

# LEUKOCYTE COPING CAPACITY AS A TOOL TO ASSESS CAPTURE- AND HANDLING-INDUCED STRESS IN SCANDINAVIAN BROWN BEARS (*URSUS ARCTOS*)

Núria Fandos Esteruelas,<sup>1,10</sup> Nikolaus Huber,<sup>2</sup> Alina L. Evans,<sup>1</sup> Andreas Zedrosser,<sup>3,4</sup> Marc Cattet,<sup>5</sup> Francisco Palomares,<sup>6</sup> Martine Angel,<sup>1</sup> Jon E. Swenson,<sup>7,8</sup> and Jon M. Arnebo<sup>1,8,9</sup>

<sup>1</sup> Department of Forestry and Wildlife Management, Faculty of Applied Ecology and Agricultural Sciences, Hedmark University College, Campus Evenstad, NO-2418 Elverum, Norway

<sup>2</sup> Research Institute of Wildlife Ecology, Department of Integrative Biology and Evolution, University of Veterinary Medicine, Savoyenstrasse 1, A-1160 Vienna, Austria

<sup>3</sup> Department of Environmental and Health Studies, Faculty of Arts and Sciences, Telemark University College, PO Box 203, NO-3901 Porsgrunn, Norway

<sup>4</sup> Department of Integrative Biology and Biodiversity Research, Institute for Wildlife Biology and Game Management, University of Natural Resources and Applied Life Sciences, Gregor Mendel St. 33, A-1180 Vienna, Austria

<sup>5</sup> Canadian Wildlife Health Cooperative, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Dr., SK S7N 5B4 Saskatoon, Canada

<sup>6</sup> Department of Conservation Biology, Estación Biológica de Doñana–CSIC, Calle Américo Vespucio s/n, Isla de la Cartuja, 41092, Seville, Spain

<sup>7</sup> Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, PO Box 5003, NO-1432 Ås, Norway

<sup>8</sup> Norwegian Institute for Nature Research, PO Box 5685 Sluppen, NO-7485 Trondheim, Norway

<sup>9</sup> Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences, Skogsmarksgränd St., SE-901 83 Umeå, Sweden

<sup>10</sup> Corresponding author (email: nfanest@gmail.com)

**ABSTRACT:** Brown bears (*Ursus arctos*) are often captured and handled for research and management purposes. Although the techniques used are potentially stressful for the animals and might have detrimental and long-lasting consequences, it is difficult to assess their physiological impact. Here we report the use of the leukocyte coping capacity (LCC) technique to quantify the acute stress of capture and handling in brown bears in Scandinavia. In April and May 2012 and 2013, we collected venous blood samples and recorded a range of physiological variables to evaluate the effects of capture and the added impact of surgical implantation or removal of transmitters and sensors. We studied 24 brown bears, including 19 that had abdominal surgery. We found 1) LCC values following capture were lower in solitary bears than in bears in family groups suggesting capture caused relatively more stress in solitary bears, 2) ability to cope with handling stress was better (greater LCC values) in bears with good body condition, and 3) LCC values did not appear to be influenced by surgery. Although further evaluation of this technique is required, our preliminary results support the use of the LCC technique as a quantitative measure of stress.

**Key words:** Animal welfare, brown bear, capture, chemical immobilization, leukocyte coping capacity, stress, surgery, *Ursus arctos*.

## INTRODUCTION

Effective wildlife research and management often require the capture and handling of animals. However, the evaluation of capture and handling effects on target animals is often overlooked, despite the high potential for significant stress (Cattet 2013). For example, data loggers are increasingly used in research to enable remote collection of physiological information. This often involves surgical implantation, which can cause pain

and distress (Hawkins 2004) or can lead to mortality (Quinn et al. 2010; Léchenne et al. 2012). Studying changes in physiological parameters due to capture is important because morbidity can cause subtle but harmful effects that might go undetected (Cattet et al. 2003) and bias research data (Powell and Pro-lux 2003; Cattet et al. 2008).

For animal welfare, objective and quantitative measures of stress are central (McLaren et al. 2007). Several techniques can be used to measure the stress response

in animals (Palme and Möstl 1997; Windle et al. 1997b; Millspaugh et al. 2000), but to date, blood concentrations of glucocorticoids (GCs) has been the most widely used parameter to assess the acute stress of capture in free-ranging wild animals (Creel et al. 1997; Arnemo and Caulkett 2007; Delehanty and Boonstra 2009). However, GC levels alone may not equate to stress levels (Sheriff et al. 2011). Using GC levels to measure stress can be complicated, as they are affected by multiple factors, including time of day, season, handling, and anesthetic drugs (Boonstra et al. 2001; Owen et al. 2005; Arnemo and Caulkett 2007). Consequently, using cortisol measurements alone to accurately measure stress in an individual can be challenging, and results should be interpreted with caution.

Recently the interaction between stress and the immune system has received attention. Stress affects the immune system by altering the quantity, composition, activity, and responsiveness of circulating immune cells (Dhabhar et al. 1995; Ellard et al. 2001). Leukocytes circulating in the blood have receptors that are sensitive to biochemical alterations linked to stress (Mian et al. 2005). In response to external stimuli, e.g., stressful situations, leukocytes (particularly neutrophils) are activated and release reactive oxygen species (ROS) via a process called respiratory burst (Ellard et al. 2001; Montes et al. 2004). During respiratory burst, oxygen uptake by leukocytes accelerates to produce ROS that destroy bacteria and other pathogens (Halliwell and Gutteridge 2007). However, the respiratory burst activity of leukocytes decreases in individuals of several animal species in association with stress caused by transport (McLaren et al. 2003), trapping and handling (Gelling et al. 2009), and housing conditions (Honest et al. 2005; Moorhouse et al. 2007) and by psychological stress in humans (Ellard et al. 2001; Shelton-Rayner et al. 2010). Also, leukocytes produce ROS in response to agonists such as bacterial peptides and the activation of protein kinase C with

phorbol myristate acetate (PMA; Hu et al. 1999). After a stressful event, there is a latent period when the neutrophils' capacity to respond to a secondary external stimulus (e.g., bacterial challenge, PMA) is reduced (McLaren et al. 2003). As a result, an animal can be immunocompromised. By quantifying the reduction in the amount of ROS released by leukocytes in response to a secondary stimulus, one can assess the effect of the known or suspected stressor (Mian et al. 2005). The response of leukocytes to PMA challenge after a stressful event is defined as the individual's leukocyte coping capacity (LCC). Therefore, animals with a higher LCC will have greater potential to produce a respiratory burst and will be better able to respond to bacterial challenge after stress. Hence, LCC is an *in vitro* assessment of the animal's current physiological status and its overall ability to cope with stress (McLaren et al. 2003).

In this study, we used the LCC technique to investigate the stress response caused by capture and subsequent abdominal surgery of free-ranging brown bears (*Ursus arctos*). Our primary goal was to evaluate LCC values in relation to life history traits (social status, body condition), capture-related variables (pursuit time, medetomidine dose, number of times the bear had been captured), and intensity of handling (surgery, no surgery). We also aimed to compare LCC results with established methods to measure and quantify acute stress: heart rate, neutrophil-to-lymphocyte (N:L) ratio, and blood glucose and cortisol concentrations. We hypothesized that 1) bears within family groups would have higher LCC values than solitary bears, 2) bears in better body condition would have higher LCC values, 3) bears with longer pursuit times during capture would have lower LCC values, 4) bears undergoing surgery would have lower LCC values, and 5) there would be a negative correlation between LCC and other physiological measures of stress.

Animal welfare is relevant for conservation biology (McLaren et al. 2007). Stress

measurements allow for the refinement of capture and handling protocols and, therefore, improvements in animal welfare. From the perspective of evaluating wildlife welfare, our broader goal with this study was to determine if the LCC technique could be used as a practical and reliable method under field research conditions to evaluate the stress response of captured brown bears. If this technique proved to be dependable, it could have future application as a basis for improving techniques of capture and handling free-ranging brown bears.

## MATERIALS AND METHODS

### Study area and animals

Field work was conducted in south-central Sweden (61°N, 15°E). Animals were captured in April–May 2012 and 2013, shortly after they exited the dens after hibernation. Ambient temperatures ranged from 2 to 5 C. Brown bears were anesthetized for GPS collaring and sampling for ecological studies within the Scandinavian Brown Bear Research Project.

### Capture methods and handling procedures

Bears were immobilized according to the biomedical protocol used for captures of free-ranging brown bears in Scandinavia (Arnemo et al. 2012). All captures were approved by the Swedish Ethical Committee on Animal Research (application number C 7/12) and the Swedish Environmental Protection Agency.

Anesthetic agents were administered by remote darting from a helicopter with a CO<sub>2</sub>-powered rifle (Dan-Inject®, Børkop, Denmark). We used a combination of medetomidine (Domitor® 1 mg/mL or Zalopine® 10 mg/mL, Orion Pharma Animal Health, Turku, Finland) and tiletamine-zolazepam (Zoletil® 500 mg/vial, Virbac, Carros, France) at standard doses depending on the estimated weight of the animal. Ketamine (Narketan 10®, 100 mg/mL, Chassot, Dublin, Ireland) was used to extend immobilization when needed based on monitoring anesthetic depth. The movement of bears with the helicopter was kept to less than 3 min, with active pursuit lasting no more than 30 s. We recorded time of pursuit, defined as the time between first observation and when the bear was immobilized on the ground (recumbency). All yearlings were naïve to capture, whereas the other bears had been captured 1–12 times previously.

Once anesthetized, we recorded the bear's capillary refill time, respiratory rate, heart rate, and rectal temperature and assessed these parameters every 15 min throughout anesthesia. We collected two heparinized blood samples from the jugular vein from each bear using a vacutainer system (BD Vacutainer®, BD Diagnostics, Preanalytical Systems, Franklin Lakes, NJ, USA). We collected the first sample as early as possible after recumbency to assess the stress of capture. We performed complete blood counts, serum biochemistry, cortisol, and LCC determination from this sample. Hematology and chemistry analysis followed standard procedures; see Græsli et al. (2014). We collected the second sample 90 min after recumbency, during or after surgery, and measured LCC to assess the stress of surgery. Our study focused on stress caused by surgical implantation or removal of radio transmitters, physiological sensors, and temperature loggers in the peritoneal cavity. For analgesia, we administered 4 mg/kg carprofen (Rimadyl® vet. 50 mg/mL, Orion Pharma Animal Health, FI-02200 Espoo, Finland) or 0.2 mg/kg meloxicam (Metacam® 5 mg/mL, Boehringer Ingelheim, Reihn, Germany) subcutaneously before the surgery started. After completing all procedures, we administered 5 mg of atipamezole (Antisedan® 5 mg/mL, Orion Pharma Animal Health, Turku, Finland) per mg of medetomidine intramuscularly and left the bears to recover undisturbed at the capture site.

### Leukocyte Coping Capacity (LCC) measurement

To measure the unstimulated blood chemiluminescence levels and provide a baseline with which to measure an individual's LCC response, we immediately transferred 10 µL of heparinized whole blood into a silicon antireflective tube (Lumivial, EG & G Berthold, Germany) and added 90 µL of 10<sup>-4</sup> mol L<sup>-1</sup> luminol (5-amino-2,3-dihydrophthalazine; Sigma A8511, Sigma-Aldrich, Oslo, Norway) diluted in phosphate buffered saline (PBS). We shook the tube gently for mixing. Luminol chemiluminesces when combined with an oxidizing agent to produce a low-intensity light reaction (Whitehead et al. 1992). To measure the chemiluminescence produced in response to challenge, we prepared another tube as above but added 10 µL of phorbol 12-myristate 13-acetate (PMA; Sigma P8139, Sigma-Aldrich, Oslo, Norway) at a concentration of 10<sup>-5</sup> mol L<sup>-1</sup>. The PMA solution had been prepared in advance by diluting 5 mg of PMA in 500 µL of dimethyl sulfoxide (Sigma D 5879, Sigma-Aldrich, Oslo, Norway), which was then diluted to a concentration of 10<sup>-5</sup> mol L<sup>-1</sup> in PBS buffer (Shelton-Rayner et al.

2012). Individual aliquots were kept in the dark at  $-20\text{ C}$  until required. For each tube, we measured chemiluminescence in relative light units using a portable chemiluminometer (Junior LB 9509, E G & G Berthold, Germany) every 5 min for a total of 30 min. The measurements were done in the field immediately after the blood sample was collected. When not in the chemiluminometer, tubes were incubated at  $37\text{ C}$  in a lightproof water bath.

### Statistical analysis

We categorized the bears according to the following criteria; social status: solitary (single animals: males, females without dependent offspring) or family groups (mothers with dependent offspring) and whether or not surgery was performed. We also estimated a sex-specific body condition index by standardizing the residuals of the regression of body mass against body length for males and females separately (Cattet et al. 2002).

To summarize the LCC measurements over a 30-min period, we calculated the area under the response curve (AUC) (Fekedulegn et al. 2007). To ensure that there was no bias in the LCC results due to individual differences, we subtracted the PMA-unstimulated from the PMA-stimulated values for each animal and used these values for the AUC calculation. We also assessed the LCC per  $10^9$  neutrophils  $\text{L}^{-1}$  to examine the effect of the number of circulating neutrophils on ROS production.

We applied generalized linear models (GLMs) to evaluate the effects of life history traits, variables of capture and surgery on LCC, leukocyte counts and composition, and N:L ratio. We performed separate GLMs for measurements of the first and the second blood samples. The response variables for the first blood sample were AUC1, LCC1, total leukocyte counts, percentage of neutrophils and lymphocytes, and N:L ratio. AUC1 was defined as the area under the response curve for the first blood sample. LCC1 was defined as the LCC peak value (mean of the maximum LCC measurements, regardless of when they occurred during the 30-min period) for the first blood sample. We used two different sets of explanatory variables for analysis relating to the first blood sample. The first set contained the variables “social status” and “body condition.” The second set contained the variables “pursuit time,” “medetomidine dose,” and the lifetime “number of captures.” We constructed four candidate models for the first set and eight models for the second set of explanatory variables *a priori*, based on our hypotheses. The candidate models contained all possible combinations of variables.

For the second blood sample, the response variables were AUC2 and LCC2 (area under the response curve and LCC peak value for the second blood sample, respectively). The explanatory variables were “social status,” “body condition,” and whether a “surgery was performed or not.” We also constructed eight *a priori* models for all possible combinations of variables for the second blood sample.

We did not include interactions among variables into the models, due to low sample size. We selected the most parsimonious model, based on Akaike’s Information Criterion corrected for small sample sizes ( $\text{AIC}_c$ ) (Burnham and Anderson 2002; Burnham et al. 2011). For model selection we used  $\Delta\text{AIC}_c \leq 2$  and Akaike model weights ( $\text{AIC}_{c\text{wt}}$ ) (Burnham and Anderson 2004). Due to model selection uncertainty, we also applied a full-model averaging approach and used the relative importance of the predictor variables (Symonds and Moussali 2011).

We used parametric statistics (Pearson’s correlation) to investigate correlations among variables and present the mean  $\pm$  standard deviation for all variables. Differences were considered significant when  $P \leq 0.05$ . For statistical analysis we used the software R 3.0.2 (R Development Core Team 2012).

## RESULTS

### Study animals

We used 24 bears in the study: six yearlings, five subadults, and 13 adults; 10 males and 14 females; and 12 were solitary and 12 were part of a family group. We conducted surgery on 19 bears (Table 1). No mortalities occurred during anesthesia or within 30 days postcapture.

### Leukocyte coping capacity (LCC)

We obtained the first and second blood samples  $30 \pm 12$  min and  $93 \pm 8$  min after recumbency, respectively. For the first sample, the AUC1 was mainly affected by the social status of a bear. Members of a family group had a higher AUC1 than solitary bears at capture (Tables 2–3; Fig. 1). For the second sample, body condition had a positive effect on AUC2 values; bears in better body condition had a higher AUC2. We also used the LCC per  $10^9$  neutrophils  $\text{L}^{-1}$  as response variable and obtained the same results.

TABLE 1. Sex, age, social status, type of handling (surgery vs. no surgery), and leukocyte coping capacity measured in 24 brown bears anesthetized in Sweden in April–May 2012 and 2013.

Bear ID	Sex <sup>a</sup>	Age <sup>b</sup>	Social status <sup>c</sup>	Surgery <sup>d</sup>	AUC1 <sup>e</sup>	LCC1 <sup>f</sup>	AUC2 <sup>g</sup>	LCC2 <sup>h</sup>
W0806	F	5	S	Y	11,015.5	556	15,070	801
W0904	F	4	S	Y	11,618	594	10,362	502
W1019	M	8	S	Y	9,635.5	605	15,576.5	860
W0820	F	5	S	Y	4,724	308	8,086	549
W0818	F	5	S	Y	3,391.5	239	4,244	362
W0716	F	11	F	Y	23,483	1,071	21,901	1,255
W1204	M	1	F	Y	31,557	1,630	24,314	1,681
W0104	F	12	F	Y	13,411	557	21,863	1,365
W0620	F	7	F	Y	16,477.5	789	21,469	1,172
W1207	M	1	F	N	12,490	704	13,375	753
W1103	M	3	S	Y	7,840.5	423	9,542	521
W0812	M	6	S	Y	9,148.5	555	13,289.5	798
W0811	M	6	S	N	16,975	854	41,329.5	2,317
W1210	M	4	S	Y	25,042	1,635	29,607	1,532
W0625	M	10	S	Y	17,572	1,103	27,321.5	1,849
W0825	F	6	S	N	9,090.5	510	25,459	1,443
W0610	F	8	S	Y	8,700.5	545	24,782.5	1,248
W1301	M	1	F	Y	15,140	978	28,450	1,619
W1302	M	1	F	Y	16,487	999	26,995	1,655
W9403	F	20	F	Y	21,507.5	1,135	29,508	2,312
W1303	F	1	F	Y	6,347	340	13,891.5	895
W1304	F	1	F	Y	15,272	861	16,346.5	1,005
W1206	F	2	F	N	17,708.5	1,123	21,728.5	1,264
W1205	F	2	F	N	17,232	909	16,068	910
Mean $\pm$ SD		5 $\pm$ 5			14,244.4 $\pm$ 6,734.58	792 $\pm$ 372	20,024.1 $\pm$ 8,562.95	1,195 $\pm$ 538

<sup>a</sup> F = female; M = male.

<sup>b</sup> In years.

<sup>c</sup> S = solitary (no other bears observed during the capture); F = family (mothers with cubs).

<sup>d</sup> Y = yes; N = no.

<sup>e</sup> Area under the response curve (in relative light units) for leukocyte coping capacity measurements obtained as soon as the animal was immobilized.

<sup>f</sup> Maximum leukocyte coping capacity value (in relative light units) obtained as soon as the animal was immobilized.

<sup>g</sup> Area under the response curve (in relative light units) for leukocyte coping capacity measurements obtained during or after surgery.

<sup>h</sup> Maximum leukocyte coping capacity value (in relative light units) obtained during or after surgery.

From LCC peaks, we found that LCC1 was produced at 15 min in 55% of bears, with other peaks produced at 5 (4%), 10 (29%), 20 (8%), and 30 (4%) min. Social status was an important variable affecting LCC1 values (Tables 2–3; Fig. 1). Bears in family groups had higher LCC1 values than solitary bears. Capture-related vari-

ables, such as medetomidine dose, pursuit time, and number of captures, did not explain the variation in LCC1 values (Tables 2–3). For the second sample, LCC2 values were produced at 15 min in 70% of cases, with other peaks produced at 10 (13%), 20 (13%), and 25 min. (4%). Body condition also influenced LCC2 values; bears in

TABLE 2. Candidate models for the stress response to capture (measured by AUC1 and LCC1) and surgery (measured by AUC2 and LCC2) of 24 brown bears anaesthetized in Sweden in April–May 2012 and 2013. The four or five models with the lowest AIC<sub>c</sub> for each response variable are presented.

Response variable	Candidate models	<i>k</i> <sup>a</sup>	AIC <sub>c</sub> <sup>b</sup>	ΔAIC <sub>c</sub> <sup>c</sup>	AIC <sub>c</sub> W <sub>t</sub> <sup>d</sup>
AUC1 <sup>e</sup>	Social status	3	491.78	0.00	0.64
	Body condition+Social status	4	494.66	2.88	0.15
	Null	2	494.78	3.00	0.14
	Body condition	3	497.56	4.78	0.06
LCC1 <sup>f</sup>	Social status	3	355.03	0.00	0.47
	Null	2	355.78	0.75	0.32
	Body condition+Social status	4	357.93	2.90	0.11
	Body condition	3	358.08	3.05	0.10
LCC1 <sup>f</sup>	Null	2	355.78	0.00	0.39
	Number of captures	3	357.26	1.49	0.19
	Pursuit time	3	357.87	2.09	0.14
	Medetomidine dose	3	358.10	2.32	0.12
	Number of captures+Pursuit time	4	359.66	3.88	0.06
AUC2 <sup>g</sup>	Body condition	3	505.45	0.00	0.28
	Body condition+Surgery	4	505.75	0.30	0.24
	Null	2	506.31	0.86	0.18
	Surgery	3	507.76	2.31	0.09
	Body condition+Social status	4	508.33	2.88	0.07
LCC2 <sup>h</sup>	Body condition	3	371.57	0.00	0.37
	Body condition+Surgery	4	372.94	1.36	0.19
	Null	2	373.45	1.88	0.14
	Body condition+Social status	4	374.15	2.57	0.10
	Social status	3	374.59	3.01	0.08

<sup>a</sup> Number of estimated parameters.

<sup>b</sup> Akaike's Information Criterion corrected for small sample sizes.

<sup>c</sup> Differences in AIC<sub>c</sub> values between the best model (lowest AIC<sub>c</sub>) and each candidate model.

<sup>d</sup> AIC weights.

<sup>e</sup> Area under the response curve for leukocyte coping capacity measurements obtained as soon as the animal was immobilized.

<sup>f</sup> Maximum leukocyte coping capacity value obtained as soon as the animal was immobilized.

<sup>g</sup> Area under the response curve for leukocyte coping capacity measurements obtained during or after surgery.

<sup>h</sup> Maximum leukocyte coping capacity value obtained during or after surgery.

better body condition had higher LCC2 values. The relative importance of social status and surgery was low and neither influenced LCC2 values.

#### Physiological variables, complete blood counts, and biochemistry

Mean values for complete blood counts and biochemistry parameters were within the reference range for the species (Græsli et al. 2014). All animals were considered to be in good health status.

Life history traits did not affect total leukocyte numbers but did affect leukocyte composition and N:L ratio (Tables 4–5). Members of family groups had a higher proportion of neutrophils, a lower proportion of lymphocytes and, therefore, a higher N:L ratio than solitary bears.

AUC and LCC peak values in both samples did not correlate with any of the other parameters used as stress indicators, such as heart rate, N:L ratio, or glucose and cortisol concentrations (Table 6).

TABLE 3. Model averaging for the stress response to capture (measured by AUC1 and LCC1) and surgery (measured by AUC2 and LCC2) of 24 brown bears anaesthetized in Sweden in April–May 2012 and 2013.

Response variable	Predictor variable	$\beta^a$	2.5% CI <sup>b</sup>	97.5% CI <sup>b</sup>	SE <sup>c</sup>	Variable importance <sup>d</sup>
AUC1 <sup>e</sup>	Intercept	16,634.4	12,614.15	20,959.78	2,099.9	
	Social status (solitary)	-6,004.2	-11,288.57	-719.81	2,546.6	0.80
	Body condition	475.9	-2,626.34	3,005.48	1,434.2	0.21
LCC1 <sup>f</sup>	Intercept	868.90	645.25	1,110.15	113.87	
	Social status (solitary)	-264.71	-570.63	41.21	147.42	0.58
	Body condition	17.77	-167.75	158.48	80.91	0.21
LCC1 <sup>f</sup>	Intercept	875.52	529.7	1,206.15	175.17	
	Number of captures	-23.69	-71.16	23.81	22.91	0.31
	Pursuit time	-6.45	-25.89	12.86	9.38	0.24
	Medetomidine dose	-1,379.25	-6,574.64	3,766.76	2,500.34	0.23
AUC2 <sup>g</sup>	Intercept	21,918	14,739.12	28,660.04	3,553	
	Body condition	3,287	-226.68	6,737.06	1,692	0.64
	Surgery (yes)	-5,783	-14,505.34	2,879.92	4,232	0.40
	Social status (solitary)	-1,144	-9,266.57	6,175.39	3,801	0.21
LCC2 <sup>h</sup>	Intercept	1,273.3	891.83	1,631.91	191.0	
	Body condition	219.9	3.60	435.49	104.6	0.70
	Surgery (yes)	-267.2	-821.62	270.34	266.4	0.30
	Social status (solitary)	-170.5	-658.26	294.46	233.4	0.25

<sup>a</sup> Model averaged coefficients.

<sup>b</sup> Confident intervals.

<sup>c</sup> Standard error.

<sup>d</sup> Relative importance of the predictor variables.

<sup>e</sup> Area under the response curve for leukocyte coping capacity measurements obtained as soon as the animal was immobilized.

<sup>f</sup> Maximum leukocyte coping capacity value obtained as soon as the animal was immobilized.

<sup>g</sup> Area under the response curve for leukocyte coping capacity measurements obtained during or after surgery.

<sup>h</sup> Maximum leukocyte coping capacity value obtained during or after surgery.

## DISCUSSION

We determined in this study that LCC values in captured brown bears were primarily influenced by their social status and body condition, but surgical effects appeared to be minimal to inconsequential. Further, LCC values did not correlate with more conventional measures of physiological stress, including serum cortisol concentrations.

### Stress of capture

Stress affects the number and distribution of circulating leukocytes rapidly and reversibly (Dhabhar et al. 1995). In our study, LCC was not affected by the number of circulating neutrophils, as shown in

McLaren et al. (2003). However, the stress of capture influenced ROS production and leukocyte composition. The bear's social status was the main evaluated factor shaping the stress response to capture in Scandinavian brown bears. Members of a family group had higher overall LCC levels (calculated as the increase of the area under the curve), as well as LCC peak levels, than solitary bears. This confirmed our first hypothesis, suggesting that mothers with dependent offspring had greater capacity to cope with capture-induced stress and might have a higher ability to combat infection after the capture event. Studies suggest that social interactions in humans (Kirschbaum et al. 1995) and affiliative

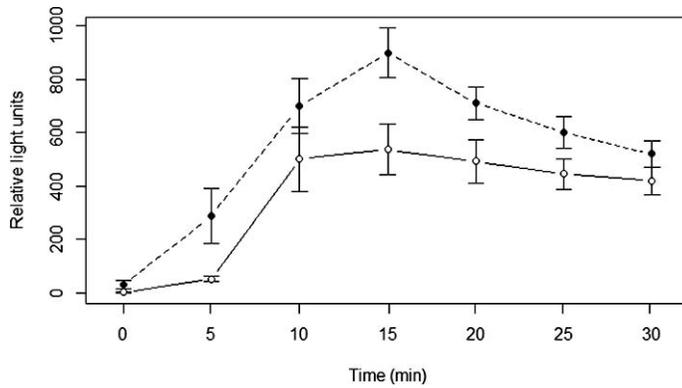


FIGURE 1. Leukocyte coping capacity measured every 5 min over 30 min time in 24 brown bears (*Ursus arctos*) captured in Sweden in April–May 2012 and 2013. The measurements represent the mean leukocyte coping capacity values (in relative light units) by social status (solitary bear or bear within a family group) for the blood sample collected as soon as possible after recumbency. The black dots connected by the dashed line represent values for bears in family groups; the white dots connected by the solid line represent solitary bears. Error bars are represented for each time point.

behaviors in animals (Giralt and Armario 1989; Smith and French 1997) could provide a buffer against stress by dampening the hypothalamic-pituitary-adrenal (HPA) axis response (Carter 1998). However, little

is known about how positive social interactions suppress corticosteroids. Some studies suggest a mechanism involving oxytocin (Cook 1997; Windle et al. 1997a), which is implicated in both the modulation of the

TABLE 4. Candidate models for the stress response to capture (measured by leukocyte counts, leukocyte composition, and neutrophil-to-lymphocyte ratio) of 24 brown bears anaesthetized in Sweden in April–May 2012 and 2013. The four models with the lowest  $AIC_c$  for each response variable are presented.

Response variable	Candidate models	$k^a$	$AIC_c^b$	$\Delta AIC_c^c$	$AIC_c Wt^d$
Total leukocyte counts	Null	2	56.09	0.00	0.68
	Body condition	3	59.09	3.00	0.15
	Social status	3	59.12	3.03	0.15
	Body condition+Social status	4	62.72	6.63	0.02
% Neutrophils	Social status	3	119.60	0.00	0.62
	Null	2	122.56	2.96	0.14
	Body condition	3	122.73	3.14	0.13
	Body condition+ Social status	4	123.07	3.47	0.11
% Lymphocytes	Social status	3	122.42	0.00	0.61
	Null	2	124.68	2.26	0.20
	Body condition+Social status	4	126.03	3.61	0.10
	Body condition	3	126.30	3.87	0.09
Neutrophil-to-lymphocyte ratio	Social status	3	74.05	0.00	0.38
	Body condition	3	74.29	0.24	0.34
	Null	2	75.80	1.76	0.16
	Body condition+Social status	4	76.40	2.35	0.12

<sup>a</sup> Number of estimated parameters.

<sup>b</sup> Akaike's Information Criterion corrected for small sample sizes.

<sup>c</sup> Differences in  $AIC_c$  values between the best model (lowest  $AIC_c$ ) and each candidate model.

<sup>d</sup>  $AIC_c$  weights.

TABLE 5. Model averaging for the stress response to capture (measured by leukocyte counts, leukocyte composition, and neutrophil-to-lymphocyte ratio) of 24 brown bears anaesthetized in Sweden in April–May 2012 and 2013.

Response variable	Predictor variable	$\beta^a$	2.5% CI <sup>b</sup>	97.5% CI <sup>b</sup>	SE <sup>c</sup>	Variable importance <sup>d</sup>
Total leukocyte counts	Intercept	5.26	4.60	5.93	0.33	
	Body condition	-0.09	-0.84	0.66	0.37	0.18
	Social status (solitary)	0.12			0.69	0.17
% Neutrophils	Intercept	70.75	64.01	77.33	3.19	
	Social status (solitary)	-10.88	-20.44	-1.70	4.61	0.73
	Body condition	2.82	-1.22	9.75	3.19	0.24
% Lymphocytes	Intercept	18.78	11.38	25.61	3.45	
	Social status (solitary)	11.31	0.56	22.07	5.01	0.71
	Body condition	-1.28	-6.65	7.63	3.64	0.19
Neutrophil-to-lymphocyte ratio	Intercept	4.55	3.03	6.02	0.72	
	Social status (solitary)	-2.14	-4.60	-0.08	1.18	0.50
	Body condition	1.09	0.01	2.42	0.64	0.46

<sup>a</sup> Model averaged coefficients.

<sup>b</sup> Confident intervals.

<sup>c</sup> Standard error.

<sup>d</sup> Relative importance of the predictor variables.

HPA axis and prosocial behaviors (DeVries et al. 2003).

### Stress of surgery

Body condition was an influential factor in the ROS production by leukocytes after capture and surgery in our study animals. Bears in better body condition had higher overall LCC and peak levels, indicating that they coped better with handling stress. This confirmed our second hypothesis, agreeing with studies in birds and mammals

that have concluded that animals in better body condition show an enhanced immune response (Alonso-Álvarez and Tella 2001; Bachman 2003).

We found no difference in LCC levels related to surgery. Therefore, we rejected our fourth hypothesis that bears undergoing surgery would have lower values of LCC. However, the conclusion that surgery was not an additional stressor at the time of sampling must be interpreted cautiously. The low sample size of the study

TABLE 6. Association among heart rate, neutrophil-to-lymphocyte ratio, glucose and cortisol concentrations, and LCC measurements in 24 brown bears anaesthetized in Sweden in April–May 2012 and 2013. Pearson correlation coefficients ( $r$ ) and  $P$  values (in parentheses) are shown.

	AUC1 <sup>a</sup>	AUC2 <sup>b</sup>	LCC1 <sup>c</sup>	LCC2 <sup>d</sup>
Heart rate	-0.47 (0.07)	0.08 (0.76)	-0.31 (0.24)	-0.004 (0.99)
Neutrophil-to-lymphocyte ratio	0.43 (0.10)	0.03 (0.89)	0.27 (0.31)	0.17 (0.52)
Glucose	0.16 (0.45)	0.11 (0.61)	0.29 (0.17)	0.10 (0.65)
Cortisol	-0.30 (0.15)	-0.04 (0.85)	-0.25 (0.24)	-0.02 (0.93)

<sup>a</sup> Area under the response curve for leukocyte coping capacity measurements obtained as soon as the animal was immobilized.

<sup>b</sup> Area under the response curve for leukocyte coping capacity measurements obtained during or after surgery.

<sup>c</sup> Maximum leukocyte coping capacity value obtained as soon as the animal was immobilized.

<sup>d</sup> Maximum leukocyte coping capacity value obtained during or after surgery.

( $n=24$ ) and the control group ( $n=5$ ), and the time the blood sample was obtained, could have influenced the results. Moreover, the administration of additional analgesic drugs to bears undergoing surgery could help explain the results.

Our second blood sample was collected  $49 \pm 14$  min after the surgery started. Although the production of ROS increases after surgical injury (Wakefield et al. 1993), the exact time at which this increase occurs is not known. Shelton-Rayner (2009) stated that neutrophils react within an hour of tissue injury during an acute inflammatory response. In studies in humans and animals, leukocytes counts increased from hours to days postoperatively (Kreeger et al. 1990; Yokoyama et al. 2005). Other parameters, such as cortisol and IL-6, a cytokine that has a major role in the early inflammatory response to surgery, also increased their levels within minutes after surgery, but the increase was not significant before 2–6 h (Desborough 2000). Therefore, time of sampling would be an important factor to account for in future studies aiming to quantify the stress response.

Analgesic drugs, which were only administered to bears undergoing surgery, can attenuate the stress response to surgery (Rademaker et al. 1992; Kehlte and Holte 2001). However, nonsteroidal anti-inflammatory drugs, such as meloxicam and carprofen, are analgesics with little effect on surgical stress responses (Kehlte and Holte 2001). In our case they provided postoperative analgesia rather than reduced the stress response to surgery.

In addition, anesthetic drugs (medetomidine+tiletamine-zolazepam), that were used in all bears, can modify the stress response by affecting the HPA axis (Desborough 2000; Ko et al. 2000; Bentson et al. 2003; Champagne et al. 2012). Nonetheless, we believe that the LCC measurements after capture were representative of the stress experienced by the bears. This is because the stressor, the capture event, occurred before the administration of the anesthetic drugs, presumably

allowing complete activation of the stress response. Thus, the effect of the anesthetic drugs, which was not immediate, was probably minimal on an already-established endocrine response. On the other hand, for the LCC measurements 90 min after the bears were recumbent, the stress response to surgery was probably blocked or diminished by the use of anesthetics  $\pm$  analgesics and were therefore not representative of the stress experienced by the bears.

#### **LCC peaks and variables of capture**

Capture variables affect an animal's physiological parameters, including body temperature and cortisol levels (Armeno and Ranheim 1999; Cattet et al. 2003). We rejected our third hypothesis that bears with longer pursuit time during capture would have lower LCC values; neither pursuit time nor medetomidine dose had a significant effect on the LCC response. Bears probably became aware of the helicopter before being observed from the air, which perhaps resulted in an inaccurate estimate of pursuit time. Additionally, the dose of medetomidine administered was estimated, as a few darts were not retrieved.

We also assessed the number of captures an animal had experienced. Shelton-Rayner et al. (2010) suggested that leukocyte reactivity exhibits habituation in humans. However, we found no effect of the number of captures on LCC levels and concluded that there was no habituation to capture. We could argue that capture is a strong negative stimulus, therefore not causing habituation in this species. A more complex analysis of the data would be necessary to properly evaluate this variable.

#### **Leukocyte number and composition**

Differences in leukocyte composition and the N:L ratio were mainly due to social status. We discovered a higher proportion of neutrophils and N:L ratio and a lower proportion of lymphocytes in members of family groups compared to solitary animals. In domestic species, a "stress leukogram"

characterized by a leukocytosis, neutrophilia, lymphopenia, and eosinopenia typically occurs following adrenal stimulation, which leads to an increased N:L ratio (Feldman et al. 2000). The N:L ratio increases after restraint in rhesus monkeys (*Macaca mulatta*; Morrow-Tesch et al. 1993) and after transport in Southern chamois (*Rupicapra pyrenaica*; López-Olvera et al. 2006). However, leukocyte profiles provide information about the number of circulating cells rather than an individual's ability to mount an immune response. Based on our results and other studies (Dufva and Allander 1995; Bachman 2003), we suggest that the observed neutrophilia exhibited by the bears occurred as preparation of the body for injury and potential bacterial infection.

#### **Correlation between LCC measurements and other stress indicators**

AUC and LCC peak values did not correlate with any of the commonly used stress indicators, e.g., heart rate, N:L ratio, or glucose and cortisol concentrations. Therefore, we rejected our fifth hypothesis that there would be a negative correlation between LCC and other variables used as stress indicators. Shelton-Rayner et al. (2012) did not find a correlation between LCC and heart rate, blood pressure, body temperature, or cortisol levels in humans. They attributed this to physiological variables and hormones being influenced by a range of factors in addition to stress, which is a plausible explanation for our findings.

#### **The effectiveness of the LCC technique to evaluate the stress of capture and handling**

Leukocytes are recognized as ideal indicators of stress because they are constantly exposed to multiple factors such as endocrine factors in plasma, changes in blood biochemistry parameters, changes in the HPA axis, etc. (Mian et al. 2003). LCC has been shown to be rapidly affected by stress and has proven to be a quick and reliable method to quantitatively measure stress in both animals and humans (McLaren et al. 2003; Honess et al. 2005; Moorhouse et al.

2007; Gelling et al. 2009; Shelton-Rayner et al. 2010). LCC measurements can be taken during or immediately after a stressful event, and the results can be obtained while the animal is still under anesthesia. Thus, the technique allows a rapid assessment of the physiological status of an animal *in situ* (McLaren et al. 2003).

#### **Animal welfare, stress, and conservation**

There are several methods to assess stress and welfare (e.g., blood parameters or behavior). Moberg (2000) stated that the biological cost of mounting a stress response is the key to determine the welfare implications of a stressor and might be more relevant than other measures of stress such as physiological or behavioral changes. The LCC technique measures the biological costs associated with the release of ROS after a stressful event (McLaren et al. 2003). Therefore, it provides a relevant measure to assess welfare. However, a combined approach using two or more stress parameters is recommended. The LCC technique can be used in combination with traditional techniques to provide a more comprehensive approach on stress and wildlife welfare.

Disentangling the stressful components of trapping and handling procedures is important as shown by previous studies (Bonacic and Mc Donald 2003; McLaren et al. 2003). The results obtained by McLaren et al. (2003) using the LCC technique indicated that the transport of badgers before capture was an additional stressor. These results led to a refinement in the capture protocol of badgers.

Given the implications that welfare has on conservation, information provided by new techniques, such as LCC, will allow researchers to better evaluate the impact of their work and plan conservation actions consequently.

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