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The effect of transport stress on neutrophil activation in wild badgers (Meles meles)

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Abstract

*Wild badgers (*Meles meles*) in Wytham woods, Oxfordshire, are routinely trapped, transported to a central field laboratory, studied and released as part of an on-going population study. These procedures have been carefully developed to minimise impact on the badgers' welfare; however they are potentially stressful, and, as part of our on-going welfare refinements, and our exploration to develop methods for quantifying stress in wild mammals, we studied the effects of transport stress on neutrophil activation in wild trapped badgers. Blood samples were obtained from 28 badgers. We compared three transport regimes: transported (n = 9), transported and rested for at least 30 mins (n = 11), and not transported (n = 8). Total and differential white cell counts were carried out and neutrophil activation was measured by the nitroblue tetrazolium test. Our goal was primarily to validate neutrophil activity as an indicator of stress, on the basis that the transport treatment was expected to be more stressful than the non-transport treatment. There were significant increases in % activated circulating neutrophils in response to transport. This study supports the proposition that stress affects circulating neutrophil numbers and the state of their activation, as determined by the nitroblue tetrazolium reduction assay, and therefore adds weight to the idea that neutrophil activation is a potential measure of stress in wild animals.*

Keywords: *animal welfare, badgers, measuring stress,* Meles meles*, neutrophil activation, transport*

Introduction

People's involvement with wild mammals, including for animal conservation or management, often involves intervention that may be stressful. For scientific and ethical reasons it is important to be able to measure such stress and aim to minimise it as far as possible. The quest for good measures of stress is therefore important and is a growing priority at the interface between the subjects of wildlife welfare and conservation (Bonacic *et al* 2003). One common type of intervention is transportation, and this offers both an easily manipulable treatment to study and an important opportunity to improve procedures in the field. To date, there have been few studies on the effects of transport stress on wild mammals, but in farm animals it has been shown that transport causes stress that affects the immune system (eg Blecha *et al* 1984; Kannan *et al* 2000).

Furthermore, there is considerable evidence to suggest that stress may reduce the effectiveness of the immune system, thus increasing the risk of infection or disease (Dhabhar *et al* 1996; Kang *et al* 1996; Raberg *et al* 1998). Our own research suggests that even short-term stressors can produce demonstrable and immediate physiological changes in heart rate, blood pressure and the activation of leukocytes (Ellard *et al* 2001). Activated leukocytes can release a whole host of mediators, which can potentially damage healthy tissue and organs (Weiss 1989; Boxer & Smolen 1998; Sanidas *et al* 2000). These mediators include cationic proteins, myeloperoxidase, lysozyme, acid hydrolases, lactoferrin (an iron-binding protein), B12-binding protein, cytochrome-b and collagenase (Rosales & Brown 1993). Other stress-induced leukocyte responses include neutrophilia and leukocytosis, although leukocytes do not always show all expected changes in response to stress: these changes depend on the nature and intensity of the stressor, the experimental design, species and a whole spectrum of other factors (Willard *et al* 1989; McLaren *et al* 2003). In humans, epidemiological studies support the idea that individuals who are more stressed are more prone to opportunistic infections (eg Graham *et al* 1986), and stress has been linked to disease in farm animals (eg Koolhaas *et al* 1999).

Stress-induced immunosuppression in wild mammals could thus have potentially serious consequences. The aim of this study was to explore further the use of neutrophil activity as a measure of stress in wild mammals. As a model system we used our long-term study of the behavioural ecology of badgers. For 16 years we have monitored badger demography through a programme of live-trapping (Macdonald & Newman 2002). Throughout this programme we have

striven to develop methodologies that minimise the stress to the badgers, and have been attentive to the impact of our intervention (Tuyttens *et al* 2002). Part of our procedure is a short journey from the capture site to a central processing area. Whilst we judge that this transportation is a helpful part of an overall system that optimises the handling of the badgers, we nevertheless expect that being transported is more stressful than not being transported. Therefore, in our test of neutrophil activation as a measure of stress, we compared badgers that were and were not transported. If our approach reveals a reliable measure of stress then we will be better equipped to improve our overall handling system in the future. We also wanted to determine if a short period of rest after transport ameliorated immune system changes brought about by transport stress.

Methods

Trapping and transporting badgers

Badgers were trapped in Wytham Woods, Oxfordshire (see Macdonald & Newman [2002] for details of the study site and its badger population), in cage traps baited with peanuts in August and November 2001. Traps were set each evening from 1600–1630h, and checked each morning between 0630–0700h. Trapped badgers were transferred to individual holding cages. Animals were then assigned to one of three experimental regimes: 1) sampling at the site of capture, without transport ('not transported', n = 8); 2) sampling immediately after transport ('transported', n = 9); or 3) transported then rested for between 30–90 mins prior to sampling ('rested', n = 11). Transport consisted of a short ride of less than 10 mins on a trailer pulled by an all-terrain quad bike. While in holding cages, and during the resting period, holding cages were covered with a blanket.

Badgers were anaesthetised using an intra-muscular injection of ketamine hydrochloride 100 mg/ml (Ketaset, Fort Dodge, USA) at a dose of 0.2 ml/kg. Blood was removed by needle venepuncture of the jugular vein, and collected into a tube containing the anticoagulant potassium EDTA (Vacutainer Systems, Plymouth, UK). This work was carried out under English Nature licence 1991537 and Home Office licence PPL 30/1826.

Leukocyte numbers

Total leukocyte counts (10^9/L) were made for each individual using a haematology analyser (Celltac MEK-5108K, Kohden, Japan). Differential cell counts were made from blood smears fixed in alcohol and stained with May-Grunwald and Giemsa stains. Leukocyte and differential cell count data were not obtained for one transported individual.

Nitroblue tetrazolium reduction assay

Nitroblue tetrazolium (NBT) (Sigma Diagnostics, catalogue number 840-10) was diluted in phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride; pH 7.4; Sigma Diagnostics, catalogue number P4417, batch 71K8201) at a concentration of 1 mg of NBT in 1 ml of PBS buffer. This solution was then stored in the dark at 4°C when not directly in use. Immediately after the blood sample was collected from the animal, a sample of 50 µl was transferred into an eppendorf tube from the EDTA vial. This tube was incubated at 37°C for 45 mins. After the incubation period, 50 µl of NBT was added to the tube, which was then incubated for a further 10 mins at 37°C. After incubation, 15 µl was taken from the tube and smeared onto a microscope slide and left to dry.

Once the slides were dry, they were flooded with 1 ml of Accustain Wright stain Modified (0.3% w/v, buffered at pH 6.9 in methanol, Sigma Diagnostics, batch 096 H4372) for 45 s. The slides were then washed with 1 ml of distilled water, allowed to stand for 1 min, washed with excess distilled water and allowed to dry.

Because this work was carried out alongside an on-going badger population study, and because we attach high priority to processing animals very quickly, there were strict time constraints that meant that the NBT test could not be carried out on all individuals. The NBT test sample sizes were: not transported: n = 5, transported: n = 9, and rested: n = 8.

Determining the % of activated neutrophils

Using the × 100 oil immersion objective, four areas of each slide were examined. The total numbers of activated and non-activated neutrophils were counted. Neutrophils were classified as active if they appeared irregular in shape and diffusely granular with intracytoplasmic formazan deposits. At least 100 neutrophils were counted on each slide, the count was repeated four times and the mean taken. The number of NBT-positive cells from these counts was considered to be the % of neutrophil activation.

Statistical analysis

All statistical analyses were carried out using SPSS for Windows, version 11.0. We used univariate General Linear Models (GLM) and *post hoc* Tukey least significant difference (LSD) tests to examine treatment effects.

Results

The levels of neutrophil activation in the three experimental groups are shown in Figure 1. Transport regime had a significant effect on neutrophil activation ($F_{2,19}$ = 12.4; $P < 0.001$), and activation was lowest in non-transported animals and highest immediately after transport. Levels of activation were significantly different between all groups (Tukey LSD: $P < 0.05$).

Table 1 shows the leukocyte number and composition data. A GLM revealed that there were significant differences between the three experimental groups in leukocyte number ($F_{2,24}$ = 5.4; $P < 0.05$), % neutrophils ($F_{2,24}$ = 3.4; $P = 0.05$) and % lymphocytes ($F_{2,24}$ = 3.5; $P < 0.05$). Treatment had a significant effect on the neutrophil–lymphocyte (NL) ratio ($F_{2,24}$ = 6.8; $P < 0.01$; Table 1). There was a significant increase in the NL ratio of transported animals compared both with non-transported and with rested animals (Tukey LSD, both comparisons: $P < 0.01$; Table 1). However, non-transported and rested badgers did not differ in their NL ratio (Tukey LSD: $P = 0.87$; Table 1). Both categories of

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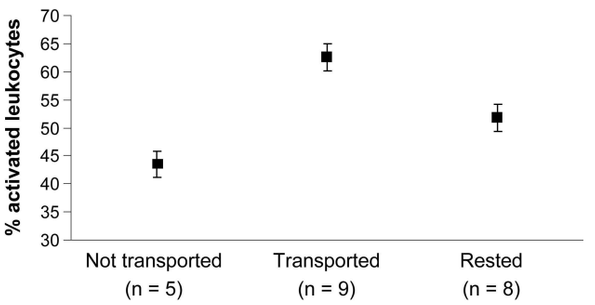
Table 1 The effect of transport regime on leukocyte numbers and composition in wild badgers. Figures in brackets are one standard error. (Blood cell data were not obtained for one transported individual.)

Transport regime	N	Leukocytes 10^9/L	% neutrophils	% lymphocytes	Neutrophil–lymphocyte ratio
Not transported	8	10.35 (0.93)	81.5 (2.4)	17.4 (2.4)	5.34
Rested	11	6.55 (0.78)	77.8 (3.4)	21.7 (3.6)	5.09
Transported	8	6.99 (0.94)	88.5 (2.1)	10.4 (2.1)	10.67

transported animals had significantly lower numbers of circulating leukocytes than did non-transported animals (Tukey LSD, both comparisons: $P < 0.05$; Table 1). Rest after transport did not affect the number of circulating leukocytes (Tukey LSD: $P > 0.05$; Table 1). For transported animals, rest had a significant effect on the NL ratio, and rested animals had a significantly greater % lymphocytes (Tukey LSD: $P < 0.05$; Table 1) and a correspondingly lower % neutrophils (Tukey LSD: $P < 0.05$; Table 1). Non-transported and transported animals did not differ in their % lymphocytes and neutrophils (Tukey LSD, both comparisons: $P > 0.05$; Table 1).

Figure 1



The effect of transport regime on leukocyte activation in wild badgers. Means and standard errors are shown.

Discussion

Transport stress caused an increase in % circulating NBT-positive neutrophils. Recent studies have demonstrated that the NBT reduction assay is a reliable measure of the activation of neutrophils in whole blood (Delano *et al* 1997; Takase *et al* 1999). Thus the significant increase in % NBT-positive neutrophils after transport reflects an activation process, perhaps preparing the body for an immune challenge. Resting brought about a reduction in the number of circulating activated neutrophils.

Transport significantly reduced the number of circulating leukocytes. Whilst it is accepted that in general a short-term stressor is usually associated with a catecholamine-associated demargination of leukocytes resulting in a doubling of cells — a finding we have also found in our laboratory (Mian *et al* 2003) — the relationship is not a simple one of acute stress equating to a simple doubling of leukocytes (Dhabhar *et al* 1996). The stress reaction depends on the nature, intensity and duration of the stressor, and on the time at which the blood sample is taken (Dhabhar *et al* 1996; Oishi *et al* 2003). In an elegant series of experiments, Oishi *et al* (2003) demonstrated that immune changes brought about by physical stimulators differed significantly from those induced by visual, olfactory and auditory stimuli in rats. The badgers in the present study would have been exposed to a multitude of stimuli as a result of transport. It is well known that activated leukocytes adhere to particular sized vessels within the microcirculation (Mian & Marshall 1993), thus the number of activated neutrophils may well be underestimated in samples obtained from the circulating pool. However, as expected there was a significant increase in the NL ratio in the transported animals, which is indicative of stress (Murata 1989; Haigh *et al* 1997; Maes *et al* 1998). Our data on the NL ratio revealed that resting reduced the ratio to pre-transport levels, and suggests that resting after transport and prior to anaesthesia leads to a reduction in stress levels at the time of sampling.

The changes in neutrophil numbers may have occurred as a result of an alteration in adhesion receptors on the neutrophils (L-selectin; integrins and PSGL-1 P-Selectin Glycoprotein Ligand-1) (Ley 1996) or the endothelium (P-selectin; Intracellular Adhesion Molecule 1). Neutrophils in the marginated pool use surface adhesion molecules, modulated by cytokines, to adhere to endothelial cells (Ley *et al* 1995; Ley 1996). Stress in some species can reduce the number of neutrophils attached in the marginal pools (and thus increase the number of circulating neutrophils). The extent to which this occurs will depend on the nature, intensity and duration of the stressor, and on the time at which the blood sample is taken (Oishi *et al* 2003). It is believed that stimuli such as adrenaline, other ß-agonists and interleukin-6 are able to stimulate this process. Rapid and reversible (within 25 mins) adrenaline and cortisol-induced leukocytosis has been reported in rats (Iversen *et al* 1994) and in humans (Toft *et al* 1994). In our study, only one blood sample was taken at the end of the stressor. It is possible that initial increases in leukocyte numbers could have occurred whilst the badgers were being transported. Recent studies by Maes *et al* (1999) have also revealed that an increase in the levels of pro-inflammatory cytokines, such as interleukin-6, interleukin-7 and tumour necrosis factor, would lead to the demargination of some neutrophils; the kinetics of interleukin-6 and interleukin-7 in badgers have not to our knowledge been studied.

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It has been recognised for some time that physiological stressors such as exercise induce leukocytosis from marginal pools (Hou *et al* 1996; Shephard & Shek 1996; Gleeson *et al* 1998). However, non-physical stressors have now also been shown to influence the number and distribution of neutrophils in the blood. Kang *et al* (1996, 1997) reported that the short-term, acute mental stress of academic examinations stimulated increases in the number and distribution of neutrophils in adolescent students. These changes were both rapid and reversible.

Two conclusions emerge from this study. Most importantly, we add weight to the proposal that neutrophil activation is a powerful tool for measuring stress in badgers and presumably in other wild mammals. Second, we use this technique to quantify the qualitatively obvious expectation that transportation is more stressful than no transportation. This is an important step in quantifying the pros and cons of different variations of our handling procedures. However, in considering our overall procedure, this result should not be taken in isolation and certainly does not indicate that transportation should be avoided. This is because the stress of transportation has to be weighed against the stress benefits it brings, such as access to a central processing facility and an overall reduction in processing time. However, armed with the neutrophil activation technique, we are now equipped to study the stress costs of each part of our procedure and to evaluate these in the wider context of costs and benefits.

This process offers opportunities to explore improvements, as illustrated by our discovery here that a 30 min rest period after transportation leads to a reduction in average neutrophil activation (from 61 to 50%) at the time of sampling — although we cannot yet say what these levels mean in terms of stress to an animal, or how important this reduction is. The pathophysiological relevance of the changes observed merit exploration. Transport *per se* is sufficient to alter the number and activation state of circulating neutrophils. Once activated, neutrophils would be unable to respond to opportunistic infections, thus rendering the host more susceptible to disease as well as to potential tissue damage from a host of proteolytic enzymes and oxygen free radicals.

Animal welfare implications

With sufficient exposure to transport stress it is possible that an animal could be more vulnerable to opportunistic infections. This could be particularly important in wild mammal studies involving transport. For example, animals that have been transported to a reintroduction site may be particularly vulnerable since transport stress will be combined with the stress of coping with a new environment. These considerations emphasise the importance of integrating studies of animal conservation and animal welfare.

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