). Although an alteration of cell numbers and adhesion molecules is not always associated with an alteration in cellular activity and function (Mian & Marshall, 1993), it is clear that leukocytes can respond rapidly to a wide range of physical and psychological stressors, and that these responses can affect the ability of the immune system to respond to ongoing or potential challenge (Dhabhar *et al.* 1995; Gleeson & Bishop, 2000). As a result, we argue that measures of stress based on leukocyte function, such as LCC, can reveal significant information about the animal's physiological status after and during stressful events.

The relationship between LCC and other components of the stress response should be further examined, but immune function during stress is a well-studied area that provides a background to further investigation of LCC. The mechanism by which the brain modulates the immune system involves the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (Neveu,

2003). 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), and cortisol levels can vary widely between individuals, obscuring the effects of stress (e.g. Montané *et al.* 2002). The immune system also plays a wide-ranging role in the stress response – for example, cytokines produced by the immune system during stress may serve as mediators of an afferent pathway to the brain and could in turn induce changes in behaviour (Buller, 2003). We have reported that immune alterations induced by stress are concomitant with increases in blood pressure and heart rate, but that the immune alterations remained long after these traditional measures of stress had returned to basal values (Mian *et al.* 2003). The most appropriate measure of stress, for a given situation, will depend upon the nature and intensity of the stressor, the experimental design and a host of other factors. We argue that stress measures based on immune system alterations are valid alternatives to measures based on the HPA axis, and may be more suitable in certain circumstances.

We have demonstrated that LCC is affected directly and rapidly by stress. The strengths of our technique, as revealed by our experiment, include: (1) the ability to take measurements during or immediately after a stressful event; (2) the direct comparability of individual responses, which themselves represent physiologically meaningful measures; and (3) the ability to take measurements in the field on whole blood (avoiding centrifugation), yielding results within minutes and without requiring baseline data from animals which have not been stressed. This method would therefore be of particular use in situations where, after or during a stressful event, a rapid assessment of the individual's ability to cope is required. In our case, LCC provided results whilst the animal was under anaesthesia. Thus, in such cases, if an individual showed an unusual response, appropriate steps could be taken well before the animal has been returned to the wild. This was demonstrated by the reduced LCC of the two individuals caught during cold conditions, indicating that, whatever the cause, these individuals had coped less well with capture than other non-transported individuals. Furthermore, the PMA challenge we present *in vitro*, is different from *in vivo* challenges such as the ACTH challenge (e.g. Goddard *et al.* 1994), because PMA challenge is a measure of the ability of the animal to respond to bacterial challenge after stress. Therefore LCC does not represent a further challenge to the individual *per se*, but is instead a measure of the animal's physiological status after a stressful event.

A rapid assessment of individual responses to the same stressor could have many other practical applications in biology, veterinary science and animal husbandry. In addition, this technique could be used in conjunction with other measures, such as faecal cortisol measurement, to indicate patterns of stress and coping in free-living vertebrates. One practical motive for our work is to disentangle the stressfulness of each component of our field procedures, so that they may be improved. This first step demonstrates the power of the coping–challenge technique. However, while we have shown that within the context of a system already carefully designed to prioritise welfare, transportation is, unsurprisingly, more stressful than no transportation, we have yet to put the magnitude of that stress into the context of the diversity of other stressors encountered by badgers either in their natural lives or during alternative handling protocols. It is exciting that our technique makes this calibration feasible, not merely in the special case of our study, but across a wide field of biological, agricultural, veterinary and medical applications.

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