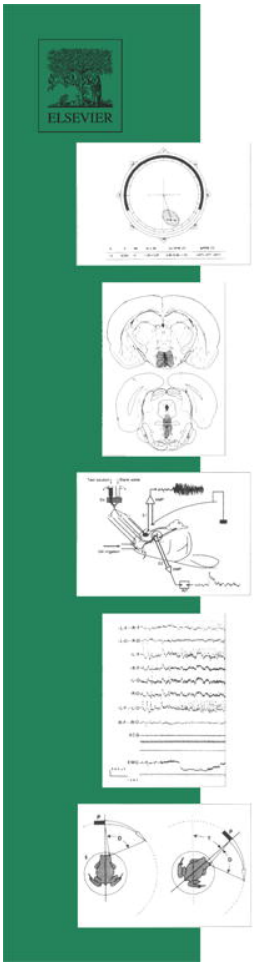

VOLUME 90 NUMBERS 2–3 FEBRUARY 28, 2007
ISSN 0031-9384

PHYSIOLOGY & BEHAVIOR

AN INTERNATIONAL JOURNAL

INCLUDES A SPECIAL SECTION ON
CHRONOBIOLOGY ASPECTS OF THE SLEEP-WAKE
CYCLE AND THERMOREGULATION

ELSEVIER

Physiology & Behavior 90 (2007) 490–495

PHYSIOLOGY & BEHAVIOR

Rank in a food competition test and humoral immune functions in male Brandt's voles (*Lasiopodomys brandtii*)

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Received 3 February 2006; received in revised form 7 October 2006; accepted 24 October 2006

Abstract

Social status can influence an animal's immune and reproductive functions, eventually leading to alterations in immunocompetence and reproductive success. Here, we report that rank assessed in a food competition test, considered as an index of social status, has significant influences on humoral immune functions in male Brandt's voles (*Lasiopodomys brandtii*) living in a group. Our data reveal a negative correlation of the spleen mass and serum antibody levels with social status, as well as a positive correlation of serum cortisol levels with social status. Males winning in food competition had a smaller spleen, a lower level of serum antibodies, and a higher level of serum cortisol than did their conspecific counterparts. These data indicate interactions between social status and humoral immune functions and might illustrate a trade-off between infection risks and reproductive success in male Brandt's voles.

Keywords: Brandt's vole; Food competition; Immune function; Social status; Stress

1. Introduction

Immune system functions play an important role in regulating animals' survival via altering their fitness to the environment. Animal immune functions and their influencing factors have become one focus of research on population ecology [1]. Social status has been proven to have significant influences on an animal's immune system [2,3]. Such influences can be dichotomous. On one hand, individuals with dominant social status are found to have higher levels of immunity, including elevated cellular and humoral immune functions and an enhanced resistance to disease [4,5]. On the other hand, these individuals could suffer from a more intensive immunosuppression in comparison to their subordinate counterparts [6,7].

Many factors may have stimulus-specific effects on interactions between social status and immune functions. For example, acute social stress (2 h of social confrontation) resulted in both a decreased number of T-cells and suppressed functional capacity per single T-lymphocyte, whereas chronic social stress (48 h of social confrontation) only reduced the number of T-cells in Long-Evans rats [8]. In another study, reduced antibodies responded to keyhole limpet hemocyanin (KLH), but no change of spleen phagocytic cell functions were reported after multiple defeat experiences, when compared with a brief social defeat [9]. Furthermore, it has been shown that because animals have specific social structures and behavioral strategies, interactions of social status and immune functions are species-specific [3].

Brandt's voles (*Lasiopodomys brandtii*) mainly live on the grasslands of Inner Mongolia of China, Mongolia, and Russia. This animal is social and displays a polygynous life strategy [10]. Over-wintering males disperse in early spring. During subsequent reproductive seasons, they usually maintain the highest social status within groups and protect their home ranges against conspecific intruders [11]. In some cases, intruders can defeat and then replace resident males [11]. Most of the previous studies on this rodent species have been

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0031-9384/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2006.10.009

focused on describing population fluctuations [12–14], behaviors [10,11,15], thermoregulation and energy metabolism [16–19], and no studies have been conducted to examine the relationship between social status and immune function. Therefore, in the present study using male Brandt's voles as a model system, we identified the individual's social status in a group-living condition using a food competition test, and then examined the relationships between social status, endocrine factors, and immune functions. We hypothesized that, in the group-living social Brandt's voles, social status has a significant impact on individual immune and endocrine functions.

2. Materials and methods

2.1. Animals

Thirty-six adult (>60 days of age, 42–52 g body mass) male Brandt's voles (*L. brandtii*) were obtained from our laboratory breeding colony established from the descendants of animals trapped in the grassland of Inner Mongolia, China. After weaning (21 days of age), animals were housed with same sex in plastic cages (40×35×20 cm, 25–30 voles/cage) where food (rabbit chow, Beijing Ke Ao Fed Co., China) and water were provided *ad libitum* except during the food deprivation period (see below). All cages were maintained under a 14L:10D photoperiod with lights on at 0600 h [20] . The temperature was maintained at 23±1 °C. Prior to experiments, animals were separated and housed individually for a week. Thereafter, they were divided into 12 groups, each with 3 mass matched (their body masses did not differ by more than 5 g) animals that had never been housed together previously in the same plastic cages.

2.2. Food competition test and social status

After 5 weeks of group housing, a food competition test was conducted. All three animals from each group were placed in an open plastic arena (40×35×20 cm). After 8–10 h of food deprivation, a block of carrot was put in the center of the arena, and then a 15 min behavioral observation was conducted. Food competition behaviors were recorded vocally on a cassette recorder. The three hungry animals were fighting for the carrot during this period. Usually while the carrot was held and ate by one animal, the other two would try to seize it. Such tri-individual food competition behaviors were broken into dyads. In one bout, if one animal seized the food from its opponents or defended its food successfully, it was scored as a winner. After the behavioral test, animals were put back into their holding cages where food and water were provided *ad libitum*. The food competition test was repeated 5 times for each group on different days. A dominance index (DI) was then calculated using the following formula described by Eden [21]:

$$DI = \frac{\sum_{i}^{N} W_i/T_i}{N}$$

where N is the total number of opponents recorded, W_i is the number of interactions won against the ith opponent, and T_i is the total number of interactions against the ith opponent. According to their dominance indices, we ranked the status of the 3 animals in each group as relatively high, middle, and low. Although an unstable relationship was sometimes found during the initial food competition tests (1st and/or 2nd test) probably due to an unestablished hierarchy in the early stage of encounters, a consistent linear relationship was always found among the three subjects following the repeated food competition tests.

2.3. Sampling

After completion of all five food-competition tests (at the end of the 6th week), each animal was weighed, received an injection of human immunoglobulin (IgG; 0.2 mg in 0.2 ml sterile saline), and then put back into the group housing condition. Five days later, the animals were sacrificed. Trunk blood (1.5–3 ml) was taken. Blood samples were then centrifuged at 1680 *g* at 4 °C for 30 min. Serum was stored at −80 °C until assayed for antibodies, testosterone and cortisol [20]. In addition, spleens and testes were removed and weighed.

2.4. Antibody assay

Enzyme-linked immunosorbent assay (ELISA) was used to assess the levels of serum antibodies against human IgG. A ninety-six-well immunoplate (Nunc MaxiSorp) was coated with antigen (0.1 mg human IgG in 0.1 ml sodium bicarbonate buffer, pH 9.6), washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T, pH 7.4), blocked with 5% calf serum in PBS, and then washed again. Thawed serum samples were diluted 1:100 in PBS, then 0.1 ml of each sample was added to the wells of the antigen-coated plate. The negative control samples were also added to the wells. Because no appropriate positive control sample was available, we used the absorbencies to represent the relative levels of serum antibodies instead of the absolute concentrations. All samples were processed in duplicate. The plate was sealed and incubated at 37 °C for 1 h, and then washed with PBS-T. A secondary antibody (horseradish peroxidase-conjugated antimouse IgG) diluted 1:1000 in PBS was added to the plate, and then the plate was sealed, incubated for 1 h at 37 °C, washed with PBS-T, and 0.1 ml of the

Table 1
Physical measurements in high, middle and low-status male Brandt's voles (*Lasiopodomys brandtii*)

	High	Middle	Low	*p*
Initial body mass (g)	47.20±0.99* (n=12)	47.11±0.80 (*n*=12)	46.63±0.75 (*n*=12)	ns
Final body mass (g)	56.12±2.56 (*n*=12)	52.12±3.12 (*n*=12)	52.87±3.21 (*n*=12)	ns
Total testes mass (mg)	703.53±94.95 (*n*=12)	511.46±120.13 (*n*=12)	606.38±89.38 (*n*=12)	ns
Spleen mass (mg)	48.44±3.59[a]** (*n*=10)	47.21±3.12[a] (*n*=10)	68.74±10.72[b] (*n*=10)	0.05

*: Data are presented as mean±SEM.
**: Alphabetic letters indicated results of a post-hoc test following a one-way ANOVA. Groups with the same letter did not differ from each other.

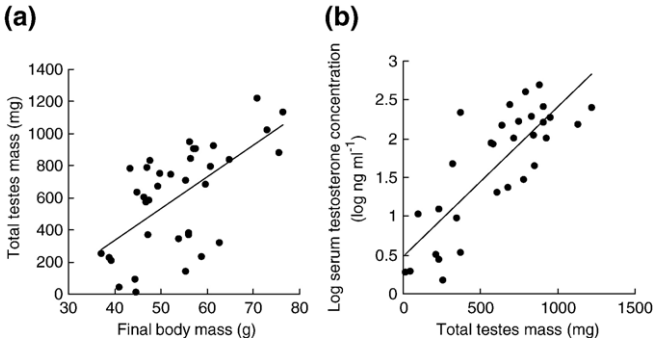

Fig. 1. (a): Correlation between final body mass and total testes mass in Brandt's voles (*Lasiopodomys brandtii*). Line is fitted by linear regression: $y=19.75x-454.70$ ($n=36$, $r^2=0.387$, $p<0.01$). (b): Correlation between total testes mass and serum testosterone concentration in Brandt's voles (*L. brandtii*). Line is fitted by linear regression: $y=0.0019x+0.4808$ ($n=33$; $r^2=0.469$; $p<0.01$).

enzyme substrate (a mixture of 3,3′,5,5′-tetramethylbenzidine and urea hydrogen peroxide in the proportion of 1:1 by volume) was added to each well. The plate was kept at 37 °C in total darkness for 20 min, followed by additions of 0.05 ml of 2 M H_2SO_4 into each well to terminate reactions. The optical density (OD) of each well was measured using a plate reader equipped with a 450 nm wavelength filter, and the average OD from the duplicates was used for data analysis [20].

2.5. Testosterone and cortisol radioimmunoassay

Serum testosterone and cortisol concentrations were measured using ^{125}I radioimmunoassay (RIA) kits provided by the Beimian Dongya Institute of Biotechnology (Beijing, China). The intra-assay and inter-assay coefficients of variation were <5% and <10% respectively [20,22]. The cortisol assay was highly specific and its cross-reactions with other steroid hormones were <0.01% [20].

2.6. Data analyses

Data are presented as the mean±SEM in the text. All statistic analyses were performed using SPSS (version 10.0 for Windows; SPSS Inc.). All variables were checked for normal distribution. One-way ANOVA and ANCOVA were used for testing the differences among high-, middle- and low-status animals (final body mass was used as a covariant) followed by Tukey's post-hoc test. In addition, relationships between variables were analyzed using Pearson's correlations and partial correlations when needed. Differences were considered statistically significant at $p<0.05$.

3. Results

No significant differences were found in body mass among the relatively high, middle and low-status animals either before ($F_{2,35}=0.127$, $p>0.05$) or after ($F_{2,35}=0.510$, $p>0.05$) the housing period (Table 1). In addition, no group differences were found in the testes mass after the housing period ($F_{2,35}=0.453$, $p>0.05$). However, a positive correlation was found between body mass and total testes mass after the group housing ($r=0.622$, $df=35$, $p<0.001$, Fig. 1a).

A significant group difference was found in the spleen mass ($F_{2,32}=3.697$, $p=0.037$; final body mass as a covariant). Spleen mass was significantly higher in low-status animals than in high and middle-status animals, and the latter two groups did not differ from each other (Table 1).

Productions of specific antibodies in response to human IgG differed significantly among the three groups ($F_{2,35}=4.061$, $p=0.027$, Fig. 2a). Low-status animals had higher levels of antibodies than did high- and middle-status animals which, in turn, did not differ from each other.

Serum testosterone levels did not differ significantly among groups ($F_{2,32}=0.029$, $p>0.05$, Fig. 2b), but showed a positive correlation with total testes mass ($r=0.685$, $df=32$, $p=0.007$, Fig. 1b).

Finally, serum cortisol levels showed significant group differences. The high-status animals had a higher level of cortisol than the middle and low-status animals ($F_{2,34}=6.515$, $p=0.004$, Fig. 2c). The latter two groups did not differ from each other.

4. Discussion

Access to limited resources, such as food, water, and mating partners, is often determined by an animal's social status in a group-living environment and is critical for the animal's

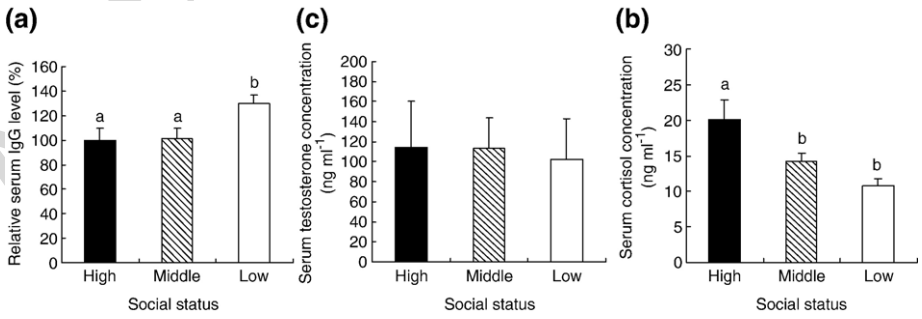

Fig. 2. Relative serum IgG levels, testosterone concentrations and cortisol concentrations of male Brandt's voles (*L. brandtii*). (a): Relative serum IgG levels are expressed as percentages of the mean value of high-status group. Males with low-status had a higher level of serum IgG than did males with high- or middle-status. (b): Males with high-, middle- or low-status did not differ in serum testosterone concentrations. (c): Males with high-status had a higher level of serum cortisol than did males with middle- or low-status. Alphabetic letters indicated results of a post-hoc test following a one-way ANOVA. Groups with the same letter did not differ from each other.

reproductive success. In laboratory conditions, competition tests for limited resources have been used to reveal an animal's social status [4,23,25]. According to the resource-defense hypothesis, the sociality of rodents can be affected by the abundance and distribution of critical resources [25]. Brandt's voles are territorial animals that live in typical steppe where they experience long cold winters [11]. These animals begin to store food in autumn, which plays an important role for their survival in winter and reproductive success in the subsequent breeding season [11]. A similar phenomenon has also been found in other sympatric rodent species such as Mongolian gerbils (*Meriones unguiculatus*) [26,27]. In the present study, we used a previously established food competition test [23] to reveal the social status of Brandt's voles living in a group condition.

Circulating levels of testosterone have been used to assess an individual's reproductive competence, and dominant males that usually have a high level of circulating testosterone are often more aggressive in competition for resources and potential mates [28–30]. Although our data seemed to imply that high-status male voles were more competitive, circulating levels of testosterone in male Brandt's voles only showed a positive correlation with testes mass, which, in turn, correlated positively with body mass, but there was no significant correlation with an individual's social status. Zhang et al [22] found that male Brandt's voles showed higher levels of testosterone under long photoperiod than under short photoperiod. It should be noted that relationships among circulating levels of testosterone, aggressiveness and social status are complicated and can be affected by many variables [30–32]. Further experiments are needed to address precisely interactions between Brandt's vole's social status and its circulating level of testosterone.

An animal's social status sometimes correlates with its morphology; in particular, larger individuals tend to win in contests and thus have higher social status than smaller ones [4,33]. However, this is not always the case. For example, group-housed male BALB/c mice did not show differences in their body mass in relation to their social status [32]. In the present study, male voles with different social status did not differ significantly in their body mass, suggesting no correlation between body size and social status in these experimental conditions. That the dominants didn't show a heavier body mass might be due to the activation of hypothalamic–pituitary–adrenal (HPA) and sympathetic axis, as the dominants' serum cortisol levels were elevated in comparison to their lower rank counterparts. It could also be a metabolic cost to maintain a dominant social status [34,35].

Serum glucocorticoid (corticosterone or cortisol) levels are often used as indices of HPA axis activity and represent an individual's response to psychosocial stress [36]. For animals in group-living conditions, a widely held view is that socially subordinate individuals usually experience psychosocial stress, and thus show an increased HPA activity indicated by elevated levels of serum glucocorticoids [3]. However, some other studies suggest that in some species, high-ranking individuals have to repeatedly and physically reassert their domination over the subordinate cohort, and thus dominant individuals may have greater physiological indices of stress than subordinates [34,35,37].

It should also be mentioned that usually corticosterone is considered to be a primary corticosteroid in rodents [38]. However, we had technical difficulties to measure corticosterone in Brandt's voles but were able to measure their cortisol levels [20]. Although cortisol may not be the most representative corticosteroid for rodents, it still provides useful information and has been used in rodent studies (e.g. [39]). In our study, high-status males had higher levels of serum cortisol than did middle or low-status males, suggesting that it can be stressful to maintain a dominant social status for male Brandt's voles. We also found that housing density had no effect on the serum cortisol levels of both male and female voles [20]. It has been demonstrated that social stress can lead to a wide range of physiological responses including suppression of immune functions [40,41], which can be at least partially responsible for the immunological differences between the male Brandt's voles with different social status in the present study.

The spleen is an internal organ with a variety of functions important for the immune system [29,36,42]. In our study, male voles with low social status had a larger spleen than did males with middle or high social status. These data indicated that male Brandt's voles with low social status might have enhanced spleen functions. However, the spleen size is a controversial parameter when used to evaluate an individual's immune function because it can be influenced by individual's prime body condition and by infections of diseases and parasites, i.e. large spleen size may represent either an elevated immune function or a hypertrophy stimulated by parasites [43]. Therefore, we also measured the levels of serum antibodies in response to a novel antigen, and integrated both measurements for evaluation of an individual's immune functions.

In the present study, we injected male voles with a novel antigen and found that males with low social status had a higher level of serum antibodies than those in the other two groups. These data, together with the data of spleen mass, indicated suppressed immune functions in high-status male Brandt's voles. Although these data are contrary to (or different from) the data from some studies in pigs [4,5] and male mice [24,44], Barnard and his colleagues [6,45] indeed reported that, in male CFTP mice, individuals that had higher social status reached a peak of infection sooner and were slower to clear infection. It was also reported that antibody titers to sheep red blood cells in dominant pigs were lower than that in either intermediate or submissive ones [7].

Under stressful and resource limited conditions, animals can choose one of two different strategies in the trade-off between survival and reproduction. One strategy is to increase the animal's own "defense capacity" (i.e. to improve their immune functions) and the other is to put more efforts into reproduction with more risks of being infected by parasites and pathogens at the same time [46]. For short-lived and gregarious animals, it may be advantage to trade-off immune defenses for reproductive success [3]. Our finding that high-status male Brandt's voles had increased HPA activity with decreased immune functions may provide further evidence to support this notion. To a dominant individual, a

significant immunosupression may not be always the case, but a life history strategy under some circumstances. Further research is needed to determine whether the negative relationship between the immunocompetence and the social status is a constant and genetic characteristic in male Brandt's voles.

Finally, it should be noted that only the spleen mass and serum antibody levels were used as indices of immune functions in the present study. Cellular immunity is another important component of acquired immunity. It has been suggested that different components of the immune system can be activated in a stimulus-specific manner [47]. Therefore, it would be useful to measure more immune parameters to assess the effects of social status on the immunity of Brandt's voles. It would also be interesting to further evaluate the current findings in field animals and to examine the relationship between immunocompetence and population dynamics.

Acknowledgements

Thanks to the valuable comments and suggestions of three anonymous reviewers. This study was supported by grants from the CAS Innovative Research International Partnership Project (CXTDS2005-4) and the National Natural Science Foundation of China to DHW (30570230).

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