

Effects of Peripheral Blood Polymorphonuclear Leukocyte Function and Blood Components in Japanese Black Steers Administered ACTH in a Cold Environment

Hiroshi ISHIZAKI and Yoshihiro KARIYA

Animal Hygiene Laboratory, Department of Grazing Animal Production, National Grassland Research Institute, 768 Senbonmatsu, Nishinasuno, Tochigi 329-2793, Japan

(Received 5 October 1998/Accepted 6 January 1999)

ABSTRACT. An examination of the effects of artificial stress induced by adrenocorticotropin (ACTH) on total and differential leukocyte counts, plasma cortisol levels, metabolic profiles and peripheral blood polymorphonuclear leukocyte (PMN) function was performed on Japanese Black steers kept in a cold environment, with the following regimes; 1) $-5^{\circ}\text{C}\cdot\text{ACTH}$ (100 IU/day for 3 days), 2) $0^{\circ}\text{C}\cdot\text{ACTH}$, 3) $15^{\circ}\text{C}\cdot\text{ACTH}$ and 4) $15^{\circ}\text{C}\cdot\text{PBS}$. Blood samples were collected before and at 1, 2, 24, 48, 72 and 96 hr prior to the application of the stressor. The plasma cortisol level was found to greatly increase at one hr after the first treatment of ACTH, particularly so in animals exposed to -5°C . Total leukocytes (-5°C and 0°C experiments, respectively), the monocytes (-5°C), neutrophils and eosinophils (-5°C , 0°C and 15°C , respectively) obviously increased just after the first administration, although lymphocyte counts at -5°C were inversely related to those described above. All of these tendencies were augmented by the cold environment except for eosinophils. The chemiluminescent (CL) response of PMN decreased in the ACTH-administered steers at an early stage of post-administration, however, it tended to recover from the lower-than-base value in the cold-affected steers. ACTH administration resulted in higher plasma glucose (Glu) compared to a control, although only steers housed at -5°C evidently showed lower plasma inorganic phosphorus (IP). No abnormal serum acute phase protein, or immunosuppressor, was noted. ACTH thus appears not only to promote physiological reactions but also to temporarily suppress PMN cellular immune function in Japanese Black steers. Although, a cold environment rapidly restored the CL activity to over the pre-administrational value, suggesting that a vital response was activated by crymo-stimuli.—**KEY WORDS:** ACTH, blood component, cold exposure, Japanese Black steer, polymorphonuclear leukocyte.

J. Vet. Med. Sci. 61(5): 487–492, 1999

In encountering various stressors, the central nervous system of livestock evokes physiological responses that ultimately result in activation of the hypothalamo-pituitary-adrenocortical axis and the sympatho-adrenal axis. The responses of these major systems are presumed to have adaptive and homeostatic value during periods of stress, and adrenocorticotropin (ACTH) regulates the synthesis and secrete of adrenal glucocorticoids. Therefore, a higher concentration of plasma cortisol indicates that animals may be in a state of stress [5]. The environmental or management conditions experienced by domestic farm animals, e.g., thermal extremes, crowding, mixing of unfamiliar animals, transportation, weaning, branding, dehorning, vaccination, tail docking and so on, influence the physiological responses and have been termed as stressors [15].

Thermal stressors have been shown to affect the secretory response of metabolic hormones such as insulin, glucagon and growth hormone. The immunosuppression of leukocytes has been shown to be disrupted by environmental stressors such as road transportation [2, 10, 16, 17] and physical exertion [3]. Prolonged exposure of ruminant livestock to low environmental temperature results in increasing heat production and conservation [33]. A decrease in environmental temperature from thermoneutral to less than 0°C does not alter adrenal responsiveness to ACTH in Holstein heifers [8]. The Japanese Black is less tolerant to a cold environment compared to the Holstein, although the influence on cellular immunity of Japanese Black steers due to cold thermal stressors is only slightly

understood. One prominent response to body stress is the synthesis and release of adrenal glucocorticoids mediated by ACTH release from the anterior pituitary gland [21]. Thus, ACTH-administration should serve as one effective mean for stimulating adrenocortical function so that stress conditions reappear in organisms.

ACTH, which is a stressor, was administered in the present study to examine the influences of continuous cold exposure on adrenocortical and cellular immune functions, especially peripheral blood polymorphonuclear leukocyte (PMN) representing neutrophil that plays a key role in essential host defense mechanisms in Japanese Black steers.

MATERIALS AND METHODS

Animals: Six Japanese Black steers, six to eight months of age and weighing 140 to 200 kg, were used. All were housed in artificial climatic chambers and kept separately in stanchion stools. An experimental design indicates in Fig. 1. Briefly, rearing temperature was 15°C (thermoneutral environment) so that the animals could accommodate themselves to new surroundings for fourteen days. Then, three steers were housed at 0°C and the others at -5°C (cold environments) for thirty-five days due to accommodation. These steers were continuously exposed to cold for the next five days due to cold experiments. After that, temperature was returned to and maintained at 15°C for ten days. And next, the three steers handled were used in a thermoneutral temperature experiment for the following

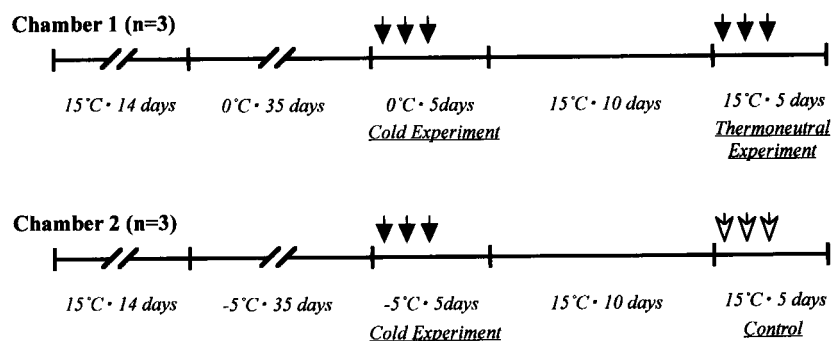


Fig. 1. An experimental design. \downarrow : ACTH (100 IU/head/day). \uparrow : PBS administration.

five days and the others served as a control with phosphate-buffered saline (PBS) administration. Chamber lights were turned on between 06:00 and 18:00. The steers were fed a diet of concentrations plus Orchard grass hay twice daily and fresh water and mineral salt block (Kouen, Nippon Zenyaku Kogyo, Co., Ltd., Koriyama, Japan) were available *ad libitum*.

Treatment and blood sampling: One hundred IU/head (suspended in 2 ml PBS) ACTH (Corticotropin A from Porcine Pituitary, Sigma, St. Louis, MO, U.S.A.) was intramuscularly injected in the neck once daily at 08:30 (just before the ACTH administration) for three continuous days. Heparinized (20 ml) and plain (10 ml) blood were collected seven times from the jugular vein before (basal level) and at 1, 2, 24, 48, 72 and 96 hr following the first ACTH-administration.

Preparation of blood smears and PMN: Blood smears for differential leukocyte-counting were prepared with Giemsa stain using heparinized blood, and the remaining blood was used for cell counting and centrifuged at $1,400 \times g$ for 20 min. Plasma and serum were stored at -20°C for subsequent hormone and metabolite determination. Other samples were obtained for isolation of PMN. The PMN was isolated by density gradient (Lymphoprep; Nycomed Pharmacia As., Norway) centrifugation at $400 \times g$ for 25 min and recovered from the packed erythrocyte layer after centrifugation. The erythrocytes were lysed by three volumes cold phosphate buffer (0.0132 M, pH 7.2) with gentle mixing for 60 sec in a 50 ml round bottom centrifuge tube. Isotonicity was restored by 1.5 volumes cold phosphate-buffered 2.7% NaCl (pH 7.2) solution. The cells recovered by centrifugation were rinsed and suspended at 5×10^6 cells/ml in Eagle's minimum essential medium. PMN was present as neutrophil at more than 95% in the preparation as determined by microscope.

Luminol-dependent chemiluminescent (CL) response in PMN: Oxidative burst activity was used as indicator of PMN in peripheral blood [13, 20] and measured by CL assay [1, 14]. Bovine opsonized zymosan was prepared by mixing zymosan A (Sigma, St. Louis, MO, U. S. A.) with fresh bovine serum as previously described [23]. A mixture of 200 μl of Hank's buffered balanced solution (HBSS) containing 1×10^6 PMNs and 10 μl luminol solution at a

final concentration of 10 mg/ml were prewarmed at 37°C in the sample chamber, and 10 μl HBSS containing opsonized zymosan at a final concentration of 0.5 mg/ml was added to it. Photon emission in the PMN mixture was live checked for 40 min and represented as a peak value. All assays were carried out in triplicate on PMN and measured with a chemiluminometer (MLR-100, Corona Electric Co., Ltd., Ibaraki, Japan). The CL activity was expressed as means for triplicate and evaluated as variation of 100% of pre-administrational base values.

Plasma biochemistries and serum protein: Plasma biochemistries were examined with an automated analyzer (Hitachi 7050, Hitachi Ltd., Tokyo, Japan). The analysis included measurement of blood urea nitrogen (BUN), aspartate aminotransferase (AST), glucose (Glu), nonesterified fatty acid (NEFA), total protein (TP), inorganic phosphorus (IP), albumin (Alb) and total-cholesterol (T-Cho) affected by stress such as transportation of dairy cattle and calves [11, 14]. Serum haptoglobin (Hp) and α_1 acid glycoprotein ($\alpha_1\text{AG}$) were measured by commercial assay kits (Saikacheck, Hp-plate and $\alpha_1\text{AG}$ -TIA, respectively, Saikinkagaku Institute, Co., Ltd., Sendai, Japan).

Plasma cortisol: Cortisol was measured using a commercially available [^{125}I] radioimmunoassay kit (Amerlex Cortisol RIA kit; Ortho-Clinical Diagnostics, Co., Amersham, UK).

Data analysis: All data were expressed as means \pm standard errors.

RESULTS

Physical features: Six Japanese Black steers were exposed to cold and administered ACTH. Shivering was noted during cold exposure. Rectal temperature did not obviously change in response to cold and thermoneutral environments.

Plasma cortisol concentration: Plasma cortisol levels in peripheral blood following ACTH administration are shown in Fig. 2. Cortisol tended to increase at 1 hr after the first ACTH injection at -5°C , 0°C and 15°C (6.5 ± 4.3 to 59.1 ± 3.9 ng/ml, 5.7 ± 2.7 to 34.7 ± 5.8 ng/ml and 6.0 ± 0.5 to 31.2 ± 6.7 ng/ml, respectively) in contrast to pre-injection and at 2 hr at 0°C (19.1 ± 4.6 ng/ml) and -5°C (22.0 ± 3.3

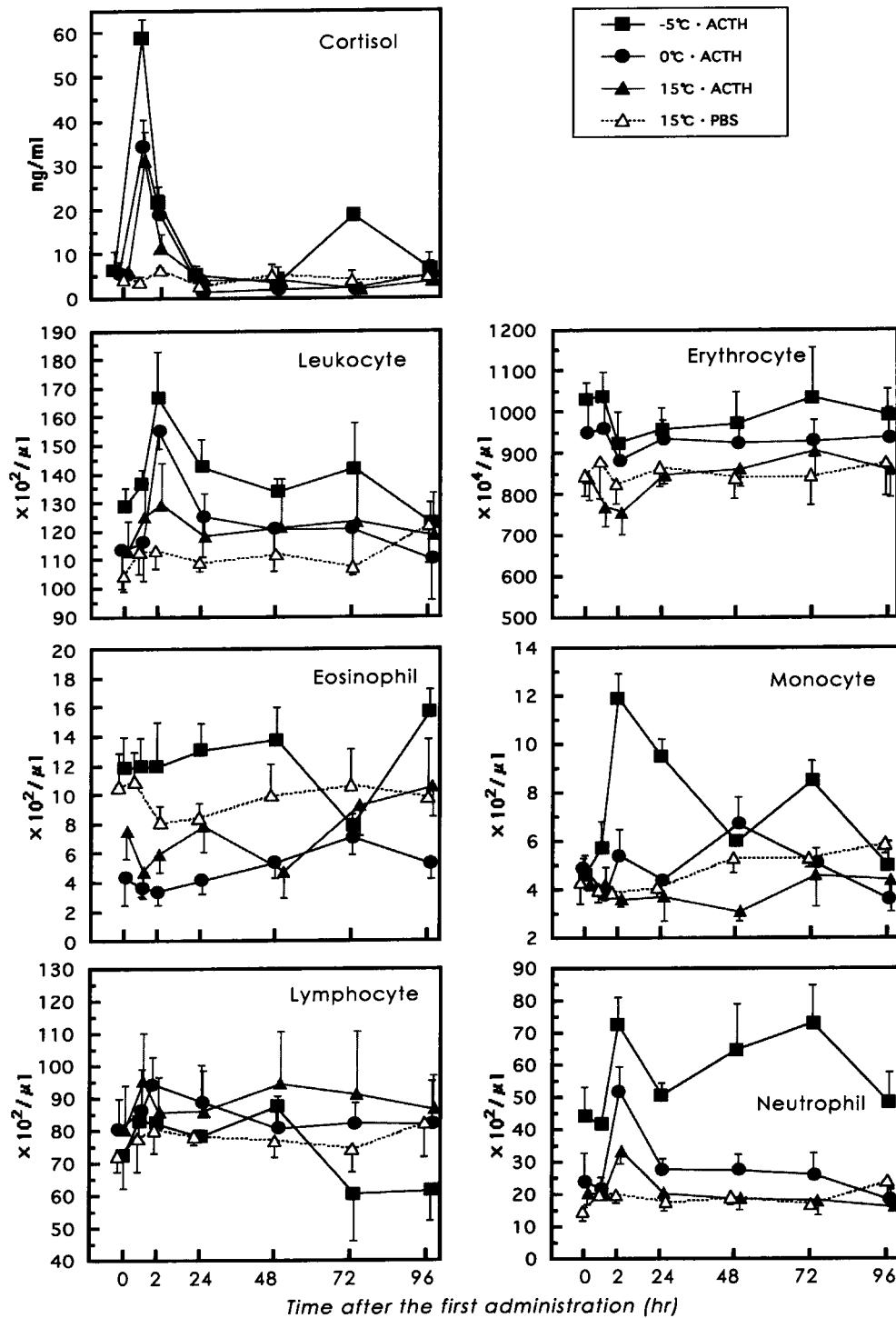


Fig. 2. Changes in plasma cortisol and numbers of erythrocytes, total leukocytes and differential counts of Japanese Black steers subjected to cold and ACTH administration. Each vertical bar represents the mean standard of error for the three animals.

ng/ml) of cold and severe cold exposure.

Hematological findings: Figure 2 shows hematological changes following the first ACTH administration. No remarkable change was observed in erythrocytes by cold

exposure and ACTH treatment. Total leukocytes at 0°C and -5°C rapidly increased to $155.3 \pm 6.1 \times 10^2/\mu\text{l}$ and $167.0 \pm 16.0 \times 10^2/\mu\text{l}$ at 2 hr after the first ACTH treatment. Monocytes changed remarkably in the severe cold

environment. Monocytes at 2, 24 and 72 hr post-administration were obviously more than before treatment ($11.9 \pm 1.0 \times 10^2/\mu\text{l}$, $9.5 \pm 0.7 \times 10^2/\mu\text{l}$ and $8.5 \pm 0.8 \times 10^2/\mu\text{l}$, respectively). Eosinophils gradually increased with

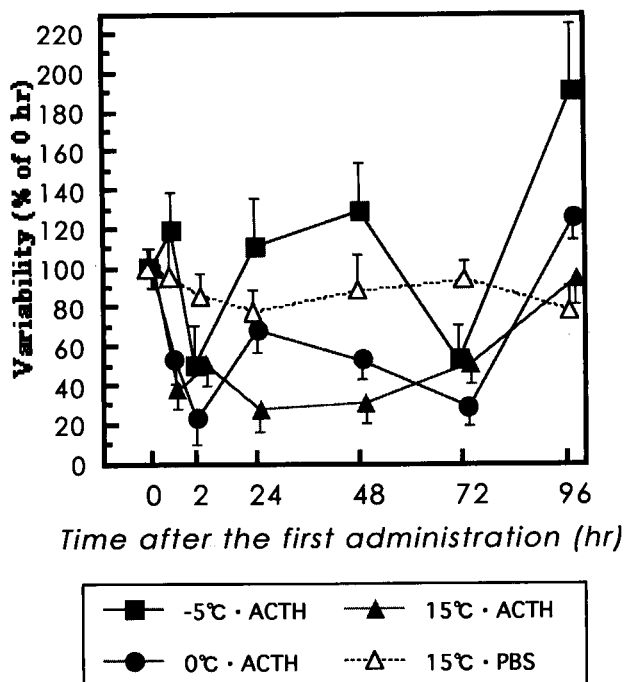


Fig. 3. Changes in CL activity of PMN from Japanese Black steers subjected to cold and ACTH administration. Means are percentages of pre-administration base values (100%). Each vertical bar represents the mean standard of error for the three animals.

slight change after ACTH-administration. Neutrophils are essential to biophylaxis, especially in early defense against inflammation and microbism. Neutrophils at -5°C , 0°C and 15°C subsequent to ACTH administration increased to $72.6 \pm 8.4 \times 10^2/\mu\text{l}$, $51.8 \pm 7.6 \times 10^2/\mu\text{l}$ and $33.4 \pm 4.0 \times 10^2/\mu\text{l}$ at 2 hr, respectively. At -5°C , evident decrease in lymphocytes was noted between 72 and 96 hr after administration ($8.5 \pm 0.8 \times 10^2/\mu\text{l}$ and $5.0 \pm 1.0 \times 10^2/\mu\text{l}$, respectively).

PMN CL response: CL response of PMN was used to monitor the oxidative burst of neutrophils. As shown in Fig. 3, the steers at 0°C and 15°C showed a greatly reduced CL response of PMN to minimum levels of $23.0 \pm 12.5\%$ and $27.7 \pm 10.8\%$ following the start of ACTH treatment, respectively. Steers, in -5°C conditions, indicated a slightly decremental CL response to $50.5 \pm 20.0\%$ (2 hr) and $52.9 \pm 18.0\%$ (72 hr) after ACTH treatment. The change to 15°C resumed the normal level at 96 hr after hormone treatment, however, those in 0°C and -5°C recovered from the decline immediately. And then, that of the control was transited without vigorous change.

Blood metabolite response: Change in plasma Glu and IP showed a consistent trend (Fig. 4). All ACTH-administered groups tended to increase and showed higher plasma Glu than in a control, and only the group reared at -5°C showed lower plasma IP than in a control during 2–72 hr of post-treatment. Other plasma metabolites remained unchanged and neither Hp nor $\alpha_1\text{AG}$ maintained normal level by ACTH treatment in cold exposure (data not shown).

DISCUSSION

Ruminant livestock are frequently subjected to cold for prolonged periods, resulting in adaptive physiological

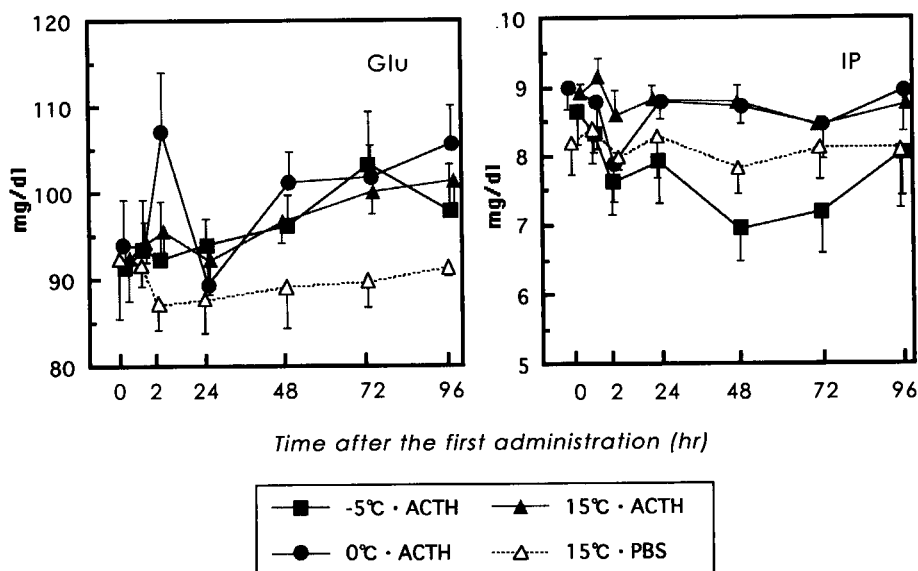


Fig. 4. Changes in plasma Glu and IP of Japanese Black steers subjected to cold and ACTH administration. Each vertical bar represents the mean standard of error for the three animals.

responses. The activity of the sympathetic nervous system increases with the release of catecholamines, adrenal corticosteroids and thyroid hormones [33], consequently enhancing substrate oxidation for energy production.

The ruminant is a homeothermic species which can maintain constant body temperature under thermal stress. Shivering was observed in steers during cold exposure which had no effect on rectal temperature. This indicates the apparent capacity to resist body cooling, cold-induced vasoconstriction, and a decrease in skin temperature that possibly would arise from reduced metabolic rate [27].

As shown in Fig. 2, plasma cortisol in ACTH-administered steers showed transient and drastic increase between 1–2 hr after the first of administration. Severe cold aggravated this condition. The same phenomenon has been noted for sheep [24, 25, 31], calves [29] and piglets [28].

The transport of animals causes rise in plasma cortisol, leukocytosis with neutrophilia, lymphopenia and eosinopenia [11, 16, 26]. ACTH has been shown to have the same effect [7, 19]. The present results demonstrate leukocytosis with neutrophilia and lymphopenia, but not eosinopenia. This phenomenon on eosinophil was at variance with the result of Wegner and Stott [32]. A possible explanation for the lack of agreement may be different interval from sample collections in the above mentioned (every hour for 24 hr). Monocytes rapidly increased after the treatment with this drug and glucocorticoids [4], though previously, it was reported to cause leukocytosis with monocytopenia in peripheral blood in a short period in piglets [6]. It suggests that this discrepancy in monocyte counts may possibly depend on animal, experimental design or blood smear staining differences [21].

The measurement of luminol-mediated CL responses has been found useful for assessing *in vitro* intracellular killing and opsonocytophagic functions of PMN. Blecha and Minocha [3] noted elevated cortisol during transportation to induce immunosuppression. Roth *et al.* [22] indicate that increase in plasma cortisol by ACTH administration has no significant effect on enhancement of bovine neutrophil activity. Similar suppression decreasing PMN-CL response at the early stage of post-ACTH-administration due to cold exposure was noted in this study and thus PMN activity associated with neutrophilia may be impaired following plasma cortisol elevation [6]. Following the early stage of post-administration, a decline of the CL responses in -5°C and 0°C conditions rapidly improved to almost the same as pre-administration level, indicating that a cold environment stimulated the hypothalamo-pituitary-adrenocortical axis and the sympatho-adrenal axis so that the physiological response was raised due to overcome immunosuppression.

Kent and Ewbank [12] previously found plasma Glu in calves rose during transportation, possibly in response to increase in circulating corticosteroids, resulting from increased serum NEFA levels. Plasma Glu in ACTH-administered groups continued to be higher than in the control throughout the present study regardless of NEFA

levels. The activation of gluconeogenic hormones, such as glucagon and cortisol, may possibly be the reason for this [29]. Plasma IP in the steers treated with ACTH was observed to decrease, indicating adrenocortical hormonal action to counter renal tubule and restrained IP resorption [30].

Serum $\alpha_1\text{AG}$, which suppresses lymphocyte proliferation, showed no significant increase in cattle with inflammatory diseases [9]. This is consistent with maintained normal serum $\alpha_1\text{AG}$ levels. Bovine Hp inhibits lymphocyte proliferative response to T cell mitogens dose-dependent by [18], though in this study, Hp showed no significant change in any case.

In a cold environment, ACTH-administration would thus appear to enhance the physiological response of Japanese Black steers and suppress cellular immune functions of PMN-CL activity with neutrophilia subsequent to plasma cortisol elevation. Furthermore, the present study reveals that it didn't take a long time to regulate immunopotential from a physiological enhancement due to a cold stimulus.

ACKNOWLEDGMENTS. The authors thank Dr. Taiichiro Fukukawa, Director of Department of Grazing Animal Production, National Grassland Research Institute, for his valuable comments on this manuscript. The authors are also grateful to Dr. Yasuhiro Aoki, Department of Grazing Animal Production, National Grassland Research Institute, for his help throughout the study.

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