



Non-invasive determination of the immune physiological state of reindeer (*Rangifer tarandus*) in the Greater Khingan Mountains, China

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ABSTRACT. Immunoglobulin and cortisol levels are good indicators of well-being and living status in animals. In this study, the concentrations of fecal immunoglobulins A ([IgA_F]), G ([IgG_F]), and M ([IgM_F]), and cortisol ([cortisol_F]) were examined by enzyme-linked immunosorbent assay in reindeer of the Greater Khingan Mountains of Inner Mongolia, China. [IgA_F] was significantly higher than [IgG_F] and [IgM_F], and [IgG_F] was significantly higher than [IgM_F] ($P < 0.05$). Both [IgA_F] and [IgG_F] were higher in the Adult group than in Aged or Infant groups, and higher in the Young than Infant group ($P < 0.05$). The four age group [IgM_F]s were not significantly different ($P > 0.05$). [IgA_F], [IgG_F], and [IgM_F] in each age group were higher in females than in males, with a significant difference in the Young group ($P < 0.05$). The Infant group had the highest [cortisol_F], and the Adult group the lowest; [cortisol_F] was significantly higher in the Infant group than in other age groups ($P < 0.05$). In each age group, [cortisol_F] was higher in females than males,

and there were significant differences among the Infant, Young, and Aged groups ($P < 0.05$). A significant negative correlation was observed between [cortisol_F] and [IgA_F] and [IgG_F] ($P > 0.05$). Overall physical condition was better in the Adult and Young groups than in the Aged and Infant groups as determined by the comprehensive analysis of fecal Ig levels in the four age groups, with the Infant group the worst.

Key words: Reindeer; Immunoglobulin; Cortisol; Ageing; ELISA; Feces

INTRODUCTION

Reindeer (*Rangifer tarandus*), also called sinomegaceros, inhabited the arctic and sub-arctic regions, the largest number of cervidae animal in the world. In China, reindeer were an alien species that were moved into the Greater Khingan Mountains of Inner Mongolia by Ewenki herders that lived to the east of Lake Baikal and upstream on the Lena river, for the purpose of escaping during fighting in 17th century (Zhong et al., 2008). At present, these reindeer, which number less than 700, are semi-domesticated and raised and managed in a traditional manner by Ewenki herders in the Greater Khingan Mountains of Inner Mongolia.

Shrinking of the habitat area, population inbreeding, and an increase in extreme weather conditions have caused the body size of reindeer to become smaller, along with a reduction in their disease-resistance and survival abilities. To protect the reindeer population in China, their well-being and living status must be monitored, and the corresponding protection strategies implemented.

Immunoglobulin (Ig) and cortisol levels are good indicators of the well-being and living status of animals. Immunoglobulin is present in the blood, tissue fluid, lymph, and external secretions in mammals, and is an important immune factor that has been shown to have many functions, including the prevention of infective agents such as bacteria and viruses from breaching the mucosal barrier, complement activation, counteracting poison, the killing of tumor cells, and disease resistance (Palmer et al., 1980). Glucocorticoids are a class of steroid hormones secreted by the adrenal cortex, which have key roles in regulating the biosynthesis and metabolism of sugars, fats, and proteins. Cortisol is the major physiological active ingredient of glucocorticoids in mammals (Gold et al., 2002).

The levels and changes in Ig and cortisol have a wide array of applications for monitoring stress (Keay et al., 2006; Rehbinder and Hau, 2006), disease (Aucan et al., 2000), and health (Pihl and Hau, 2003; Volkmann and Weekes, 2006), and are good indicators of health and living conditions. The levels of secretion of Ig and cortisol are controlled not only by genetic factors but also by age, gender, and environment, and have been studied in Steller sea lions (Mashburn and Atkinson, 2004), David's deer (Zhang et al., 2011), rats (Pihl and Hau, 2003), and swine (Sutherland et al., 2005). However, no studies on fecal immune physiological indices on reindeer living in the Greater Khingan Mountains of Inner Mongolia under the basal metabolic conditions have yet been conducted. In order to monitor health condition from the perspective of immune physiology and provide a reference for management of the reindeer population in China, in this study we examined the concentrations of fecal Igs (IgA, IgG, and IgM) and cortisol in various age groups and genders, and compared the differences between groups.

MATERIAL AND METHODS

Animals and grouping

The 80 reindeer monitored in this study were free-ranging within the Greater Khingan Mountains of Inner Mongolia, and did not receive any supplementary feeding. The reindeer were divided into four age groups according to age structure (Du et al., 2007): Infant (0-1 years), Young (2-3 years), Adult (4-9 years), and Aged groups (more than 10 years). Each age group comprised 10 male and 10 female reindeer.

Sample collection and preservation

Samples were collected using the tracking method from December 25, 2013 to January 10, 2014. After the animals defecated, the fecal pellets were collected in a Ziplock bag using disposable plastic gloves, details of the individual's serial number and date of sampling were recorded thereupon, and samples were promptly stored in a -20°C freezer.

Extraction of fecal Ig and cortisol

The method used for saline extraction of fecal Ig was adapted from that used by Peters et al. (2004) and Zaine et al. (2011). The frozen fecal samples were thawed at room temperature, and mixed samples were composed of an equivalent quantity of feces from the same individual. Approximately 10 to 20 g (wet weight) of mixed fecal samples were placed in a mortar and ground into a mixture; from this, 1 g well-mixed wet feces was placed in a 15-mL centrifuge tube containing 10 mL phosphate buffer saline (PBS) buffer (pH 7.2, 0.5% Tween-20) and vortexed for 5 min, placed for 20 min at room temperature and then centrifuged at 2000 rpm for 20 min. The supernatant was transferred to a new tube and centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatants were transferred to clean Eppendorf tubes and stored at -20°C.

The method used for the extraction of fecal cortisol was adapted from that used by Capezzuto et al. (2008). An aliquot (1 g) of well-mixed wet feces was placed in a 15-mL centrifuge tube containing 10 mL 90% methanol, and vortexed for 5 min at room temperature. The tubes were incubated in a thermostatic oscillator for 20 min at 60°C and then centrifuged at 2000 rpm for 20 min at room temperature; the supernatant was transferred to clean Eppendorf tubes and stored at -20°C.

Approximately 10 to 15 g well-mixed wet feces were heated at 105°C to constant weight and the content of dry matter of each individual fecal sample was calculated.

Quantification of fecal Ig and cortisol

Double antibody sandwich method enzyme-linked immunosorbent assays (ELISAs) were used to quantify the total concentrations of IgA, IgM, and IgG in feces. Capture ELISAs were used to quantify the concentrations of fecal cortisol with a Thermo Multiskan FC Micro Plate Reader (Thermo Fisher Scientific, USA); plates were washed with a Thermo Wellwash Microplate Washer.

Ig concentrations were measured using a commercial immunoglobulin enzyme im-

munoassay kit (EIA DSL-10-4000, Quantization Set, Bethel Laboratories Inc.; Montgomery, TX, USA). Cortisol concentrations were measured using a Cortisol Enzyme Immunoassay kit (Yueyan Biological Reagent Co. Ltd., Shanghai, China). Each sample was measured in triplicate.

Data analysis

Results are reported as means \pm standard deviation (SD), and in units of nanograms of Ig or cortisol per gram dried feces. The Kolmogorov-Smirnov test was used for determining normal distribution. If the data were normally distributed, then one-way analysis of variance combined with *post-hoc* tests was used to compare different concentrations in each age and gender. If the data were not normally distributed, a Kruskal-Wallis test was used to compare differences. The level of significance was set at $P < 0.05$. All statistical analyses were carried out using SPSS 20.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Comparisons of concentrations of fecal IgA, IgG, and IgM in different ages and genders

In order to study the differences in fecal IgA, IgG, and IgM between different ages and genders, Ig from fecal samples of 80 reindeer was extracted and measured. Fecal Ig concentrations were compared among the four ages of reindeer sampled in this study (Table 1).

Table 1. Measurements of fecal IgA, IgG, and IgM concentrations from reindeer in different age groups.

Item	Infant (N = 20)	Young (N = 20)	Adult (N = 20)	Aged (N = 20)	Total (N = 80)
IgA (ng/g)	3837.95 \pm 489.42 ^a	4332.16 \pm 714.09 ^{bc}	4651.32 \pm 614.50 ^c	4106.52 \pm 617.57 ^{ab}	4265.45 \pm 672.61
IgG (ng/g)	334.87 \pm 64.26 ^a	428.32 \pm 71.04 ^b	506.11 \pm 66.76 ^c	407.28 \pm 46.74 ^a	419.15 \pm 87.03
IgM (ng/g)	105.26 \pm 13.95	114.70 \pm 14.39	111.38 \pm 18.08	104.78 \pm 12.97	109.03 \pm 15.28

Superscripted letters indicate that significant differences exist between those respective groups ($P < 0.05$).

The concentration of fecal IgA was significantly higher than that of IgG and IgM, and IgG concentration was significantly higher than that of IgM ($P < 0.05$). Across the different age groups, the change of concentration of fecal IgA was the same as that of fecal IgG. The concentrations of fecal IgA and IgG were the highest in the Adult group, followed by the Young and Aged groups; the Infant group had the lowest concentration. The concentrations of fecal IgA and IgG were significantly higher in the Adult than in the Aged and Infant groups, and the concentrations in the Young group were higher than those in the Infant group ($P < 0.05$). Fecal IgM concentrations in the four age groups were not significantly different ($P > 0.05$).

Fecal Ig concentrations were compared between female reindeer and male reindeer in the four age groups in this study (Figures 1, 2, and 3).

The concentrations of fecal IgA in each age group were higher in female reindeers than in male reindeers (Figure 1). From the Infant, Young, and Adult groups to the Aged group, the concentrations of fecal IgA in females were higher than in males by 7.69, 31.16, 8.68, and 8.54%, respectively, and there was a significant difference in the Young group ($P < 0.05$).

The concentrations of fecal IgG in each age group were higher in female than in male

reindeer (Figure 2). There was a significant difference in the Young group ($P < 0.05$), similar to that of IgA.

As shown in Figure 3, the concentrations of fecal IgM in each age group were slightly higher in female than in male reindeer, and there was no significant difference in each age group ($P > 0.05$).

In summary, the concentrations of fecal IgA, IgG, and IgM were higher in the Adult and Young groups than in the Aged and Infant groups, and the overall level of fecal Ig was the highest in the Adult group and the lowest in the Infant group.

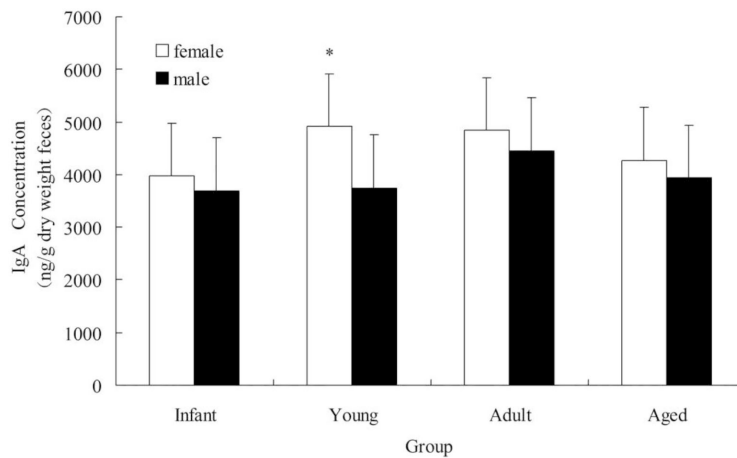


Figure 1. Comparison of fecal IgA concentrations of male and female reindeer in different age groups. Error bars show the standard deviation. Statistically significant differences are indicated by an asterisk above the female bars for different genders in the same age group ($P < 0.05$).

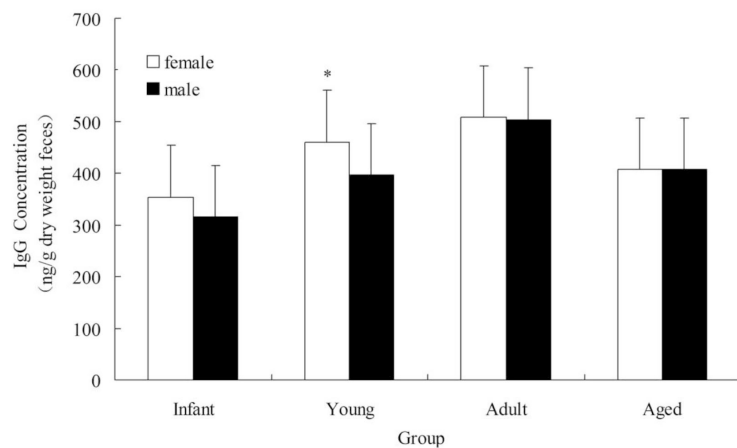


Figure 2. Comparison of fecal IgG concentrations of male and female reindeer in different age groups. Error bars show the standard deviation. Statistically significant differences are indicated by an asterisk above the female bars for different genders in the same age group ($P < 0.05$).

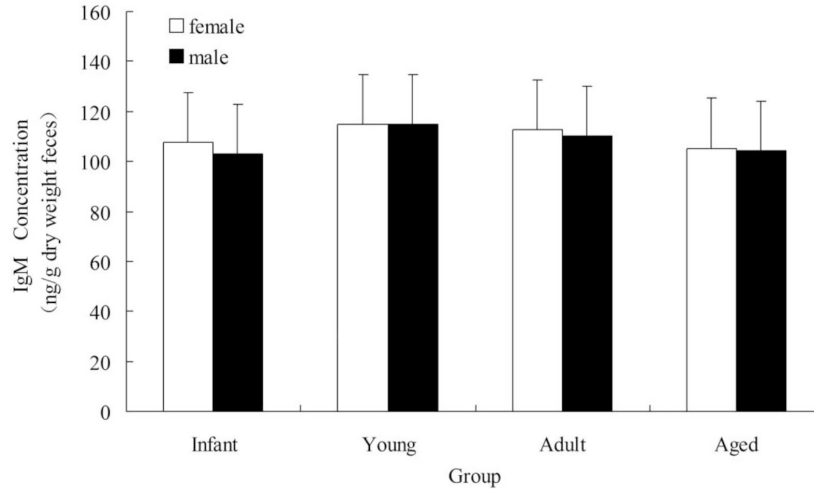


Figure 3. Comparison of fecal IgM concentrations of male and female reindeer in different age groups. Error bars show the standard deviation. Statistically significant differences are indicated by a star above the female bars for different genders in the same age group ($P < 0.05$).

Comparison of fecal cortisol concentrations in different ages and genders

In order to study the differences of fecal cortisol in different ages and genders, cortisol from fecal samples of 80 reindeer was extracted and measured. Fecal cortisol concentrations were compared among the four age groups of reindeer sampled in this study (Figure 4).

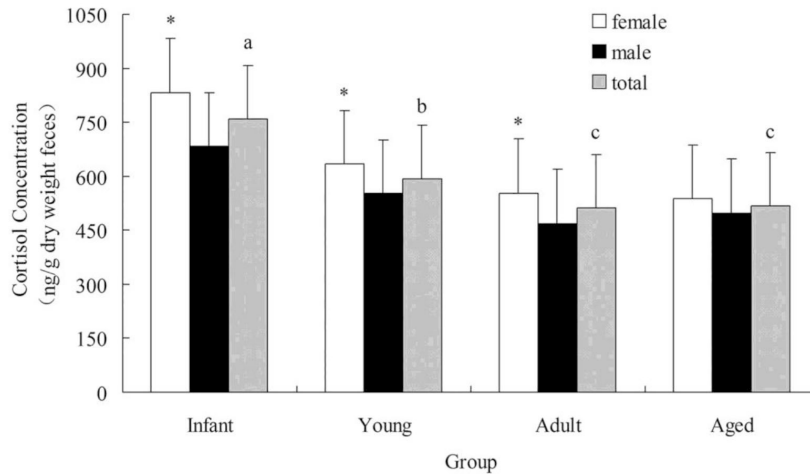


Figure 4. Comparison of fecal cortisol concentrations of male and female reindeer in different age groups. Error bars show the standard deviation. Statistically significant differences are indicated by an asterisk above the female bars for different genders in the same age group; different letters above the total immunoglobulin bars reflect significant differences between different age groups ($P < 0.05$).

The changes in concentration of fecal cortisol within female and male reindeer were synchronous in the four age groups, and declined gradually with increasing age. Across the ages, the concentration of fecal cortisol was significantly greater in the Infant than in the Young group, and was significantly greater in the Young than in the Adult and Aged groups ($P < 0.05$). There was no significant difference between the Adult and Aged groups ($P > 0.05$).

The fecal cortisol concentration was significantly greater in female reindeer than in male reindeer, and this was particularly prominent in the Infant, Young, and Adult groups.

Correlation analysis of fecal IgA, IgG, IgM, and cortisol concentrations

Immunoglobulins are the main immune factors in the humoral immune response, and they have multiple immune functions because of the differences in their molecular structures. Cortisol is a type of glucocorticoid secreted by the adrenal gland that plays an important role in dealing with stress. Elevated cortisol concentration has been shown to improve blood pressure and blood sugar levels, and to weaken the activity of the immune system.

The relationship between the concentrations of Ig and cortisol in feces was assessed by examining their correlation in all of the samples analyzed (Figure 5).

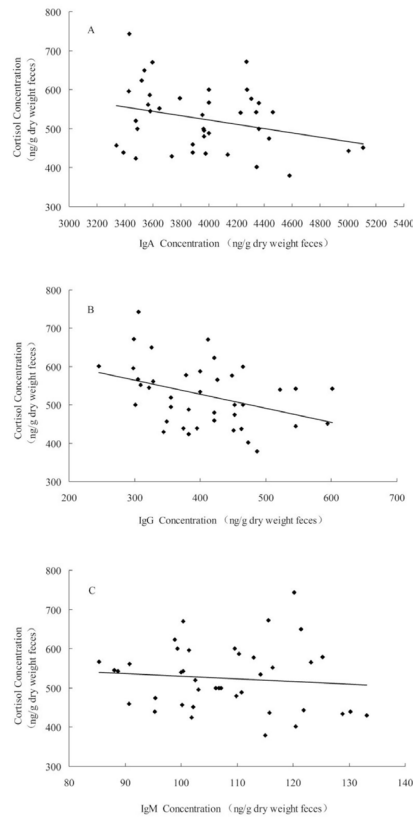


Figure 5. Relationship between fecal cortisol and immunoglobulin concentrations. **A.** Fecal cortisol and IgA concentrations; **B.** fecal cortisol and IgG concentrations; **C.** fecal cortisol and IgM concentrations.

The concentration of fecal cortisol was significantly correlated with IgA and IgG concentrations (IgA, $r_p = -0.293$, $P = 0.008$; IgG, $r_p = -0.379$, $P = 0.001$), but was not significantly correlated with that of IgM ($r_p = -0.100$, $P = 0.378$). These results imply that a higher cortisol level would inhibit the production of Ig, thereby reducing the immune responsiveness of the organism.

DISCUSSION

The function of the immune response is the defense against harmful factors that might damage the body. Another way of looking at this response is that the adaptability to the environment can be estimated via changes in immune status and their influencing factors (Xu and Wang, 2012). Ig level is an important index that reflects immune status, and IgA, IgG, and IgM are the main indices (Schatz and Palme, 2001). In China, reindeer are only distributed in the cold temperate zone coniferous forest in the Greater Khingan Mountains of Inner Mongolia, and have been semi-domesticated and managed. IgA, IgM, and IgG were identified in reindeer fecal materials, and the concentrations of fecal IgA were found to be significantly higher than those of IgG and IgM. Our results demonstrated the predominance of IgA in feces and are in agreement with findings from captive forest musk deer (Lang, 2013) and swine (Franz and Corthier, 1981), which described IgA as the most important Ig in adult intestinal fluid, which plays an important role in gastrointestinal immune responses.

There are large differences in Ig concentrations because of their distribution and biological activities in different body fluids. IgA is the main antibody in mucosal defense against local infection, and has a higher content in feces. IgA possesses antibiotic activity against some viruses, bacteria, and antigens that forms a barrier of organismal mucosal immunity. Abundant IgG exists in the blood, tissue, and lymphatic fluids. IgG is the main component of the animal serum Ig that passes through the placenta, and plays an important role in prevention of infection in newborn animals. After contact with an antigen, IgM is synthesized first during the humoral immune response and is the vanguard of the body's resistance to infection. The synthetic quantity of IgM is less, and a rapid catabolism.

The immune response progresses gradually from low to high in all types of biological species. With respect to the ontogeny of immune response, immune tolerance has been shown to appear in the fetal period, and improve and develop gradually with the growth and development of the body. As the organism ages, the organs of the immune system degenerate and immunity is reduced (Chen et al., 2006).

Our present analyses suggested that the concentrations of fecal IgA and IgG exhibited significant variation among the four age groups, with the Adult group showing the highest levels, followed by the Young and Aged groups, with the lowest concentration in the Infant group. Reindeer were found to be widespread in the Greater Khingan Mountain areas of Inner Mongolia in Northeast China. The infant reindeer were weaned in October in this region, and their immune system did not appear to be fully developed by December and January of the following year, as evidenced by their lower Ig concentrations. We believe that the Ig level of aged reindeer declined because of the atrophy of immune organs and recession of immunological function due to ageing. The young reindeer grew quickly, and their Ig levels were close to adult levels for each function. The adult reindeer, which had optimal immune function, were the strongest individuals and occupied the dominant

position in the population, and had higher immune factor levels and enhanced ability to resist disease. The concentrations of fecal IgM showed less difference among the four age groups. IgM is an antibody that is synthesized and secreted early in the development of the individual, and is generated in the fetus during late embryonic development, reaching the level of an adult reindeer at two years of age. The results obtained herein conformed to those expected for normal growth and metabolism based on the physiological characteristics of reindeer.

Stress status is one of the important physiological indices of living conditions, and reflects an organism's ability to adapt to environmental conditions (Clark et al., 1997). Cortisol is a type of glucocorticoid that is secreted by the adrenal gland; its level was the highest in the Infant group and lowered gradually with increased age. The fecal samples were collected in January, which is the coldest month with a mean temperature below -35°C . The continued cold weather presents a chronic environmental stressor for reindeer. Infant reindeer, with lower immune ability, were predicted to have had a strong stress reaction to the environmental changes, causing the central nervous system to produce a nerve impulse prompting the secretion of the glucocorticoids, leading to the elevated fecal cortisol concentration. As adult and aged reindeer are expected to have a strong tolerance to the natural changes of the environment, in these animals, the secretion of sugar cortical hormone is not activated and therefore the concentration of fecal cortisol remains low.

In the reindeer population, the levels of immunity were shown to differ between the genders, with the general finding that the function of cellular immunity and humoral immunity of the females were higher than those of the males (Li et al., 2002). This study found that the concentrations of fecal Igs (IgA, IgG, and IgM) were higher in female reindeer than in males, and this was particularly prominent in the young age group. Reindeer are highly social animals with a strict social hierarchy, and the adult males hold a dominant position in reindeers. The aged males become eliminated gradually because of the decline of their reproductive and immunity functions, and often are active only in the periphery of the herd. With the development and maturation of sexual organs, the young male reindeer have a strong sexual desire and close in frequently to the female deer. They are defended and attacked severely by adult males; however, whether this leads to the levels of the fecal Ig and cortisol of young female reindeer being significantly higher than those of young males has yet to be determined.

Animals perceive the changes of the body's internal environment from physiological stress and adjust the body's immune system accordingly. The specific immune response was shown to be induced through information processing and integration of the changes (Grayson et al., 1981; Yuan et al., 2005). The mixed sample of each individual was comprised of single fecal samples over each of 15 days that were obtained with no interference; thus, the results represent the basal metabolic level of the reindeer in winter. There was a significant negative correlation between the fecal cortisol and IgA, IgG levels in reindeer, which was most likely due to the continuous low temperature environment that prompted the secretion of the sugar cortical hormone and suppressed the synthesis of Igs, and was consistent with results obtained from studies on rats (Royo et al., 2004).

In summary, the comparative studies of the levels of fecal Ig and cortisol in different age groups can effectively reflect the immune condition of a reindeer. The management of the breeding of infant reindeer should be enhanced in winter in the Greater Khingan Mountain areas of Inner Mongolia in Northeast China.

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