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Influence of enclosure size and animal density on fecal cortisol concentration and aggression in Père David's deer stags

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Abstract

We investigated the impact of enclosure size and animal density on behavior and adrenocortical secretion in Père David's deer in Dafeng Nature Reserve, China. From February 15 to April 16 in 2004, we conducted two experiments. First, we studied maintenance behavior and conflict behavior of Père David's deer stags in a large enclosure (200 ha) with low animal density (0.66 deer/ha) and a small display pen (0.75 ha) with high animal density (25.33 deer/ha). The maintenance behavior we recorded included standing, locomotion, foraging and rest. During the behavioral observations, we collected fresh voided fecal samples from the stags periodically, and analyzed the fecal cortisol concentrations in those samples using radioimmunoassay technique. Second, we monitored the fecal cortisol concentrations of one group of stags (12 deer lived in an enclosure of 100 ha) before and after transferred into a small pen (0.5 ha). We found that in the first experiment: (1) there were significant differences in standing and rest whereas no significant differences of locomotion and foraging between the free-ranging group and the display group; (2) frequency of conflict behavior in the display group was significantly higher than those in the free-ranging group; and (3) fecal cortisol concentration of the display group (326.17 ± 16.98 ng/g dry feces) was significantly higher than that of the free-ranging group (268.98 ± 15.21 ng/g dry feces). In the second experiment, there was no significant difference of the fecal cortisol concentrations among sampling days, but the mean fecal cortisol concentration of the day after transferring (337.46 ± 17.88 ng/g dry feces) was significantly higher than that of the day before transferring (248.44 ± 7.99 ng/g dry feces). Comparison with published findings, our results indicated that enclosure size and animal density affect not only behaviors, but also adrenocortical secretion in Père David's deer. Small living space with high animal density may impose physiological stress to captive Père David's deer. Moreover, long-term physiological stress and increase of conflict behavior may subsequently affect survival and reproduction of the deer.

Keywords: *Elaphurus davidianus*; Aggression; Adrenocortical hormone; Glucocorticoid; Captive effect; Stress

1. Introduction

Mammals react to captive environments with behaviors modulated by adrenocortical response (e.g. Carlstead, 1996; Wells et al., 2004; Cockrem, 2005; Carlstead and Brown, 2005). Activation of the hypothalamic–pituitary–adrenal (HPA) is considered to be associated with physiological stress (Sapolsky, 1992; DeVries, 2002). Increase of cortisol may influence behavior and physiological functions in mammals (Wingfield and Romero, 1999; Möstl and Palme, 2002). Stress-related behaviors in captive environment show that captive environment is a source of stress (Lindburg and Fitch-Snyder, 1994; Carlstead, 1996).

Studies indicated that prolonged high cortisol levels cause body weight loss, reproductive failure, immuno-suppression and shorter life span (Cassinello and Pieters, 2000; Creel, 2001; Clubb and Mason, 2003; Carlstead and Brown, 2005). Thus, adrenocortical and behavioral responses to captive stress have been emphasized in *ex situ*

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conservation and animal welfare (Barnett and Hemsworth, 1990; Goymann et al., 1999; Möstl and Palme, 2002; Cockrem, 2005). Indeed, cage size and animal density are the two main factors which result in high adrenocortical hormone level and elevated agonistic interaction, low survival probability and poor reproductive capability in captive animals (e.g. Koontz and Roush, 1996; Cassinello and Pieters, 2000; Crockett et al., 2000; Clubb and Mason, 2003; Wells et al., 2004; Weingrill et al., 2004; Carlstead and Brown, 2005).

Père David's deer (*Elaphurus davidianus*), which originally lived in Northeast and East-Central China, Korea and Japan, was extinct in the wild in the late 19th century or early (Beck and Wemmer, 1983; Cao et al., 1990). However, Père David's deer had been kept in the royal hunting garden in south suburb of Beijing for more than two hundred years (Cao et al., 1990). Nowadays, there are about two thousands of Père David's deer living in captivity or free-ranging situation all over the world (Jiang et al., 2000). Despite of the long captive history, behavior, growth and reproduction of Père David's deer is influenced by captive situation. For example, birth rate of Père David's deer show density-dependent patterns (Jiang et al., 2001a). Comparing with those living in large enclosure with low density, Père David's deer live in smaller enclosure with high density express more agonistic behavior, and produce fewer fawns (Collins, 1983; Jiang et al., 2001a,b; Li, 2004). All of those reports implied that captive situation may bring stress to Père David's deer. However, no study investigated whether enclosure size and animal density inside enclosure influence maintenance behaviors, social stability and cortisol secretion in Père David's deer. We do not know the behavioral and endocrinological mechanism adopted by Père David's deer to respond to confinement in enclosures.

The first aim of this study was to investigate if Père David's deer stag adopt different behavioral pattern when they live in enclosure of different sizes and animal densities. Maintenance behaviors play a very important role in animal survival, and conflict behaviors reflect social stress and social stability (Hurnik et al., 1995). We chose maintenance and conflict behaviors to assess the behavioral response to captive situation.

Changes of adrenocortical cortisol secretion reflect if animal react to captive stress (Möstl and Palme, 2002). Therefore, the second aim of this study was to determine if Père David's deer stag live in small pen suffer more physiological stress than those living in large enclosure. We collected fecal samples for subsequent analysis of fecal glucocorticoid metabolite by radioimmunoassay (Huber et al., 2003; Millspaugh and Washburn, 2004). By monitoring the cortisol concentrations in feces, we compared the fecal cortisol concentration between Père David's deer stag in the free-ranging group and the display group.

However, the deer live in either large enclosure or in small pen, all have being kept in captive environment for many years. We did not know if acute stress, such as deer being transferred from a large enclosure into a small pen will elicit physiological stress on the transferred deer. Therefore, the last aim of this study was to test the instant effects of transfer on adrenocortical reaction in Père David's deer stags. We monitored fecal cortisol concentration in a group of deer stags before and after being transferred from a large enclosure into a small pen.

2. Methods

2.1. Study area and animals

Dafeng Père David's Deer Nature Reserve (32.°59′–33.°03′N, 120.°47′–120.°53′E) is located in coastal region of Yellow Sea, Jiangsu Province, China (Jiang et al., 2000). The reserve has an altitude of 2–5 m. Annual average temperature is 14.1 °C. Average annual precipitation is about 1068 mm. The reserve was established after 39 individuals that were introduced from England in 1986. Since then, the deer in this group lived in a large enclosure of 200 ha. They grazed on natural vegetation in spring, summer and autumn and had supplementary feeds in winter. In all seasons except winter, the vegetations in large enclosure can satisfy needs of living and reproduction of Père David's deer (Ding, 2004). 131 deer (36 stags, 53 hinds, 18 sub-adults and 24 calves) live in the large enclosure at the time of commence of study. Thus, the density of the free-ranging group was 0.66 individuals/ha. In addition, tourist visit the large enclosure was forbidden. However, keepers could enter the large enclosure once every two weeks. This free-ranging group in the large enclosure was one of our experimental groups.

Another experimental group was a display group that was separated from the deer lived in the large enclosure in 1998. The small pen was 0.75 ha in size. Père David's deer in small pen fed on supplementary feeds all year round. The supplementary diet, including corn silage and mixed with barley, corn, bran, soy straw and bean cake, which was suggested nutritionally equivalent to the natural forage of the free-ranging deer (Ding, 2004). In spring of year 2004, there were 19 deer (7 stags, 6 hinds, 3 sub-adults and 3 calves) in the small pen. Thus, the density in the display group was 25.33 individuals/ha. In contrast to large enclosure, during spring in 2004, the deer were on display during day time every day, the number of visitor varied from 1 to 22 people per day. Visitors stay outside fence and watch the deer whereas deer keepers could enter the pen to tend the deer. Except wild birds, wild small mammals and few Chinese water deer (*Hydropotes inermis*), no other large mammal species (including predator or potential predator) were found in the large enclosure. As for in the small pen, only few birds, such as Eurasian tree sparrow (*Passer montanus*), spotted dove (*Streptopelia chinensis*) and cattle egret (*Bubulcus ibis*) were found.

2.2. Behavioral observation

To avoid seasonal fluctuation of behavior and cortisol level in deer (Ingram et al., 1999; Li et al., 2001), we carried out our experiment in the large enclosure (free-ranging group) and the small pen (display group) from February 15 to April 16 in 2004, which was not the rut season of stags. Only one observer was involved in the observations, who normally stayed in a distance of about 80–100 m from the deer. We observed each group from 6:00 to 18:00. After one day observation on the free-ranging group and another day on the display group, we took a break for three days, and then we resumed a new bout of observations. During each observation day, we scanned 8 stags identified by ear tags or the shape of antler in the free-ranging group, or all 7 stags in the display group to record the maintenance behavior and conflict behavior of all objects sequentially (i.e. each stag was observed for two minutes) with SJ-1 Event Recorder (Jiang, 1999). Following behavioral variables were recorded:

Maintenance behaviors: standing, locomotion, foraging and rest (include sitting and lying down).

Conflict behaviors: fighting and chasing other individuals.

2.3. Feces sampling and extraction of cortisol from feces

During the observation, we collected fecal samples from 3 stags in each group every 6 days. From 6:00 to 8:00, we collected one sample from each stag on each sampling day. Freshly voided fecal samples were collected and sealed in plastic bags which were marked the date, name of the pen and object animals with a permanent mark pen. We stored the fecal samples in a cooler instantly. After 30 min, we transferred the fecal samples into a refrigerator and stored them at −20 °C until laboratory analyses.

Wasser et al. (1996) recommended using well-mixed, dried fecal powder from premixed wet samples for hormone metabolite analysis. However, Terio et al. (2002) reported a decrease in glucocorticoid concentration in feces after drying. Thus, we quantified the cortisol concentration in wet fecal samples, and then transferred the steroid concentration in wet fecal sample to dry fecal sample. We first removed all foreign materials from the fecal samples, and then weighed the samples. For measuring the dry matter content of the fecal samples, we divided each sample into two halves, one half for measuring moisture content at 120 °C to constant weight, another half for cortisol assay. The dry substance content of fecal sample was calculated as $\alpha = C/G$ (G is the gross weight of the sample, and C is the constant weight of the sample.). We used the index of $\beta = 1/\alpha$ as a correction factor to transfer the steroid concentration from wet fecal sample to dry fecal sample.

We used a technique as described by Huber et al. (2003) and Millspaugh et al. (2001) to extract fecal cortisol from the wet fecal samples. We placed 0.5 g wet fecal samples into a tube, added 4 ml mixture of analytically pure methanol and distilled H_2O ($V_{Methanol} : V_{H_2O} = 8 : 1$), homogenized and vibrated the tube for 2 min. For lipid extraction, we added 2 ml analytically pure petroleum ether and vibrated the tube for 1 minute, after centrifugation at 1500g for 15 min at room temperature, we transferred 1 ml methanol layer to another tube and dried it at 70 °C. For future analysis, we redissolved the dried sample with 1 ml phosphate buffer solution (0.1 M, pH 7.0) in a tube, and vibrated the tube for 2 min, to form the last samples.

2.4. Cortisol radioimmunoassay

We used ^{125}I cortisol radioimmunoassay (RIA) kits (Beijing Chemclin Biotech Co., Ltd.) to quantify the cortisol concentration in the processed fecal samples. This assay has been validated for fecal glucocorticoid assessment in other cervids (Millspaugh et al., 2001; Washburn and Millspaugh, 2002). Although there was no report of fecal cortisol radioimmunoassay in Père David's deer, the radioimmunoassay of other fecal steroid hormones showed valid in this species (Li et al., 2001). We validated the assay for fecal cortisol in this species by comparing parallelism in a serial dilution of processed fecal samples with the cortisol standard curve ($r = 0.983$). Then we followed the protocol in the manufacturer's guidebook for the ^{125}I cortisol RIA kits, except that we assayed fecal sample in duplicate. Standard curves were produced from seven standards (0–500 ng/ml). We used a SN-682 radioimmunoassay γ counter (Shanghai Hefu Photoelectricity Instrument Co., Ltd.) to count the radioactivity.

The manufacturer's reported cross-reactivity of ^{125}I cortisol antiserums was 100% with cortisol and less than 0.5% for other steroids. Intra- and inter-assay coefficient of variation of cortisol was less than 5.0% ($n = 10$) and 10.0% ($n = 10$), respectively. For all samples from deer, we made estimation of loss of steroids only during the extraction procedure by the addition of ^{125}I- cortisol prior to extraction and measurement of radioactivity in the appropriate methanol (or dichloromethane) fraction after separation. Average recovery rate of cortisol was 81.4 ± 4.4% ($n = 10$).

2.5. Experimental design for acute captive effect on fecal cortisol secretion

For monitoring the acute captive effect on fecal cortisol secretion, we also use wheat seedlings to bait a group of deer (4 stags, 6 hinds and two one-year calves) from another large enclosure of 100 ha with the density of deer of 0.68 individuals/ha into a new small pen of 0.5 ha. Before and after transferring, we collected, stored feces and extract fecal cortisol of those 4 stags with the method described in Section 2.3. The method we used to quantify the fecal cortisol concentration was the same as described in Section 2.4.

2.6. Statistic methods

We pooled the frequency of behaviors at 20-min intervals and presented the data as means ± standard error. When the distribution of data differed significantly from the normal distribution (one sample Kolmogorov–Smirnov test, $P < 0.05$), we used Mann–Whitney U test to check the differences in the frequency of maintenance and conflict behaviors between free-ranging group and display group. We used the independent samples t-test to check the difference of fecal cortisol concentrations between free-ranging group and display group. In this independent samples t-test procedure, we used Levene's test to estimate the equality of variances (when $P > 0.05$, variances was equal, when $P < 0.05$, variances was unequal). In transfer experiment, the distribution of data differed significantly from the normal distribution (one sample Kolmogorov–Smirnov test, $P < 0.05$); we used the Friedman nonparametric repeated measures ANOVA to check the difference of fecal cortisol concentration of the deer among sampling days. Because the variables of fecal cortisol concentration of the deer in transfer experiment were related, we used Wilcoxon signed ranks test to check the difference of fecal cortisol concentration of the deer before and after transferring. The difference at $P < 0.05$ was taken as significantly different for all statistical tests.

3. Results

3.1. Difference of maintenance and conflict behaviors between the free-ranging and the display group

Maintenance behavior and conflict behavior were significant different between the free-ranging group and the display group. Frequency of standing and conflict in display group were significantly higher than those in free-ranging group (Mann–Whitney U test, $N_{\text{Free-ranging group}} = 8$, $N_{\text{Display group}} = 7$. For standing, $Z = -5.113$, $P < 0.05$; for conflict, $Z = -3.569$, $P < 0.05$. Fig. 1). Frequency of rest in display group was significantly lower than that >in free-ranging group (Mann–Whitney U test, $N_{\text{Free-ranging group}} = 8$, $N_{\text{Display group}} = 7$, $Z = -2.579$, $P < 0.05$. Fig. 1). There were no significant differences of locomotion and foraging between free-ranging group and display group (Mann–Whitney U test, $N_{\text{Free-ranging group}} = 8$, $N_{\text{Display group}} = 7$. For locomotion, $Z = -0.294$, NS; for foraging, $Z = -0.652$, NS. Fig. 1).

3.2. Difference of fecal cortisol concentration between the free-ranging and the display group

Fecal cortisol concentration of the free-ranging group was 268.98 ± 15.21 ng/g dry feces, and 326.19 ± 16.98 ng/g dry feces for the display group. Levene's test (in independent samples t-test) indicated that the variances of fecal cortisol concentration was equal ($N_{\text{Free-ranging group}} = 24$, $N_{\text{Display group}} = 30$, $F = 1.89$, $P > 0.05$). Fecal cortisol concentration of the display group was significantly higher than that of the free-ranging group (Independent samples t-test, $t = 2.339$, df = 52, $P < 0.05$. Fig. 2).

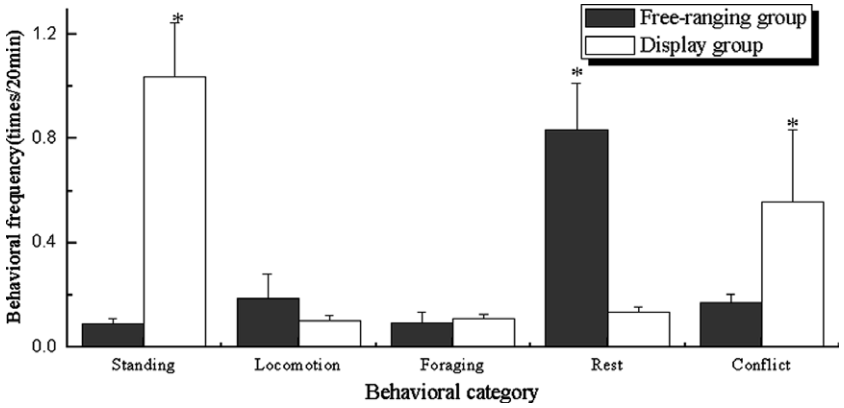

Fig. 1. Maintenance behaviors and conflict behaviors of Père David's deer stag in the free-ranging group ($N = 8$) and the display group ($N = 7$). Each bar represents the means ± SE for each group. Behavioral data marked with an asterisk are significant different between the two groups. See text for statistical analyses.

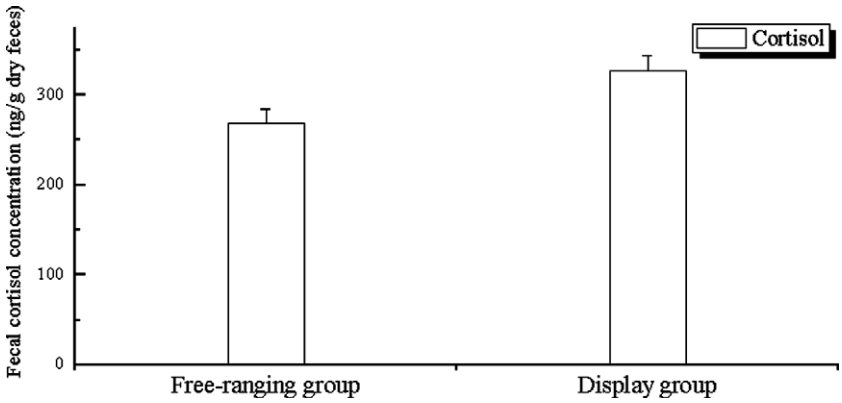

Fig. 2. Fecal cortisol concentrations of Père David's deer stag in the free-ranging group ($N = 8$) and the display group ($N = 7$). Each bar represents the means ± SE for each group. The mean fecal cortisol concentration of the display group was significantly higher than that of the free-ranging group. See text for statistical analyses.

3.3. Changes of fecal cortisol concentration before and after transfer

There was no significant difference of the mean fecal cortisol concentrations among sampling days (Friedman test, $df = 8$, $\chi^2 = 11.73$, $P > 0.05$. Fig. 3). The peak value of the mean fecal cortisol concentration was 425.80 ± 75.16 ng/g dry feces (on March 16, after transfer). The nadir value of the mean fecal cortisol concentration was 242.66 ± 15.15 ng/g dry feces (on February 24, before transfer).

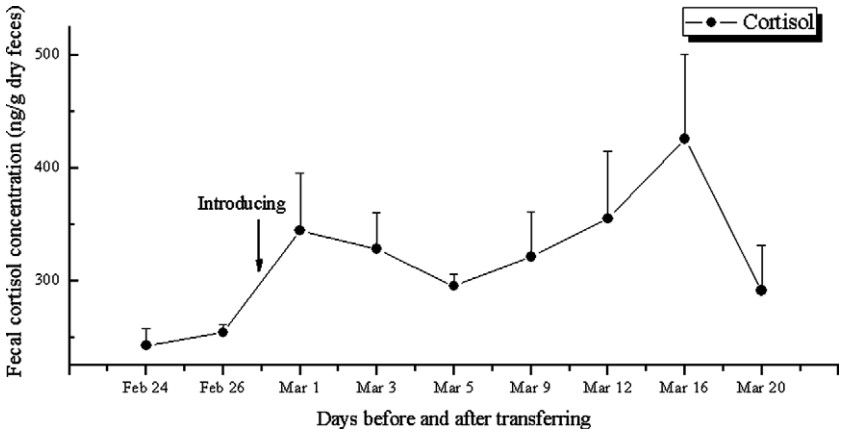

Fig. 3. Fecal cortisol concentrations of Père David's deer stag ($N = 7$) among sampling days in the transfer experiment. Each dot represents the means ± SE of fecal cortisol concentrations for each sampling day. There was no significant difference of the mean fecal cortisol concentration among sampling days. See text for statistical analyses.

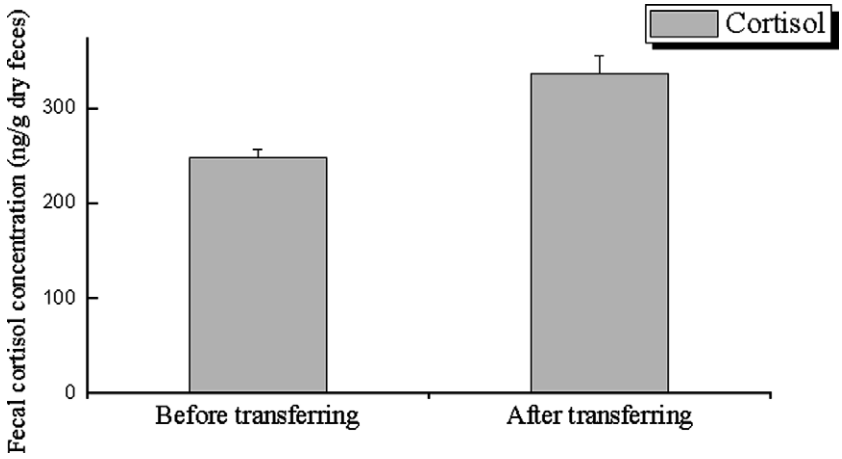

Fig. 4. Fecal cortisol concentration of Père David's deer stag before and after transferred into a small pen ($N = 7$). Each bar represents the means ± SE for each sampling period of time. The mean fecal cortisol concentration of the day after transferring was significantly higher than that of the day before transferring. See text for statistical analyses.

The mean fecal cortisol concentrations of the day after transfer was significantly higher than that of the day before transfer (Wilcoxon test, $N_{\text{Before transferring}} = 8$, $N_{\text{After transferring}} = 28$, $Z = -2.521$, $P < 0.05$. Fig. 4).

4. Discussion

4.1. Effects of captive situation on behavior and adrenocortical action

Our data reveal that Père David's deer living in different captive situation showed different patterns of maintenance and conflict behavior. Moreover, our data demonstrate that fecal cortisol concentration in the display group was higher than that of the free-ranging group. Thus, captivity situation has significant effects on not only behaviors, but also cortisol secretion in Père David's deer. Carlstead (1996) suggested that, for some wild animal species, captivity was considered to be stress because of its limited space, deficient environmental elements and additional human disturbance. For Père David's deer, captive environment were characterized by limited space, high population density, supplementary feed, low herbage cover and human disturbance (Beck and Wemmer, 1983; Jiang et al., 2001b; Li, 2004). Our present study confirms that limited living space, high animal density and human visitors impose stress on the captive Père David's deer.

Many studies show that wild animals may perform different behavioral pattern when they are transferred to restrained enclosure (Martin and Bateson, 1993; Carlstead, 1996). When Père David's deer lived in small pen with high animal density, they stood up longer and rest less, at same time they challenged, chased or even fought more often. Similar results were found in other animal species, such as pigtailed macaques (*Macaca nemestrina*, Crockett et al., 2000), black rhinoceros (*Diceros bicornis*, Carlstead et al., 1999), wild-ranging carnivores (Clubb and Mason, 2003), and some other ungulate species (Del Thompson, 1989).

Why did Père David's deer live in small pen stood up longer but rest less? Presumably, Père David's deer stood up longer for vigilance due to visitor disturbance. Although Père David's deer have been bred in captivity for more than two hundred years, they still are vigilant on human presence (Cao et al., 1990; Li et al., 2006). Our data of changes of behavioral pattern imply that Père David's deer need more time consumption to cope with stress of captivity of small pen.

Conflict behavior, which is considered to be the result of competition for resource and social dominance, is largely related with social stress, whereas captivity usually amplifies the social stress in animals (Koontz and Roush, 1996; Carlstead, 1996). It was suggested that tension caused by a stressful stimulus result in increased social aggression, or even aggression directed towards humans (e.g. Hoff et al., 1997; Goymann et al., 1999; Cassinello and Pieters, 2000; Mitchell and Hosey, 2005). Furthermore, conflict among individuals is largely associated with glucocorticoid secretion. The increase of cortisol may results in increase of conflict behavior (Sapolsky, 1992; Möstl and Palme, 2002), whereas Creel (2001) pointed out agonistic interactions provoke large and persistent increases in cortisol secretion. In our study, although we can not distinguish causality between aggression and cortisol secretion, the simultaneous increase of conflict behavior and fecal cortisol level implies that social conflict of Père David's deer is correlated with adrenocortical action.

In addition, our data of Père David's deer differed from those of farmed red deer (*Cervus elaphus*) in rut season. We found that captive stress of small pen resulted in increased social stress of Père David's deer and led cortisol secretion to high level. However, the chronic social stress in rut season with its increased aggression (Suttie, 1985). Ingram et al. (1999) has been reported to reduce plasma cortisol concentrations in red deer

stags. An explanation is that, testosterone concentrations which peak in stags during the rut were considered to inhibit cortisol secretion directly by influencing steroidogenic pathways (Hornsby, 1982; Miller, 1988). We suppose that social stress caused by captivity and rut differed substantially, and animals responded to different stress with in different pathway.

When animals live in a cage, they may behave differently from their wild counterparts, but no single behavioral variable adequately indicate the animal are enduring stress (e.g. Barnett et al., 1984; Barnett and Hemsworth, 1990). Thus, many researchers suggested that adrenocortical hormones are indicators of stress (see review by Möstl and Palme, 2002). Except behavioral changes, we also find that Père David's deer that live in small pen with high density showed high fecal cortisol concentration. This is the further evidence for confinement or high animal density has brought physiological stress to Père David's deer. In addition, prolonged periods of high cortisol concentrations and social turbulence caused by severe chronic stress are regarded as factors that inhibit growth and suppress reproductive function (Morberg, 1985; Rideout et al., 1985; Wingfield and Sapolsky, 2003). Together with changes of behavioral patterns, we suggested that confined space and high animal density may lead Père David's deer to physiological stress and cause subsequently problems of survival and reproduction.

4.2. Adrenocortical response to acute stress

Our present manipulating experiment show that, after being transferred into a small pen, fecal cortisol level of Père David's deer elevated significantly. Previous studies indicated that capture, yarding and transport result in physiological stress in red deer (Jago et al., 1997; Ingram et al., 1999; Waas et al., 1999). Bubenik et al. (1983) also found that cortisol levels in male white-tailed deer (*Odocoileus virginianus*) are much higher during cold months than during the rest of the year. Millspaugh et al. (2001) reported that human activity might have elevated summer glucocorticoid concentrations in red deer. These reports indicate that deer response to acute stress with endocrine modulated behavior.

Generally, glucocorticoid responses are thought to help animals cope with acute stressors (Sapolsky, 1992; Sapolsky et al., 2000). Short-term response can improve fitness by energy mobilization by changing behavior (Raynaert et al., 1976; Korte et al., 1993). However, when high level of glucocorticoid persists for a long time (e.g. more than two weeks), the short-term benefits will become long-term pathologies, and then will do harm to immune system, growth and reproduction (Morberg, 1985; Rideout et al., 1985; Creel, 2001; Wingfield and Sapolsky, 2003). All these characteristics are indicative of chronic stress that may directly compromise animal's survival and reproduction. Thus, we suppose that long-term stress of small pen may be the reason for the fact that growth and reproduction of Père David's deer were influenced by bad captive situation (for literatures, see Collins, 1983; Jiang et al., 2001a,b; Li, 2004).

4.3. Conclusions

The main finding of this study is that enclosure size, animal density and human disturbance significantly influence behavior and adrenocortical reaction of Père David's deer. Père David's deer react to acute stress in endocrine response; small pen may bring negative effects on survival and reproduction of the deer.

For animal propagate in captivity, it is important to understand the captive effects on behavior and its endocrine mechanisms (Curio, 1996; Sutherland, 1998; Cockrem, 2005). Previous researches revealed that captive situation may affect behavioral expression and reproductive strategy of Père David's deer (Jiang et al., 2001b; Li et al., 2004, 2005, 2006, 2007). We elicit the implications for captive Père David's deer management: large enclosure and high environmental heterogeneity will benefit survival and reproduction of Père David's deer in captive environment.

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