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Effects of *Pinus koraiensis* Bark Substrate on the Behavioural Responses of Captive Giant Pandas

Ming-Yue Zhang*, Bo Yuan, Xiao-Hui Zhang, James Ayala and Rong Hou*

Chengdu Research Base of Giant Panda Breeding, Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu, 610081, China

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Authors' Contribution
MYZ, BY, JA and RH conceived the study and participated in design, coordination and drafted the manuscript. XHZ collected behavioral data. MYZ analysed the data statistically.

Key words
Captive giant pandas, *Pinus koraiensis* bark, Environmental enrichment, Stereotypic behavior, Behavioral diversity

ABSTRACT

Environmental enrichment plays a very important role in improving the behavioral selectivity, health and welfare of captive giant pandas. The increase in specific behaviors and the reduction of abnormal behaviors are considered to be important indicators of the physical health of giant pandas, and the ideal result of environmental enrichment is to make captive giant pandas appear close to nature. To understand the effect of *Pinus koraiensis* bark substrate on the behavioural responses of captive giant pandas, we selected 6 adult female giant pandas and 6 juvenile giant pandas from Chengdu Giant Panda Breeding Research Base, and performed a one-month behavioral study during the non-mating season, focusing on observing the performances of stereotypes and behavioral diversity. The results showed that both adult and juvenile giant pandas preferred to feed in places where substrate was placed; additionally, juvenile giant pandas living in an outdoor playground that was covered with substrate were more active than those without substrate ($p<0.01$); moreover, the social and the play behaviors were significantly more frequent in the juvenile giant pandas with substrate ($p<0.01$). The *P. koraiensis* bark substrate significantly reduced the frequencies of stereotypic behaviors of adult captive giant pandas ($p<0.01$) and increased the expression of behavioral diversity among captive giant pandas. It is concluded that the use of this environmental enrichment program of laying *P. koraiensis* bark substrate in an outdoor playground could increase the behavioral adaptability of captive giant pandas, facilitate the expression of behavioral diversity, and improve the welfare of captive giant pandas.

INTRODUCTION

Substrate refers to a variety of natural and artificial materials that covers the surface of an enclosure and provides a comfortable (dry, warm, soft) habitat for the animals (Wang, 2015). Substrate was originally used in livestock production and has been subsequently widely used in experimental animal breeding; it is also currently used in wildlife management. In the captive environment, substrate is currently used mainly in primates but is less used for other animals: adding substrate in the primate living environment allows the animal to fully express its natural behavior (providing chimpanzees with straw, shavings and branches show the natural behavior of making a bed before going to bed) (James *et al*., 2019), reduces the frequency of fighting behavior, increase the time of spending in activities on the ground, and prolong the foraging time; over time, the substrates can effectively inhibit bacteria (Forthman and Bakeman, 1992). Other animals, such as elephants, use river sand as a substrate to fully demonstrate their natural behaviors, such as digging and dust-bathing (Schiffmann *et al.*, 2018). Bears use bark, sawdust, hay and other substrates to reduce stereotypes (Wagman *et al*., 2018). In addition, the substrates not only reduce the occurrence frequency of animals' stereotypic behaviors but can also play a better role in keeping the enclosure dry and warm and buffering the animal's joint pressure.

According to research in the wild, in the estrus period, giant pandas like to be active in areas where *Pinus armandii* is dominant (Liu, 2001). In the Wolong National Nature Reserve, giant pandas prefer to inhabit the broad-leaved forests area dominated by *Picea purpurea*, *Abies faxoniana*, *Sabina saltuaria*, *Betula albosinensis* and *Betula utilis* (Taylor, 1989). During the estrus period, the scent-marked tree is mainly *Abies faxoniana* (Donald and Hu, 1991). In addition, Fengtongzhai and Foping giant pandas use tree dens and caves as nests and use the wood chips, bamboo or sawdust from the fir tree holes (more prefer to use sawdust) as nesting substrates (Wei *et al.*, 2019). From these observations we found that pine trees are the main tree species serving as the natural habitat of giant pandas in the wild, and foreign zoos (such as the

* Corresponding author: zmy6611@126.com; hourong2000@panda.org.cn
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ZOO PARC DE BEAUVEL) have begun to use pine bark as the indoor substrate for captive giant pandas. However, there have been no reports of laying *Pinus koraiensis* bark substrate in an outdoor playground, and it is unclear whether it would affect the welfare and behavioral adaptability of captive giant pandas. Therefore, to study the effects of laying *P. koraiensis* bark substrate in an outdoor playground on the behavior of captive giant pandas, in this study we selected adult and juvenile captive giant pandas as the research object and observed the behavioral changes in non-estrus periods from October to November (mainly observe normal and stereotypic behaviors) to evaluate the effects of the *P. koraiensis* bark substrates on the behavioral expression of captive giant pandas and to provide an important scientific basis for improving the welfare of captive giant pandas.

MATERIALS AND METHODS

Animals

Six adult female giant pandas (Cheng Da, Ke Lin, Ya Yun, Xiao Yatou, Qing He and Miao Miao) and six juvenile pandas (Da Mei, Yn Wen, Chuan Zai, Run Jiu, Qi Cheng and Xiao Xin) came from the Moon House and Sun House in Chengdu Giant Panda Breeding Research Base, and their behavioral responses were observed during non-breeding period in two different outdoor playgrounds (one with *P. koraiensis* bark substrate laid and the other without *P. koraiensis* bark substrate laid), from October 29, 2018, to November 30, 2018. None of the experimental pandas had any previous exposure to the enrichment item (*P. koraiensis* bark substrate).

Feeding and management

The two outdoor playgrounds of the Moon house were equipped with the same environmental enrichment facilities, which included trees, shrubs, lawns, climbing shelves, pools and rockeries. The temperature of the two outdoor playgrounds was maintained at 9.5 ± 3.2°C with a relative humidity of 68 ± 2% from October 29, 2018, to November 30, 2018. A subset of adult female giant pandas and their nurtured cubs were living in the substrate group, while the other subset of adult female giant pandas and their nurtured cubs were in the non-substrate group; see Table I for specific groups. The management modes of the two venues were exactly the same, and the giant pandas were raised and managed by the same breeder. The giant pandas were released into the outdoor playgrounds at 8:30 every day and were taken indoors at 17:30. The feeding times were 8:00 and 14:00 every day, and the bamboo supply was sufficient. During the interval, intermittent feeding of "Wowotou", apples, honey water and medicine was performed. The carbonized *P. koraiensis* bark substrate was purchased from the Huilong Construction Engineering Team in Chenghua District, Chengdu. The *P. koraiensis* bark was laid under the panda climbing shelves in outdoor playground 1 of the moon house. The length of the substrate was 2-5 cm, and the thickness was approximately 1 cm.

Behavioral observations

During the non-mating season, behavioral observations were conducted every three days from October 29, 2018, to November 30, 2018. We continuously recorded and watched the videos from 6:00 h to 18:00 h every three days in October 29, 2018, to November 30, 2018, avoiding the half hour that the breeder cleaned manure, which occurred from 14:00 h to 14:30 h. The key observation times were from 9:00 h to 11:00 h and from 14:00 h to 16:00 h. The experimental site was recorded using a Digital Video system (HDR-CX680, SONY, Japan) for data acquisition. To observe the occurrence of stereotypes caused by hunger before feeding, it was recommended that the stereotypes of giant pandas be recorded from 14:00 to 15:30 in the afternoon.

The collected images were observed by visual inspection using the scanning sampling method with 1 min as the time unit over a 4-h observation period with 240 behavioral record points that constituted the behavioral records of all of the treatment groups. The statistical state behaviors that were observed included standing and movement behaviors, resting behavior, feeding and drinking behaviors and excretion behavior. The statistical event behaviors that were observed included stereotypic behavior, exploring behavior, vigilance behavior, playing behavior and grooming behavior, and the behaviors were recorded once for each occurrence (for specific behaviours and definitions, see Table II). State behavioral data were converted to a percentage of observed time, and event behaviors were calculated as the actual number of occurrences of the behavior. The behavioral data of each experimental panda on the grass, concrete floors, trees, stakes and rockeries in their respective outdoor playgrounds were recorded.

Statistical analysis

The collected data of the behavior tests were analysed with the Statistical Package for Social Sciences (SPSS 23.0; software IBM Institute Inc., Chicago, USA). Data from the behavior observations were analysed for agreement with a normal distribution with the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) was used to examine the effects of the *P. koraiensis* bark substrate on the behavioral responses in adult and juvenile captive giant pandas. Multiple comparisons were performed using the least-significant difference (LSD) method. Differences

Table I. Experiment grouping design.

	Name	Studbook	Sex	Birthday	Location
Substrate group					
Adult	Cheng Da	824	Female	2011-8-15	CHENPANDA
	Ke Lin	678	Female	2007-8-13	CHENPANDA
	Qing He	537	Female	2001-9-24	CHENPANDA
Juvenile	Da Mei	1073	Female	2017-6-27	CHENPANDA
	Chuan Zai	1121	Female	2018-5-20	CHENPANDA
	Qi Cheng	1131	Female	2018-7-02	CHENPANDA
Non-substrate group					
Adult	Xiao Yatou	635	Female	2006-8-13	CHENPANDA
	Miao Miao	855	Female	2012-9-04	CHENPANDA
	Ya Yun	796	Female	2010-9-10	CHENPANDA
Juvenile	Xiao Xin	1085	Female	2017-7-26	CHENPANDA
	Run Jiu	1145	Female	2018-7-31	CHENPANDA
	Yun Wen	1124	Female	2018-6-05	CHENPANDA

CHENPANDA, Chengdu Research base of Giant Panda Breeding.

Table II. Behavioral categories and their definitions.

Behavioural categories	Definitions
State behaviors	
Stand and locomotion (%)[a]	Individuals in various non-stationary states, any kind of directional travel between points including walking, running and climbing without placing feet in the same position each time and following the same path. Stand and Locomotion totally called active state
Resting (%)[a]	Individuals remain stationary in various postures, lying, sitting or standing without changing position, totally called non-active state.
Feeding and drinking (%)[a]	Consumption, or processing for consumption, of provisioned food, including bamboo and supplementary diet items in a variety of postures, including lateral lying, ventral lying, lying and sitting.
Excretion (%)[b]	Urinating and defecating while in a squat, leg cock, handstand, or standing posture on the wall or ground.
Event behaviors	
Stereotypic behaviors (times/min)[c]	Animal moves to and fro or circles in a repetitive way for at least three times. including pacing (continuous walking back and forth following the same path); circling (walking following a defined route placing feet in the same position each time); self-mutilation (self-inflicted physical harm, such as biting or chewing the tail or leg, or hitting the head against a wall); head bobbing (standing in one place and continuously moving the head up and down); and standing bipedally at the window of the door or the fence, often seemingly in expectation of food delivery.
Exploring (times/min)[a]	Investigating an object with a distance of 0.1 m or more between the nose and the object.
Vigilance (times/min)[d]	Turn its head and look at the direction of the sound source or vigilant to look up.
Grooming (times/min)[a]	Scratching and licking of the pelage.
Social play (times/min)[a]	Reciprocal play fighting: Two or more individuals who have been squirming, pushing, pulling, or pushing each other with their feet for a long time, with no harm or no sound; Non-reciprocal play fighting: Two individuals have long-term contact, one of them pushes, pulls or clap, but the other does not react, and there is no sound.
Object play (times/min)[a]	Self-motion playing and locomotive play: These include rolling and swinging limbs, rolling forward, turning around objects, moving the body while climbing walls and railings, and biting branches or other plants.

[a]Based on Liu *et al*. (2003); [b]Based on Swaisgood *et al*. (1999); [c]Based on Liu *et al*. (2006); Liu *et al*. (2017); [d]Based on Du *et al*. (2012).

between groups were considered to be statistically reliable if the associated p-value did not exceed 0.05. The values in the text are expressed as the means ± standard errors of the mean (SEM).

RESULTS

Upon comparing the locations and duration times of feeding behavior throughout the day, it was found that the duration times of feeding and drinking behaviors of juvenile giant pandas on *P. koraiensis* bark substrate were significantly higher than those in other places after laying substrate and that the adult female giant pandas had the longest feeding times on the substrate and the climbing shelves (Adult: F (4,145)= 44.214; p<0.01; Juvenile: F(4,145)=8.695; p<0.01) (Fig. 1).

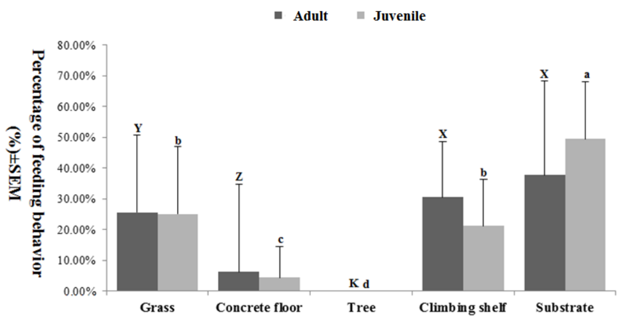

Fig. 1. Feeding time distribution of adult and juvenile captive giant pandas in different locations. a, b, c, d: Different superscripts indicate significant differences in the feeding times of adult giant pandas between different locations (p<0.01). X, Y, Z, K: Different superscripts indicate significant differences in the feeding times of juvenile giant pandas between different locations (p<0.01). The data are presented as the mean ± SEM.

The juvenile giant pandas that lived in the outdoor playground where *P. koraiensis* bark substrate was laid were active for a significantly longer time than the adult giant pandas and the juvenile giant pandas that did not live in the outdoor playground where the substrate was laid (Activity: F (2, 87) =895.979; p<0.01). Moreover, the time of inactivity was significantly lower than that of adult giant pandas (Inactivity: F (2, 87) =486.256; p<0.01) (Fig. 2).

Adult female giant pandas preferred to express various event behaviors on the climbing shelves in the outdoor playground where no *P. koraiensis* bark substrate was laid (F (3,116) =5075.690; p<0.01), while the juvenile giant pandas preferred to be on the tree (F (3,116) =2691.116; p<0.01). After laying the *P. koraiensis* bark substrate, the juvenile giant pandas chose to spend most of their feeding and activity time on the substrate (F (4,145) =802.544; p<0.01), and juvenile giant pandas preferred to show greater frequencies of event behaviors in places with substrate than did adult giant pandas (F (1,58) =606.977; p<0.01) (Fig. 3).

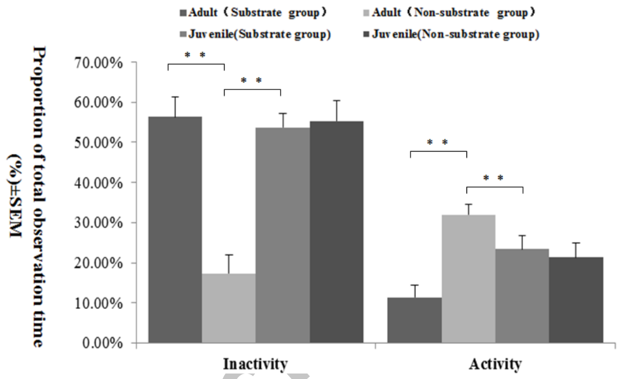

Fig. 2. Effects of laying substrate on the state behaviors of adult and juvenile captive giant pandas. The data are presented as the mean ± SEM; * p<0.05; ** p<0.01.

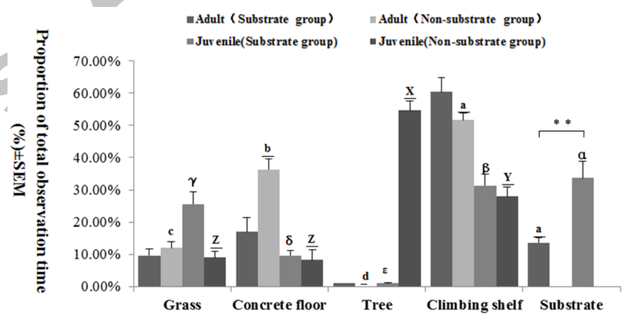

Fig. 3. Event behavioral performance distribution of adult and juvenile captive giant pandas in different locations. a, b, c, d: Different superscripts indicate significant differences in expressing event behaviors of adult giant pandas between different locations (p<0.01). X, Y, Z: Different superscripts indicate significant differences in expressing event behaviours of juvenile giant pandas between different locations (p<0.01). α, β, γ, δ, ε: Different superscripts indicate significant differences in expressing event behaviors of juvenile giant pandas between different locations (p<0.01). The data are presented as the mean ± SEM; * p<0.05; ** p<0.01.

After laying the *P. koraiensis* bark substrate, there was a significant effect on the frequency of playing behavior occurrence among the juvenile giant pandas. The frequencies of social playing and object playing occurrence in the juvenile giant pandas with substrate laid were significantly greater than those of other juvenile giant pandas without substrate laid (social play: F (1,58) =62.179; p<0.01; object play: F (1,58) =119.729; p<0.01)

(Fig. 4).

After laying the *P. koraiensis* bark substrate, there was a significant effect on the frequency of the stereotypic behaviors among the adult giant pandas. The frequencies of stereotyping occurrence in the adult giant pandas with substrate laid were significantly lower than those of other adult giant pandas without substrate laid (F (1,58) =610.261; p<0.01). The *P. koraiensis* bark substrate had no obvious effect on the frequency of stereotyping occurrence in juvenile giant pandas (F (1, 58) =2.551; p=0.116) (Fig. 5).

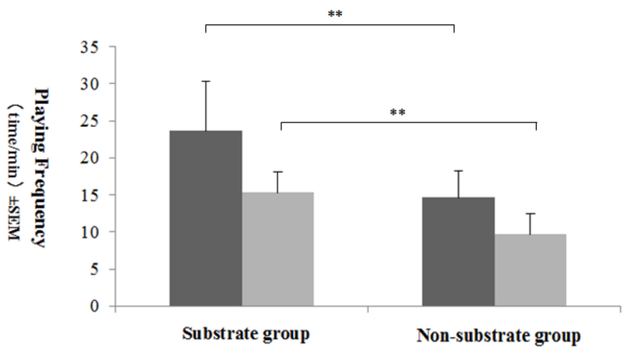

Fig. 4. Effects of laying substrate on the playing behavior of juvenile giant pandas. The data are presented as the mean ± SEM; * p<0.05; ** p<0.01.

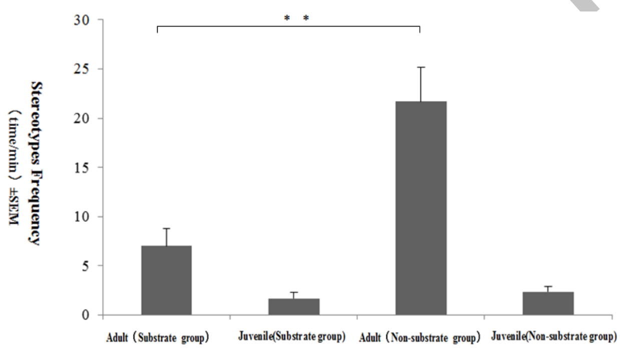

Fig. 5. Effects of laying substrate on the stereotypic behavior of adult and juvenile giant pandas. The data are presented as the mean ± SEM; * p<0.05; ** p<0.01.

DISCUSSION

In this study, we found that laying carbonized *P. koraiensis* bark substrate in an outdoor playground significantly increased the activity time and the frequency of playing occurrence in juvenile giant pandas and significantly reduced the frequencies of stereotyping occurrence in the adult giant pandas. This showed that laying carbonized *P. koraiensis* bark substrate in an outdoor playground can not only increase the environmental richness and reduce occurrence of stereotypic behaviors of captive giant pandas, but can also effectively increase the activity time of juvenile giant pandas and the social playing behaviors between dams and their cubs. Our results were also in agreement with Swaisgood *et al.* (2001), who argued that adult and sub adult captive giant pandas might spend significantly more time active; and display a greater variety of object- and nonobject-directed behaviors when enrichment is present and might also display greater behavioral diversity when enrichment is present (Swaisgood *et al*., 2001). In the early days, researchers achieved consistent results with this experiment through different enrichment treatments (mainly object items and food items), which could reduce the frequencies of stereotyping occurrence in captive giant pandas (Swaisgood *et al*., 2001, 2005; Liu *et al*., 2006; Liu *et al*., 2003). However, we also found that the environmental enrichment treatment of laying *P. koraiensis* bark substrate on the outdoor playgrounds had different effects on adult and juvenile captive giant pandas; for example, environmental enrichment had no effect on the activity time of adult giant pandas but significantly increased the activity time of juvenile giant pandas. Of course, some studies have shown that age also had a significant effect on the frequencies of stereotyping occurrence in captive giant pandas. Swaisgood *et al.* (2001) found that the frequencies of the stereotypic behaviors in captive adult giant pandas were significantly higher than those of sub-adults (Swaisgood *et al*., 2001). However, the effects of captive duration on the stereotypic behavioral responses in captive adult giant pandas were unclear, though the results of studying confined sows demonstrated that long-term environmental confinements could significantly increase the frequencies of stereotypic behaviors in confined sows (Zhang *et al*., 2017). We suspected that the results of this study may be similar to those of captive giant pandas. Environmental and food enrichment had different effects on adult and juvenile captive giant pandas. Our results were also in agreement with Swaisgood *et al.* (2001) and Wang *et al.* (2008). Swaisgood *et al.* (2001) found that adult, but not subadult captive giant pandas, displayed a significant preference for feeding enrichment (Swaisgood *et al*., 2001). Wang *et al.* (2008) also found that the enrichment treatment of laying a tire in the outdoor playground significantly increased the frequencies of exercise-related behaviors in captive adult giant panda (Bing Dian) and reduced the frequencies of feeding-related behaviors, while another captive adult giant panda (Xiao Shuang) showed greater frequencies of feeding-related behaviors and lower frequencies of exercise-related behaviors. A “frozen food” enrichment program significantly reduced the frequency of stereotypic behaviors in captive adult giant panda (Bing Dian), but it did not have a significant effect on the stereotypic behavioral response in another captive adult giant panda (Xiao Shuang) (Swaisgood *et al*., 2003).

The results of this study also demonstrated that laying carbonized *P. koraiensis* bark substrate in an outdoor playground can significantly reduce the frequency of stereotyping occurrences in adult captive giant pandas and increase the behavioral diversity of juvenile giant pandas (including playing behaviors). It has been reported that laying substrates in an artificial denning box could significantly improve the expressions of maternal behaviors in captive female giant pandas during the cub-rearing period and increased the survival rate of twin panda cubs (Heiderer *et al*., 2018). These results showed that the housing environment was relatively simple and the activity space was limited, which seriously hindered the expression of normal behaviours of captive giant pandas and affected the performance of behavioral diversity. The laying of carbonized *P. koraiensis* bark substrate provided the captive giant pandas with a new environmental stimulus, which also promoted natural species-typical behaviours, especially the expression of exploring and playing behaviors, and reduced abnormal or stereotypic behaviors (Swaisgood *et al*., 2001). At the same time, we also found that the stereotypes of captive adult giant pandas were more frequent than those of captive juvenile giant pandas, meaning that long-term environmental confinements significantly increased the frequencies of captive giant pandas' stereotypic behaviors. This was consistent with our previous conclusions on the study of stereotypic behavior among captive sows (Zhang *et al*., 2017). Therefore, the emergence of stereotypes was considered to be a behavioral pattern exhibited by captive giant pandas to adapt to the pressures brought about by environmental changes (Liu *et al*., 2017). To accurately measure the performance of stereotypes, we focused on the feeding anticipation time of captive giant pandas, because this activity of feeding anticipation was closely associated with the development of stereotypies (Hughes and Duncan, 1988; Mason, 1991). From the perspective of adaptability, stereotypes are caused by poor environmental conditions; i.e., the animals are normal, but the environmental conditions in which they are located are not suitable to some extent (such as insufficient environmental enrichment) (Lawrence and Terlouw, 1993). Environmental enrichment is considered to be a dynamic work process that builds and changes the living environment of captive animals to allow as much normal behavior as possible, providing them more opportunities for behavioral development (Mason *et al*., 2007).

Based on the results of this experiment, we believed that the enrichment treatment of laying carbonized *P. koraiensis* bark substrate in an outdoor playground was effective for improving the welfare of captive giant pandas because good enrichments were thought to offer animals opportunities to perform activities more often and to perform the stereotypic behaviors less frequently (Mason *et al*., 2007). The expression of stereotypies in captive animals is generally believed to reflect poor wellbeing, although the mechanism of stereotypic behaviors is still unclear. However, we know that the occurrence of stereotypic behaviors is the result of the interaction between environment and genes (Mason, 1991). Once the adverse environment exceeds the tolerance of the body, a specific physiological response occurs that cause changes in the expression of related proteins encoded by the animal's DNA, resulting in changes in the behavioral phenotype (Maes *et al*., 1997). Therefore, in the future, we should pay attention to the molecular mechanism of stereotypic behaviors, especially in research on epigenetics, with the hope of making some contribution to improving the welfare of captive giant pandas.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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