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Effect of Group Density on the Physiology and Aggressive Behavior of Male Brandt's Voles (*Lasiopodomys brandtii*)

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Xin Dai, Ling-Yu Zhou, Jie-Xia Cao, Yan-Qi Zhang, Feng-Ping Yang, Ai-Qin Wang, Wan-Hong Wei, and Sheng-Mei Yang (2018) Population density is well known to influence animal physiology and behavior. How population density affects the aggressive behavior of the Brandt's vole (Lasiopodomys brandtii) is, however, little known. The aim of this study was to investigate the effect of group density on physiologic responses and aggressive behavior of male Brandt's voles and their potential underlying neuro-mechanism. The results show that increasing group density led to elevated serum corticosterone levels and increased spleen weight; it also induced more male-male aggressive behavior. By contrast, it had a negative effect on body growth and the weight of testis and epididymis. Aging also increased male-male aggressive behavior. Higher density reduced mRNA levels of tryptophan hydroxylase 2 (TPH2), 5-hydroxytryptamine receptor 1A (5HT1A), and 5-hydroxytryptamine receptor 1B (5HT1B) in the amygdala and the dorsal raphe nucleus (DRN). Our results demonstrate that higher population density can intensify stress reactions and male-male aggressive behavior in Brandt's voles at the price of inhibiting body growth and reproduction. Serotonergic systems in the amygdala and the DRN may take part in the control of aggressive behavior among male voles. Our results provide novel insights into the neuro-mechanism underlying the influence of population density on aggressive behavior in Brandt's vole, and imply that aggressive behavior may play an important role in the population fluctuation of the animal.

Key words: Brandt's vole, Group density, Aggressive behavior, Physiological response, 5-hydroxytryptamine.

BACKGROUND

Population dynamics are tightly correlated with animal population density (Guckenheimer et al. 1977; Zeng et al. 1980; Lee and McDonald 1985; Zhang 1996; Christian 1980). The behavioral-physiological hypothesis postulates that an increase in population density can enhance behavioral interactions between individuals, cause corresponding physiological responses, and eventually result in population fluctuations (Geller and Christian 1982). Aggressive behavior is in fact an important regulator for populations and plays an important role in the biosocial mechanism underlying density-dependent effects (Nie and Liu 2005; Boonstra and Boag 1992; Rogovin et al. 2003). However, the population dynamics of animals can be simultaneously determined by density-dependent and density-independent factors (Leirs et al. 1997; Karels and Boonstra 2000). It is therefore difficult to investigate the exact effect and mechanism of density-dependent aggressive behavior in individuals and wild populations. A number of ecologists have conducted laboratory

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studies on rodents in order to get insights into wild populations and found that variations in group density affects levels of aggressive behaviors (Van Loo et al. 2001; Butler 1980) and induces physiological and immunological changes (Brain and Nowell 1970; Schuhr 1987; Barrett and Stockham 1963; Peng et al. 1989; Newman et al. 2015); however, little is still known about the neuromechanism of density-dependent aggressive behavior.

The Brandt's vole (Lasiopodomys brandtii) is a small, gregarious and mainly polygynous, seasonally reproductive mammal that is common and abundant in the grasslands of Inner Mongolia, China (Xie et al. 1994; Shi et al. 1999; Wan et al. 2002). Its population fluctuates tremendously each year (Wan et al. 2002; Zhang et al. 2003). The Brandt's vole is therefore a suitable model to study the physiological and behavioral responses to population density and its mechanisms, and to illuminate the relationship between population density and fluctuations. However, due to the difficulties of fieldwork, little is known about the effect of population density on this animal's aggressive behavior and its underlying neuro-mechanism. The neurotransmitter 5-hydroxytryptamine and its receptors (5HT1A and 5HT1B) play an important role in the regulation of aggressive behavior (Nelson and Chiavegatto 2001: Olivier 2004: de Boer and Koolhaas 2005: Takahashi et al. 2011; Edwards and Kravitz 1997; Carkaci-Salli et al. 2011; Nelson and Trainor 2007), especially inter-male aggression (Simon et al. 1998). Several members of the serotonin receptor family are expressed in the dorsal raphe nucleus (DRN), medial prefrontal cortex (mPFC), and amygdala (Bortolato et al. 2013), which are components of brain circuits implicated in aggression (Bortolato et al. 2013; Takahashi and Miczek 2014). We therefore hypothesized that the serotonergic system within the central nervous system is involved in density-dependent aggression in the male Brandt's vole.

In this study, we housed captive male Brandt's voles at different densities and investigated growth, reproduction, immune organs, serum corticosterone (CORT) levels, and duration of aggressive behavior. We compared expression differences between *TPH2*, *5HT1A*, and *5HT1B* in the mPFC, the DRN, and the amygdala. Our aim was to investigate changes in physiology and aggressive behavior in response to population density in male Brandt's voles and to explore the neuro-mechanism underlying the aggressive behavior. The results of the current study thus help us better understand the role of aggressive behavior in the process of population density influencing population dynamics.

MATERIALS AND METHODS

Animals and Procedure

Brandt's voles captured from the grasslands of Inner Mongolia were bred as the F0 generation in the animal group facility at Yangzhou University, Jiangsu Province, China, under controlled environmental conditions at a temperature of $22 \pm 1^{\circ}$ C, a relative humidity of $50 \pm 5\%$, and a photoperiod of 12 h light/12 h dark (light on at 6:00 am and off at 6:00 pm). At 21 days of age, F1 generation male voles were weaned and housed in polypropylene cages for another 13 days until 5 weeks of age. Thereafter, the male voles were transferred to larger cages $(48 \times 35 \times 25 \text{ cm}^3)$ and randomly divided into groups of three (threevoles group) or five (five-voles group; six cages of each group size), guaranteeing that the male voles in each individual cage were not siblings. The experiment lasted for 10 weeks. The male voles were individually marked on the fur of different body parts (left ear, right ear, neck, waist, and hip) with a bright red hair dye. The mark was renewed weekly and the cages were also cleaned weekly. All voles were provided with filtered tap water and standard rodent chow ad libitum before and throughout the experimental period. All procedures were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University.

At the age of 6 weeks, the male voles' behavior was recorded in their home cages at 8:30 am for a period of 30 min using an Embedded Net XVR system with a high-definition infrared camera (HIKVISION, Hangzhou, China) in a specific video room. The male voles were acclimated for 30 min before recording and weighed after recording. Thereafter, their behavior was recorded weekly, while their weight was measured again at the age of 9 weeks and 15 weeks. The duration of aggressive behavior (other behaviors were not analyzed) was scored as in Van Loo et al. (2001) using The Observer XT 7.0 program (Noldus Information Technology, Wageningen, Netherlands). We calculated the means of the duration of aggressive behavior and body weight in each individual cage, yielding six measurements

of aggressive behavior duration for each group in each week, and six measurements of body weight at the ages of 5, 9, and 15 weeks each. According to the agonistic behavior description in Hofmann et al. (1982), the animals' behavior was interpreted as aggressive when there were several offensive behaviors, as described in the following (1) threat: the vole raises its forefeet off the floor, extends its head toward the other vole, bares and sometimes chatters its teeth; (2) attack: the vole either thrusts its head and the front of its body toward the other vole or jumps at it with all of its feet leaving the floor, and actual bites may or may not occur; the vole in an upright posture strikes at the head and shoulders of the other vole with its forefeet; (3) chase: one vole pursues the other; the chase may end with a leaping attack from the rear. Behavior duration data in two contiguous weeks were recognized as one variable, so there were five variables for each group in the statistical analysis.

At the age of 15 weeks, all animals were weighed and decapitated after anesthetizing with ether. Serum samples were collected as previously described (Dai et al. 2016). Spleen, paired testes, and paired epididymis were collected and weighed. The relative weight of testis, epididymis, and spleen was calculated as paired testes, paired epididymis, and spleen weights (g), divided by body weight (g). Furthermore, we calculated the mean for these three parameters in each individual cage, yielding six measurements for each parameter for each group. The brains were removed and dissected on ice to extract the two fragments of the hypothalamus in a mouse brain matrix. Referring to the mouse brain atlas (Franklin and Paxinos 1997) and earlier reports (Van De Werd et al. 2010; Van De Werd and Uylings 2014), three brain fragments (mPFC, amygdala, and DRN) were obtained by making coronal cuts, horizontal cuts, and two bilateral parasagittal cuts. These brain fragments were stored individually in a sample protector for RNA (TaKaRa) at -20°C. The mRNA levels of TPH2, 5HT1A, and 5HT1B were determined in individual fragments.

Enzyme-Linked Immunosorbent Assay (ELISA) For Serum Hormones

Serum hormones, namely corticosterone (CORT) and testosterone (T), were quantified in duplicate using a double antibody sandwich ELISA mouse kit (Jingke Chemical Technology Limited Company, Shanghai, China) according to the manufacturer's instructions and as described in a previous report (Dai et al. 2016). Standard curves constructed for each of the assayed hormones had a regression value higher than 0.99. The purity of the CORT and T standard preparations were all > 95%. The intra- and inter-assay coefficients of variation were < 9 and < 11% for T and < 9, and < 15% for CORT. We calculated the mean level for both hormones in each individual cage, yielding six concentrations for each hormone for each group.

Quantitative Real-Time Polymerase Chain Reaction (Qpcr)

Total RNA was extracted and stored using the procedure established in our previous study (Dai et al. 2016). RNA samples of 1 μ g were reverse-transcribed using a PrimeScript 1st strand cDNA synthesis kit (TaKaRa, Dalian, China). Gene expression was measured by gPCR. The sequences of qPCR primers for β -actin were adopted from previous studies (Hegab et al. 2014; Zhang et al. 2014), while those for 5HT1A, 5HT1B, and TPH2 were determined according to the same procedure described in our previous study (Dai et al. 2016). An amino acid sequence from vertebrate animals corresponding to 5HT1A, 5HT1B, and TPH2 was retrieved from the National Center for Biotechnology Information (NCBI) database and aligned using Clustal X (Larkin et al. 2007). Two degenerate primer pairs suitable for cDNA amplification for each gene were designed using CODEHOP (Rose 2005). The synthesized cDNA was cloned into pMD18-T vectors and sequenced by Sangon Biotech Company (Shanghai, China). The sequences were submitted to GenBank as partial mRNA sequences for each gene (accession numbers for 5HT1A, 5HT1B, and TPH2 are MF536087, MF536088, and MF536089, respectively) and analyzed using the NCBI Primer Blast tool to design qPCR primers. The specificity of the primers was checked using PCR and the melt curve of qPCR to ensure that no primer dimers or non-specific products were formed (Table 1). PCR reactions were conducted in a real-time PCR system (Applied Biosystems, Grand Island, NY, USA) using SYBR Premix EX Taq II (TaKaRa, Dalian, China). From each sample, 10 µL cDNA was retrieved and qPCR was conducted in a 10-µL reaction volume using the procedure described in Dai et al. (2016). Each sample was analyzed in triplicate. Thermal cycling conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 34 s, and 72°C for 30 s. Amplification efficiency of cDNA was tested using

standard curves (Dai et al. 2016), which ranged between 0.9 and 1.1 and indicated the validity of the comparative quantification method. The fold change of gene expression was calculated using the 2^{- $\Delta\Delta$ Ct} method (Livak and Schmittgen 2001), using β -actin (O'Shaughnessy et al. 2002) as a housekeeping gene for brain gene expression. The expression level of each gene in the DRN for each three-voles group was regarded as an expression level of 1. We calculated the mean relative mRNA expression level for all three genes in three brain regions in each individual cage. This yielded six relative mRNA expression levels for each gene for each group in each brain region.

Statistical Analysis

All variables were tested for normality and homogeneity using the Shapiro-Wilk test and the Levene test, and were log10- or square roottransformed when necessary. The effect of density on body mass of the voles was studied using repeated-measures analysis, with body weight at 5 weeks of age as the covariate. The effect of density and age on duration of aggressive behavior was studied using two-way analysis of variance (ANOVA) followed by Tukey's HSD or Dunnett's T3 post hoc tests. The duration of aggressive behavior among the five age groups were both determined using one-way analysis of variance (ANOVA) followed by Tukey's HSD or Dunnett's T3 post hoc tests. Independent-samples t-tests were used to compare the differences in serum hormones, in relative weight of testis, epididymis, and spleen, and in gene expression between the three-voles and five-voles groups. Statistical significance was determined at P < 0.05. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Differences in body and organ weight

The body weight of the voles was higher in the three-voles group than in the five-voles group ($F_{1,9} = 5.440$, P = 0.045; Fig. 1A). Similarly, the relative weight of testis (Fig. 1B) and epididymis (Fig. 1C) were both higher in the three-voles than in the five-voles group (P < 0.001, P = 0.017, respectively). In contrast, relative spleen weight was higher in the five-voles than in the three-voles group (P = 0.048; Fig. 1D).

Differences in serum hormones

The concentrations of serum CORT were higher in the five-voles compared with the three-voles group (P = 0.014; Fig. 2A). The concentrations of serum T did not differ significantly between the two groups (P = 0.769; Fig. 2B).

Duration of aggressive behavior

The interaction effect between density and age on aggressive behavior duration within 30 min was not significant ($F_{4, 50} = 1.182$, P = 0.333). Effects of density ($F_{1,54} = 29.751$, P < 0.001) and age ($F_{4,54} = 7.537$, P < 0.001) on aggressive behavior were significant, with the five-voles group showing longer durations of aggressive behavior than the three-voles group (P < 0.001; Fig. 3), and with the voles at 6-7 weeks of age showing shorter durations of aggressive behavior than the voles at 10-11, 12-13, and 14-15 weeks of age (P = 0.024, P = 0.002, P < 0.001, respectively; Fig. 3).

Expression of tryptophan hydroxylase and serotonin receptor genes in the brain

The mRNA levels of *TPH2* in the DRN and the amygdala were higher in the three-voles group than in the five-voles group (P < 0.001, P = 0.003, respectively; Fig. 4A), but there was no difference

Table 1. Primers for c	quantitative real-time	polymerase chain	reactions used in	the present study	'
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	Forward(5'-3')	Reverse(5'-3')	Reference
TPH2	TTGCCCGTCCCTTCTCAGTA	TTTATTCAGGGCATCGCACAC	Designed in present study
5HT1A	TAGAAAAGAAGGGAGCGGGC	CGCACCATTAGCGCATGAAG	
5HT1B	CGTCGGATATCACCTGTTGC	AGAAGACCCACACCAGCGTTA	Hegab et al.(2014)
β-actin	TTGTGCGTGACATCAAAGAG	ATGCCAGAAGATTCCATACC	



Fig. 1. Body weight (A), and relative weight of testis (B), epididymis (C), and spleen (D) of male Brandt's voles (*Lasiopodomys brandtii*) in the three-voles and the five-voles groups. Error bars indicate standard error. *indicates significant differences at P < 0.05 and ***indicates significant differences at P < 0.001 between two groups (n = 6). Note: Relative weight of testis, epididymis, and spleen was calculated as paired testes, paired epididymis, and spleen weights (g), respectively, divided by body weight (g).



Fig. 2. Concentration of corticosterone (CORT) (A) and testosterone (T) (B) in the serum of male Brandt's voles (*Lasiopodomys brandtii*) in the three-voles and the five-voles groups. Error bars indicate standard error. *indicates significant differences between two groups at P < 0.05 (n = 6).

in the mPFC between the two groups (P = 0.338).

The mRNA levels of *5HT1A* (Fig. 4B) and *5HT1B* (Fig. 4C) in both the DRN (P = 0.015, P = 0.007, respectively) and the amygdala (P < 0.001, P = 0.002, respectively) were higher in the three-voles group than in the five-voles group, while there was no significant difference in the mPFC between the two groups in the expression of these two serotonin receptors (P = 0.203, P = 0.128, respectively).

DISCUSSION

Our study demonstrates that group density significantly affects growth, physiology, and aggressive behavior of male Brandt's voles, and shows in an innovative way that serotonergic systems in two areas of the brain are involved in the regulation of density-dependent aggressive behavior in these animals. In our study, although cage density was different from the density selected in the research of Li et al. (2003), the same trend of decreasing body weight with increasing cage density was observed. Higher cage density consistently inhibited weight gain in rats (Armario et al. 1984; Gamallo et al. 1986) and Microtus ochrogaster under short-day photoperiods (Nelson et al. 1996). In the present study, the relative weight of testis and epididymis also decreased with increasing cage density, which is again in line with previous laboratory (Li et al.



Fig. 3. Duration of male Brandt's voles (*Lasiopodomys brandtii*) engaging in aggressive behavior during a 30-min period in the three-voles and the five-voles groups. Error bars indicate standard error. Same letters indicate no significant differences between age groups at P < 0.05 (n = 12).

2003) and field (Zhou et al. 1992) research on the Brandt's vole. In addition, *M. ochrogaster* (Nelson et al. 1996) and mice (*Mus musculus*) (Brain and Nowell 1970; Christian 1955) have shown decreases in testis weight with group density elevation.



Fig. 4. Relative mRNA expression levels of *TPH2* (A), *5HT1A* (B) and *5HT1B* (C) in the dorsal raphe nucleus (DRN), amygdala (AMY), and medial prefrontal cortex (mPFC) of male Brandt's voles (*Lasiopodomys brandtii*) in the three-voles and the five-voles group. Error bars indicate standard error. Same letters connect bars with no significant differences at P < 0.05 (n = 6).

Higher cage density led to more aggressive behavior, indicating that male-male aggressive behavior levels in the Brandt's vole are positively density-dependent, which is in accordance with reports that group size positively correlates with aggression in mice (Van Loo et al. 2001; Butler 1980). Mice in higher-density groups show a more unstable hierarchy than mice in lower-density groups, and dominance status changes between animals more often in the former, which might result in more aggressive behavior in higherdensity groups (Poole and Morgan 1973). Indeed, even after 10 weeks of caging in the current study, durations of aggressive behavior were not reduced, and it was difficult to confirm the hierarchical status of each male vole due to their rambling behavior in the process of the experiment. We propose that each male vole's status was more unstable in the five-voles group, which resulted in more aggressive behavior than in the three-voles group. We conclude that density is an important factor affecting male-male aggressive behavior of the Brandt's vole.

It has been documented that an animal's age can affect its level of aggressive behavior, and that the duration and frequency of aggression thus increases with population growth in a group of mice (Van Loo et al. 2001). In this study, we consistently found that aggression durations showed an increasing trend with age, with aggression durations in the sub-adult period (6-7 weeks of age) being lower than in the adult period (older than 9 weeks of age). We therefore propose that age is also an important factor determining levels of aggressive behavior in male Brandt's voles, and that aggressive behavior levels increase with the animals' growth and development; this could explain the phenomenon of only adult or sub-adult male voles, not younger male voles, often migrating among colonies in the field (Shi et al. 1999).

It is well documented that high population density can activate the hypothalamic-pituitaryadrenal (HPA) axis and result in elevated serum levels of corticosterone (Barrett and Stockham 1963; Lee and McDonald 1985; Geller and Christian 1982). In this study, when cage density increased from three to five voles per cage, serum corticosterone concentrations increased as anticipated. In contrast, group density did not affect serum corticosterone concentration in male Brandt's voles (Li et al. 2003). The voles grouped in each cage were siblings or were familiar with each other before the experiment. This reduced aggression among voles in the study by Li et al. (2003). In our study, the voles were not siblings and had not met each other before the experiment. This may explain the differences in serum corticosterone concentration. In general, elevated corticosterone levels are considered to inhibit the immune system (Peng et al. 1989; Khansari et al. 1990). The spleen is well known to be an important immune organ in mammalians (Nelson et al. 1996), but the relative spleen weight increased with cage density in our study, corresponding to increasing corticosterone concentrations. Similarly, the spleen weight of attacked mice was positively correlated with aggression (Van Loo et al. 2000). Although in this study we did not estimate the parameter of wounds caused by male-male attacking, it has been reported that the number of wounds was higher in groups of five mice than in groups of three mice (Van Loo et al. 2001). Thus, more aggressive behavior should lead to a higher occurrence of wounds in the five-voles group and stimulate the immune system repeatedly and thus make the spleen, which is not simply regulated by serum corticosterone concentrations, grow heavier. However, a heavier spleen does not necessarily indicate higher immune function in male Brandt's voles (Li et al. 2003). Since serum antibodies were not measured in this experiment, we should be careful with attributing the heavier spleen to higher immune function in male Brandt's voles in higher cage density.

The relative testis weight decreased in the five-vole group, although the concentration of serum testosterone did not consistently decrease in the same group. Similarly, an increase in group density can reduce the testis index of Brandt's voles (Li et al. 2003). It has been documented that aggressive male-male encounters can induce elevations in androgen levels (Gleason et al. 2009; Wingfield, 2005) and that high levels of testosterone are associated with aggression in adolescent male non-human primates (Higley et al. 1996). Androgens can promote the intermale aggressive behavior in many species (Nelson and Trainor 2007; Simon et al. 1998). We thus propose the existence of a positive-feedback loop in which aggression in the five-vole group led to an elevation in testosterone levels, and elevated testosterone levels in turn facilitated a further increase in aggression. This resulted in no observed decrease in testosterone level in the fivevole group, where the testosterone level should have otherwise been lower due to the sharp decrease in the weight of the reproductive organs.

This phenomenon also suggests that testosterone levels in Brandt's voles may not always exactly be consistent with the reproductive status, and that they may be affected by many variables such as male-male antagonistic encounters. Our group density manipulation in male voles was limited by the fact that, in the field, Brandt's vole families consist of several male and a larger number of female voles (Shi et al. 1999). In fact, finding three or more adult male voles in one burrow unit is usually rare in the reproductive season (Xie et al. 1994). Xie et al. (1994) also found that two male voles housed with two or three females could incur intense struggles between males, resulting in one male vole killed and breeding failure in those groups. Hence, we believed that, if female voles were added, the male-male aggression in our study would be even stronger. This would partly explain the absence of a large number of adult male voles in natural colonies that is observed in natural settings. Aggressive behavior is considered to play a role in controlling population fluctuations in M. oeconomus (Nie and Liu 2005). Taken together, we infer from our physiological and behavioral results that male-male aggressive behavior also contributes to the regulation of population dynamics in Brandt's voles. As the population is growing, aggressive behaviors among male Brandt's voles intensify and thus enhance stress reactions and inhibit growth and reproduction, which may impact population growth and gradually lead to a population decrease. Another important insight from our study is that when captive Brandt's voles are used to conduct experiments in which the density is not an experimental variable, it is important to ensure that the same group density is used for all groups; otherwise, changes in the physiological status of the animals could affect the experiment.

In this study, expression levels of *THP2*, *5HT1A*, and *5HT1B* in both the DRN and the amygdala decreased sharply in the five-voles group with higher aggressive behavior compared to the three-voles group, which supports the role of serotonergic systems in the regulation of aggression (Carkaci-Salli et al. 2011; Popova et al. 2005; Olivier et al. 1995; Bortolato et al. 2013). This indicates that *THP2*, *5HT1A*, and *5HT1B* of serotonergic systems in the DRN and the amygdala are involved in the neuro-pathway for male-male aggressive interactions in the Brandt's vole, thus supporting our hypothesis that the serotonergic system is involved in density-dependent aggression in these

animals. In accordance with our study, activation of 5HT1A and 5HT1B in the DRN by microinjection of selective receptor agonists reduced aggressive behavior in rats and mice (Mos et al. 1993; Bannai et al. 2007; Faccidomo et al. 2008). 5HT1A and 5HT1B expression has been found in amygdala nuclei (Aznar et al. 2003; McDonald and Mascagni 2007). However, 5HT1B expression increases in the basolateral amygdala of rats showing aggressive behaviors (Suzuki et al. 2010). In this study, we collected the entire amygdala, not only its basolateral region. 5HT1B is thought to have brain region-specific roles in regulating aggressive behavior (Suzuki et al. 2010). We thus infer that 5HT1B expression in other regions of the amygdala would also be involved in the regulation of male-male aggressive behavior and decrease with increasing male Brandt's vole population density.

Expression of 5HT1A and 5HT1B was also detected in the mPFC of Brandt's voles, indicating a serotonergic system in the mPFC of these animals. 5HT1A and 5HT1B were consistently found to be localized in neurons of the PFC (Santana et al. 2004; Bortolato et al. 2013). Furthermore, the serotonergic system in the PFC has been considered to be involved in aggressive behavior in rodents and humans (Davidson et al. 2000; Biver et al. 1996; Takahashi and Miczek 2014), while activation of 5HT1B in the mPFCreduced species-typical territorial aggression in male mice (Faccidomo et al. 2012). By contrast, in this study, expression levels of 5HT1A and 5HT1B in the mPFC did not differ between the two density groups. In addition, expression levels of both 5HT1A and 5HT1B in the mPFC were lower, only 20-40 % of those occurring in the DRN and the amygdala. Along with 5-HT receptor 1, members of other 5-HT receptor families such as 5-HT receptor 2 were also expressed in the PFC of rats (Abi-Saab et al. 1999; Liu et al. 2007) and were shown to take part in regulating aggressive behavior in mice (Sakaue et al. 2002). Several subareas in the PFC, such as the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), are involved in male-male aggressive behavior in rats and mice (Haller et al. 2006; Halász et al. 2006; Wall et al. 2012; Centenaro et al. 2008). Besides, it has been suggested that the PFC may modulate several types of aggressive behavior in different ways (Takahashi and Miczek 2014). Taken together, we propose that 5HT1A and 5HT1B in the mPFC may either play a weak role in the regulation of this kind of aggression among polygynous male Brandt's voles, or that other subtypes of the 5-HT receptor in the mPFC serotonergic system may be involved in the regulation of aggressive behavior in the animal. Alternatively, other subareas of the PFC may be implicated in this kind of aggressive behavior.

Based on our study, we infer that increasing male vole population density could decrease the expression of serotonin and its receptors in the DRN and amygdala, thus intensifying malemale aggression in Brandt's voles. However, the neural pathway related to the aggressive behavior is complicated (Suzuki et al. 2010; Miczek et al. 2002). Therefore, further investigation into other areas of the brain and other neurotransmitters involved in this kind of male-male aggression is required to clarify its underlying neural mechanism.

CONCLUSIONS

In summary, increasing group density in male Brandt's voles led to elevated corticosterone levels, more male-male aggressive behavior, and inhibited weight gain and reproductive functions, but promoted immune organ growth. Age affected levels of aggressive behavior, with aggression duration increasing with the growth of the animal. We show that group density can influence the expression levels of TPH2, 5HT1A, and 5HT1B, which are involved in the serotonergic systems in the DRN and amygdala and may take part in the regulation of male-male aggressive behavior in the Brandt's vole. Our results provide novel insights into the neuro-mechanism of male-male aggressive behavioral responses to variation in population density and imply an important role of aggression among males in the process of population density regulating population dynamics in the Brandt's vole.

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Authors' contributions: Prof. Sheng-Mei Yang and Wan-Hong Wei designed the experiment and revised the manuscript. Dr. Xin Dai did statistical analyses and wrote the manuscript. Master students Ling-Yu Zhou, Jie-Xia Cao and Yan-Qi Zhang performed the experiment. Dr. Feng-Ping Yang and Prof. Ai-Qin Wang provided technological support for this experiment.

Competing interests: All eight authors declare that they have no conflict of interest.

Availability of data and materials: The sequences we cloned have been submitted to GenBank as partial mRNA sequences for each gene (accession numbers for *5HT1A*, *5HT1B*, and *TPH2* are MF536087, MF536088, and MF536089, respectively).

Consent for publication: Not applicable.

Ethics approval consent to participate: All procedures in our experiment were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University.

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